Alcohol in Health and Disease

edited by

Dharam P. Agarwal
Helmut K. Seitz

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Dharam P. Agarwal
University of Hamburg
Hamburg, Germany

Helmut K. Seitz
Salem Medical Center
Heidelberg, Germany

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Preface

Alcohol use and abuse, alcohol intolerance, alcohol dependence, and other alcohol-related health effects are some of the most challenging public health problems facing our modern-day society. The biochemical and pathological disturbances observed in humans after acute and chronic intake of alcohol are exceedingly complex and many biological and environmental factors may influence their outcome. Both epidemiological and clinical studies have implicated the excessive use of alcohol in the risk of developing a variety of organ, neuropsychiatric, and metabolic disorders. Family, twin, and adoption studies suggest that alcoholism is familial, with a significant proportion attributable to genetic factors besides cultural and familial environmental factors. Recent investigations have shown that genetically determined variations in alcohol and acetaldehyde metabolism may be responsible for individual and racial differences in acute reactions to ethanol, alcohol drinking habits, and vulnerability to alcohol-related end-organ damage. Research on the biomedical effects of alcohol intake, particularly with respect to the putative benefits of moderate drinking on the cardiovascular system, has attracted considerable attention in recent decades. Epidemiological studies have consistently shown that light to moderate intake of alcoholic beverages is associated with a reduced incidence of, and mortality from, coronary heart disease.

Keeping in tradition of the past Titisee Conferences on relevant scientific issues, we organized an International Titisee Symposium on Health Effects of Alcohol Intake, in Titisee, Germany, from December 9 to 12, 1999. Based on issues covered at the symposium, this volume examines multidisciplinary topics (molecular biochemistry, genetics, epidemiology, pathophysiology, neurobiol-

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ogy, as well as cardiovascular aspects of alcohol use and abuse. The volume has been compiled into six main parts. Part I deals with alcohol metabolism, genes, and their possible role in alcohol sensitivity, alcohol drinking habits, and alcohol dependence. In Part II, genetic and epidemiological consumption-related morbidity are grouped. Part III deals with general health issues related to alcohol consumption. Part IV contains contributions related to hepatic, metabolic and nutritional disorders of alcoholism—in particular, alcoholic liver diseases. Part V covers topics related to alcohol and cancer. Finally, Part VI contains papers related to alcohol and cardiovascular disorders, including the French paradox.

We are convinced that this volume will prove useful not only to basic scientists and physicians interested in the area of alcoholism research but also to clinicians, geneticists, pharmacologists, toxicologists, psychiatrists, biochemists, and those involved in health-policy–making decisions.

We thank all the participants of the Titisee Symposium for making it a scientifically exciting and socially pleasant meeting. We also thank the Volkswagen–Stiftung, Hannover, Falk Foundation, Freiburg, and Merck AG, Darmstadt for their financial support for organizing the Titisee Symposium. Last, but not least, we thank the staff of Marcel Dekker, Inc., for their cooperation in bringing out this timely volume.

Dharam P. Agarwal
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Contributors

Junko Adachi Department of Legal Medicine, Kobe University School of Medicine, Chuo-ku Kobe, Japan

Dharam P. Agarwal Institute of Human Genetics, University of Hamburg, Hamburg, Germany

Sohail Ahmed Department of Neurochemistry, Regional Neurosciences Centre, Newcastle General Hospital, Newcastle upon Tyne, England

Abdellah Allali-Hassani Department of Biochemistry and Molecular Biology, Universitat Autònoma de Barcelona, Bellaterra, Barcelona, Spain

Tatjana Arslic Department of Medicine, Salem Medical Center, Heidelberg, Germany

Christiane Bode Department of Physiology of Nutrition, Hohenheim University, Stuttgart, Germany

J. Christian Bode Department of Internal Medicine, Robert-Bosch Hospital, Stuttgart, Germany

Emma Borràs Department of Biochemistry and Molecular Biology, Universitat Autònoma de Barcelona, Bellaterra, Barcelona, Spain

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Michael H. Criqui Department of Family and Preventive Medicine, School of Medicine, University of California–San Diego La Jolla, California

Howard Edenberg Departments of Biochemistry and Molecular Biology, Indiana University School of Medicine, Indianapolis, Indiana

Jaume Farrés Department of Biochemistry and Molecular Biology, Universitat Autònoma de Barcelona, Bellaterra, Barcelona, Spain

Ulrich Finckh Institute of Human Genetics, University Hospital Hamburg–Eppendorf, University of Hamburg, Hamburg, Germany

Tatiana Foroud Department of Medical and Molecular Genetics, Indiana University School of Medicine, Indianapolis, Indiana

John Howard Foster Department of Health, Environmental and Biological Sciences, Middlesex University, Enfield, Middlesex, England

David M. Goldberg Department of Laboratory Medicine and Pathobiology, University of Toronto, Toronto, Ontario, Canada

Minna L. Hannuksela Department of Internal Medicine and Biocenter Oulu, University of Oulu, Oulu, Finland

Andreas Heinz Department of Addictive Behavior and Addiction Medicine, Central Institute of Mental Health, Mannheim, Germany

Anders Helander Alcohol Laboratory, Department of Clinical Neuroscience, Karolinska Institute and Hospital, Stockholm, Sweden

Victor M. Hesselbrock Department of Psychiatry, University of Connecticut School of Medicine, Farmington, Connecticut

Nils Homann Department of Gastroenterology, Medical University of Lübeck, Lübeck, Germany

Hiromasa Ishii Department of Internal Medicine, School of Medicine, Keio University, Tokyo, Japan

Kalle Jokelainen Research Unit of Alcohol Diseases, Helsinki University Central Hospital, Helsinki, Finland

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Shinzo Kato Department of Internal Medicine, School of Medicine, Keio University, Tokyo, Japan

Arthur L. Klatsky Senior Consultant in Cardiology, Kaiser Permanente Medical Center, Oakland, California

Charles S. Lieber Alcohol Research Center and Section of Liver Disease and Nutrition, Mount Sinai School of Medicine and Bronx Veterans Affairs Medical Center, Bronx, New York

Karl Mann Department of Addictive Behavior and Addiction Medicine, Central Institute of Mental Health, Mannheim, Germany

David Mantle Department of Neurochemistry, Regional Neurosciences Centre, Newcastle General Hospital, Newcastle upon Tyne, England

Michael Martin Department of Anaesthesiology and Operative Intensive Care Medicine, Campus Mitte, University Hospital Charité, Humboldt University of Berlin, Berlin, Germany

Susana E. Martínez Department of Biochemistry and Molecular Biology, Universitat Autònoma de Barcelona, Bellaterra, Barcelona, Spain

Sílvia Martras Department of Biochemistry and Molecular Biology, Universitat Autònoma de Barcelona, Bellaterra, Barcelona, Spain

Katsuya Maruyama Department of Internal Medicine, National Institute on Alcoholism, National Kurihama Hospital, Kanagawa, Japan

Onni Niemela Department of Clinical Chemistry, University of Oulu, Oulu, and EP Central Hospital Laboratory, Seinäjoki, Finland

Tim Neumann Department of Anaesthesiology and Operative Intensive Care Medicine, Campus Mitte, University Hospital Charité, Humboldt University of Berlin, Berlin, Germany

John I. Nurnberger, Jr. Department of Psychiatry, Indiana University School of Medicine, Indianapolis, Indiana

Carl M. Oneta Department of Internal Medicine, Division of Gastroenterology and Hepatology, University Hospital of Lausanne, Lausanne, Switzerland

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Xavier Parés Department of Biochemistry and Molecular Biology, Universitat Autònoma de Barcelona, Bellaterra, Barcelona, Spain

Seppo Parkkila Department of Clinical Chemistry, Anatomy and Cell Biology, University of Oulu, Oulu, and EP Central Hospital Laboratory, Seinäjoki, Finland

Alexandr Parlesak Department of Physiology of Nutrition, Hohenheim University, Stuttgart, Germany

Timothy John Peters Department of Clinical Biochemistry, School of Medicine, King's College, University of London, London, England

Victor R. Preedy Department of Nutrition and Dietetics, King's College, University of London, London, England

Carol A. Prescott Virginia Institute for Psychiatric and Behavioral Genetics, Department of Psychiatry, Medical College of Virginia of Virginia Commonwealth University, Richmond, Virginia

Theodore Reich Department of Psychiatry, Washington University School of Medicine, St. Louis, Missouri

John P. Rice Department of Psychiatry, Washington University School of Medicine, St. Louis, Missouri

Albert Rosell Department of Biochemistry and Molecular Biology, Universitat Autònoma de Barcelona, Bellaterra, Barcelona, Spain

Mikko Salaspuro Research Unit of Alcohol Diseases, University Central Hospital of Helsinki, Helsinki, Finland

Markku J. Savolainen Department of Internal Medicine and Biocenter Oulu, University of Oulu, Oulu, Finland

Christian G. Schütz Psychiatric Hospital, University of Munich, Munich, Germany

Helmut K. Seitz Department of Medicine, Salem Medical Center, Heidelberg, Germany

Ulrich A. Simanowski Department of Medicine, Salem Medical Center, Heidelberg, Germany

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George J. Soleas Liquor Control Board of Ontario, Toronto, Ontario, Canada

Michael Soyka Psychiatric Hospital, University of Munich, Munich, Germany

Claudia Spies Department of Anaesthesiology and Operative Intensive Care Medicine, Campus Mitte, University Hospital Charité, Humboldt University of Berlin, Berlin, Germany

Ann P. Streissguth Department of Psychiatry and Behavioral Sciences, University of Washington School of Medicine, Seattle, Washington

Paolo M. Suter Department of Internal Medicine, Medical Policlinic, University Hospital, Zurich, Switzerland

Julia Vaglenova Department of Biochemistry and Molecular Biology, Universitat Autònoma de Barcelona, Bellaterra, Barcelona, Spain

Xiang-Dong Wang Gastrointestinal Nutrition Laboratory, Jean Mayer USDA Human Nutrition Research Center on Aging, Tufts University, Boston, Massachusetts

John B. Whitfield Department of Clinical Biochemistry, Royal Prince Alfred Hospital, Sydney, Australia

Simon Worrall Alcohol Research Unit, Department of Biochemistry, The University of Queensland, Brisbane, Queensland, Australia

Shih-Jiun Yin Department of Biochemistry, National Defense Medical Center, Taipei, Taiwan, Republic of China

Akira Yokoyama Department of Internal Medicine, National Institute on Alcoholism, Kurihama National Hospital, Kanagawa, Japan

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Titisee Symposium Participants *(left to right)*

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Row 2 Kalle Jokelainen, Nils Homann, Christian Bode, Arthur Klatsky, Mikko Salaspuro, Michael Soyka, Helmut Seitz, Dharam Agarwal, Paolo Suter

Row 3 Shih-Jiun Yin, Shinzo Kato, John Whitfield, Ulrich Simanowski, Timothy Peters, Markku Savolainen, Michael Criqui, Anders Helander, Tim Neumann, Serge Renaud, Carl Oneta, Victor Hesselbrock, David Goldberg, Victor Preedy

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Functional Polymorphism of Alcohol and Aldehyde Dehydrogenases

Alcohol Metabolism, Alcoholism, and Alcohol-Induced Organ Damage

Shih-Jiun Yin
National Defense Medical Center, Taipei, Taiwan, Republic of China

Dharam P. Agarwal
University of Hamburg, Hamburg, Germany

I. INTRODUCTION

Alcoholism is widely thought to be a multifactorial, polygenic disorder involving complex gene-with-gene and gene-with-environment interactions (1,2). Alcohol metabolism is one of the biological determinants that can significantly influence drinking behavior and the development of alcoholism and alcohol-induced organ damage (3–7). Most ethanol elimination occurs by oxidation to acetaldehyde and acetate, catalyzed principally by alcohol dehydrogenase (ADH) and aldehyde dehydrogenase (ALDH) (6,8). Other ethanol oxidation pathways, including catalase and microsomal ethanol-oxidizing system, as well as the nonoxidative pathway, which forms fatty acid ethyl esters, appear to play a minor role (9). Both ADH and ALDH exhibit genetic polymorphism and ethnic variation (3,10). This chapter reviews recent advances in the understanding of the functional polymorphisms of ADH and ALDH and their metabolic, physiological, and clinical correlations.

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II. ALCOHOL AND ALDEHYDE DEHYDROGENASES

A. Gene Families

Human alcohol dehydrogenases constitute a complex gene family (11,12). To date, seven genes have been identified. *ADH1* through *ADH5* and *ADH7* encode α, β, γ, π, χ, and μ (or designated σ) polypeptides, respectively. The *ADH6*-encoding subunit has not been designated a Greek letter. Three allelic variants occur at the *ADH2* locus: *ADH2*1*, *ADH2*2*, and *ADH2*3*, which encode the subunits of β_1, β_2, and β_3, respectively (10). Two variants occur at the *ADH3* locus: *ADH3*1* and *ADH3*2*, which encode the subunits of γ_1 and γ_2, respectively. *ADH2*1* is the predominant allele among most world populations thus far studied (~90%), and *ADH2*2* is the predominant allele in East Asian populations (~70%) (3). *ADH2*3* exists in populations of African origin (~20%) but appears to be very rare among the other ethnic groups. *ADH3*1* is the predominant allele among East Asians and Africans (~90%), whereas in Caucasians it is about equally distributed with *ADH3*2*. The above allelic variations are produced by single nucleotide substitutions that occur in exon 3 of *ADH2*2*, exon 9 of *ADH2*3*, and exons 6 and 8 each of *ADH3*2* (10). All of the human *ADH* genes lie in the same region on chromosome 4q22-24, spanning 367 kb (S.-F. Tsai, personal communication, 2000). *ADH1*, *ADH2*, and *ADH3* are tandemly arrayed within 77 kb. The clustering of *ADH* genes suggest that they belong to the family resulting from gene duplication and diversification.

The human aldehyde dehydrogenase family is complex in a different way (13). Twelve *ALDH* genes have been identified with a wide range of divergency in deduced amino acid sequence (>80%–<15% positional identity) and in catalytic function. There is a functional single nucleotide polymorphism (SNP) occurring within exon 12 of the *ALDH2* gene, resulting in a glutamic acid/lysine exchange at position 487 (10). About half of several East Asian populations, including Han Chinese, Japanese, and Koreans, carry the variant *ALDH2*2* allele, which is rarely seen in the other ethnic groups so far examined (4). The genes of *ALDH1* and *ALDH2*, showing 68% identity in the amino acid sequence, have been mapped to chromosome 9q21 and 12q24, respectively (10).

B. Kinetic Properties

Alcohol dehydrogenase, a NAD-dependent, zinc-containing dimeric enzyme, functions as rate-limiting step in the ethanol metabolism. Primarily based on homology of the primary structure and also on electrophoretic mobility, Michaelis constants of ethanol, and sensitivity to pyrazole inhibition, human ADH family members have been categorized into five classes (11). The interclass and intraclass sequence similarities at the amino acid level are approximately 90% and 60%, respectively. Featured kinetic constants for ethanol oxidation of ADH fam-

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ily are summarized in Table 1. Class I ADHs are composed of α-, β-, and γ-subunits having low K_m values (<5 mM) for ethanol oxidation and class II ππ and class IV μμ have intermediate K_m(~30 mM). Class III χχ is not saturable with ethanol and virtually functions as glutathione-dependent formaldehyde dehydrogenase (6,8). Kinetic constants for the class V enzyme, encoded by *ADH6*, have not yet been reported. Interestingly, both $\beta_2\beta_2$ and $\beta_3\beta_3$ ADHs exhibit 30–40-fold greater V_{max} for ethanol oxidation than $\beta_1\beta_1$, and the V_{max} for $\gamma_1\gamma_1$ is about twice that of $\gamma_2\gamma_2$ (8,14). The kinetic differences of the ADH allozymes can be attributed to a single amino acid substitution in the coenzyme binding domain (in β_2, histidine for arginine-47 in β_1; in β_3, cysteine for arginine-369 in β_1; and in γ_1, arginine for glutamine-271 in γ_2) that may affect dissociation of NADH, a rate-determining step in catalysis (8,14). The second isoleucine/valine-349 exchange for γ_1/γ_2 appears not to alter enzyme activity, because it is located away from the active site.

Among the human ALDH family members, which exhibit widely diverse substrate specificities (13), the low-K_m mitochondrial ALDH2 (0.20 μM) and cytosolic ALDH1 (33 μM) are the major enzymes responsible for oxidation of acetaldehyde in the liver (Table 1). Since the catalytic efficiency (V_{max}/K_m) of ALDH2 was found to be 160-fold greater than that of ALDH1, the mitochondrial ALDH2 may play a predominant role in the ethanol metabolism, particularly at low concentration of acetaldehyde in vivo. Recent reports, using propionaldehyde as substrate, have shown that the recombinant variant ALDH2 displayed a 260-fold increase in K_m for NAD and an 11-fold reduction in V_{max} as compared with the normal enzyme (18). The variant subunit also results in decreased activity of the tetrameric enzyme (19,20), and accelerated degradation of the enzyme in transformed cell lines (21). The X-ray structure of ALDH2 has revealed that the lysine substitution for glutamic acid-487 may affect ion pairing with arginine-475 from across the dimer interface, thereby indirectly diminishing the enzyme activity (22). Therefore, the appearance of dominance of the variant *ALDH2*2* in expression of enzyme activity on starch gel electrophoresis (23,24) can be explained by the activity of the homo- and heterotetrameric enzyme forms in the heterozygous *ALDH2*1/*2* liver samples being below the detection limit of staining of the gels.

C. Tissue Distribution

Human alcohol dehydrogenase exhibits tissue-specific expression (14,25). Class I ADHs are detected in the liver, kidney, lungs, and the mucosa of stomach and lower digestive tract. Class II ππ appears solely in the liver. Class III χχ is ubiquitously expressed. Class IV μμ displays a limited distribution to the mucosa of upper digestive tract and stomach. The class I α-, β-, and γ-ADHs and class II enzyme, as well as the class I γγ and class IV enzyme, are the major forms

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Table 1 Human ADH and ALDH Family Members Involved in Alcohol Metabolism

Enzyme	Class	Gene locus	Allelic variant	Subunit composition	K_m(coenzyme) (μM)	K_m(substrate) (μM)	V_{max} (min^{-1})
ADH	1	*ADH1*		αα	13	4200	27
		ADH2	*ADH2*1*	$\beta_1\beta_1$	7.4	49	9.2
			*ADH2*2*	$\beta_2\beta_2$	180	940	400
			*ADH2*3*	$\beta_3\beta_3$	710	36000	320
		ADH3	*ADH3*1*	$\gamma_1\gamma_1$	7.9	1000	87
			*ADH3*2*	$\gamma_2\gamma_2$	8.7	630	35
	II	*ADH4*		ππ	14	34000	20
	III	*ADH5*		χχ	25	NS	—
	IV	*ADH7*		μμ(σσ)	260	29000	1500
ALDH	I	*ALDHI*		Tetramer	3.9	33	35
	II	*ALDH2*	*ALDH2*1*	Tetramer (E_4)	42	0.20	33
			*ALDH2*2*	Tetramer (K_4)	8100	4.6	0.94

Kinetic constants were determined at pH 7.5, 25°C, except for χ-ADH at pH 10. Data for usual ALDH2 (E_4) and variant ALDH2 (K_4) were measured using the recombinant enzymes (S.-J. Yin et al., unpublished result). K_m (coenzyme), NAD; K_m (substrate), ethanol for ADH and acetaldehyde for ALDH; NS, not saturated; —, not determined. *Source*: Adapted from Refs. 6, 17.

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involved in the metabolism of ethanol in the liver and stomach, respectively (14). High-K_m ADH forms, such as the class II enzyme in liver and the class IV, and possibly class III, enzymes in stomach, are effective in contribution to the first-pass, or presystemic, metabolism of ethanol (15,16). The distribution of ALDH1 and ALDH2 appears to be ubiquitous in the human tissues, with the highest activity in liver, except that *ALDH2* is negligibly expressed in erythrocytes and the mucosa of aerodigestive tract (3,26).

III. ETHANOL METABOLISM AND ALCOHOL SENSITIVITY

A. Functional Polymorphism and Ethanol Metabolism

The pharmacological and toxicological effects of alcohol are dependent upon the duration of exposure and the concentrations of ethanol and its metabolite acetaldehyde attained in body fluids and tissue within that period. An individual's exposure to ethanol/acetaldehyde after alcohol consumption is best described by the area under the blood ethanol/acetaldehyde concentration-time curve (AUC).

The functional polymorphism of *ALDH2* gene can significantly influence AUC of the blood acetaldehyde following alcohol ingestion in East Asians (27–29). A recent report (30), in which the age, body mass index, drinking habit, and the genotypes of both *ADH2* and *ADH3* were controlled for, demonstrates that after alcohol challenge (0.2 g/kg body weight) young healthy men with homozygous *ALDH2*2* variant alleles show significantly persistent higher blood acetaldehyde concentrations than do the heterozygous and homozygous *ALDH2*1* individuals during a period of about 2 hr (Fig. 1B). The *ALDH2*1/*2* individuals also exhibit significantly higher acetaldehyde levels than the *ALDH2*1/*1* individuals. Notably, at 130 min after alcohol consumption, the *ALDH2*2* homozygotes still showed blood acetaldehyde levels (17 μM) similar to that of the peak concentration (24 μM) of the heterozygotes. The *ALDH2*1* homozygotes exhibited barely detectable peak acetaldehyde ($<$2.5 μM). The extremely small AUC of *ALDH2*1/*1* genotype (Fig. 1B) indicates that the mitochondrial ALDH2 activity in normal homozygotes is sufficient to oxidize acetaldehyde derived from ethanol via cytosolic ADH in liver. Hence virtually no buildup of blood acetaldehyde is found in normal homozygous individuals. Since heterozygotes exhibited only one-fifth of the AUC exhibited by the *ALDH2*2* homozygotes (Fig. 1B), it suggests that some of the heterotetrameric ALDH2 enzymes in heterozygote individuals have residual activity. Cytosolic ALDH1 appears to be responsible for acetaldehyde removal in both the *ALDH2*2* homozygotes and the heterozygotes, especially in the former case because of the extremely low activity of the ALDH2 enzyme in these subjects (18,19). The above explanations are primarily based on the enzymatic properties of human ALDH1 and ALDH2, which differ widely in K_m (33 μM vs. 0.20 μM, respectively) as well as in catalytic efficiency (V_{max}/K_m:

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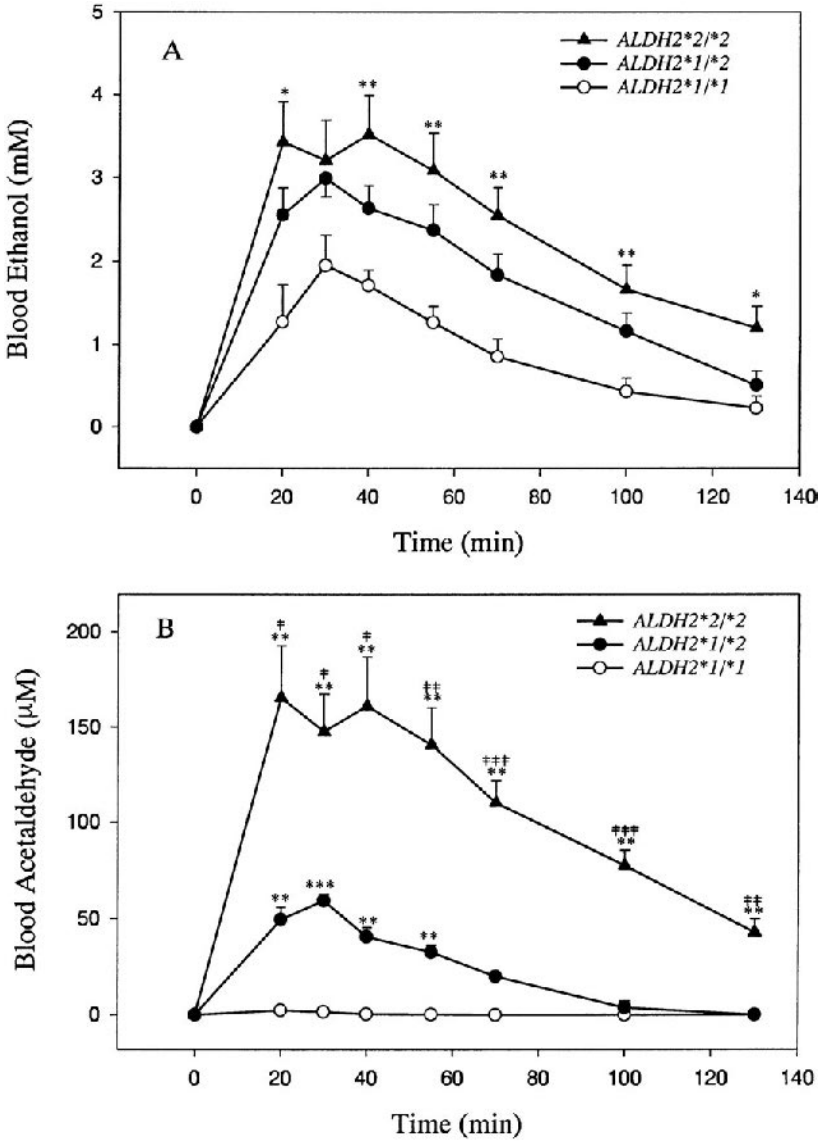

Figure 1 Blood ethanol (A) and acetaldehyde (B) concentrations after administration of an oral dose of ethanol (0.2 g/kg) to men with different *ALDH2* genotypes. All subjects were homozygous *ADH2*2* and *ADH3*1*. *$p < 0.05$ vs. *ALDH2*1/*1*; **$p < 0.01$ vs. *ALDH2*1/*1*; ***$p < 0.001$ vs. *ALDH2*1/*1*; †$p < 0.05$ vs. *ALDH2*1/*2*; ††$p < 0.01$ vs. *ALDH2*1/*2*; †††$p < 0.001$ vs. *ALDH2*1/*2*. (Adapted from Ref. 30.)

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1.1 $\mu M^{-1}min^{-1}$ vs. 170 $\mu M^{-1}min^{-1}$, respectively) for oxidation of acetaldehyde (Table 1).

Interestingly, after alcohol intake the homozygous *ALDH2*2* persons exhibited more than twofold increase of AUC of blood ethanol compared with the *ALDH2*1* homozygotes (Fig. 1A), indicating slower alcohol elimination and longer and heavier exposure in the former subjects. This is likely caused by a reduction in ADH activity due to product inhibition by acetaldehyde in the liver (14). It is worth noting that during alcohol consumption allelic variation at *ADH2* does not seem to cause significant elevation of blood acetaldehyde level, which is actually close to zero, in the homozygous *ALDH2*1* Asian subjects (28), although the high-activity β-ADH variant may significantly increase the alcohol elimination rate (31). This reflects that the hepatic ALDH2 and ALDH1 can be very efficient and sufficient to remove acetaldehyde that are formed from ingested alcohol through the ADH pathway.

B. Functional Polymorphism and Alcohol Sensitivity

Alcohol sensitivity, including facial and upper limb/trunk flushing, palpitation, nausea, vomiting, drowsiness, headache, and other discomforting symptoms (32,33), has been ascribed to the deficiency of mitochondrial ALDH2 activity (34) and high acetaldehyde concentrations in blood (35,36). The alcohol flush reaction is very similar to the aversive reaction caused by alcohol ingestion in patients being treated with ALDH2 inhibitors such as disulfiram (Antabuse) and calcium cyanamide (Temposil) (37). Cardiovascular responses are the most conspicuous physical symptoms seen in Asians showing alcohol sensitivity. It has recently been reported that with low-dose ethanol challenge (0.2 g/kg), the homozygous *ALDH2*1* persons displayed no noticeable hemodynamic effects. This is compatible with the observed low blood ethanol (<3 mM) and extremely low blood acetaldehyde (~ 1 μM) (Fig. 1). In contrast, the homozygous *ALDH2*2* persons showed the greatest cardiovascular hemodynamic effects due to the high blood acetaldehyde levels (Figs. 1 and 2). Over 100% increases in the flow rate of the facial artery, as well as of the common carotid and internal carotid arteries, were found at peak times of blood acetaldehyde in the *ALDH2*2* homozygotes. These homozygous subjects also persistently exhibited significant increases (35–70%) in heart rate and cardiac output as well as persistent decrease (20–50%) of systemic vascular resistance during a period of 130 min (30). The heterozygotes showed an intermediate hemodynamic responses between those of the *ALDH2*1* and *ALDH2*2* homozygotes (30). Interestingly, at 30 min following alcohol ingestion, the increase in heart rate and cardiac output in the heterozygotes is about 60% that seen in the *ALDH2*2* homozygotes (Fig. 2), whereas the corresponding blood acetaldehyde level is only about 30% that of the latter (Fig. 1B). This indicates cardiovascular hemodynamic responses are highly sensi-

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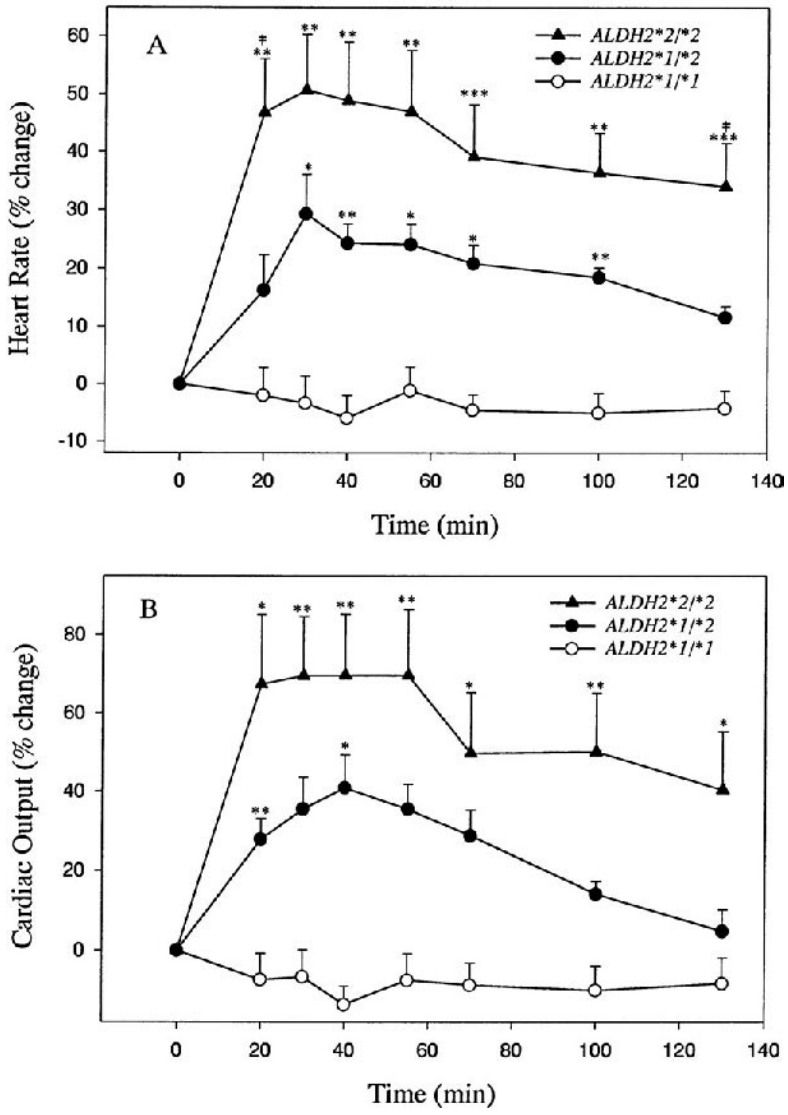

Figure 2 Alterations in heart rate (A) and cardiac output (B) in men with different *ALDH2* genotypes following an oral dose of ethanol (0.2 g/kg). All subjects were homozygous *ADH2*2* and *ADH3*1*. *$p < 0.05$ vs. *ALDH2*1/*1*; **$p < 0.01$ vs. *ALDH2*1/*1*; ***$p < 0.001$ vs. *ALDH2*1/*1*; †$p < 0.05$ vs. *ALDH2*1/*2*; ††$p < 0.01$ vs. *ALDH2*1/*2*. (Adapted from Ref. 30.)

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tive to the low acetaldehyde concentrations. Furthermore, the decline of the cardiovascular effects is slower than the disappearance of blood acetaldehyde. This is consistent with the residual acetaldehyde concentration at 130 min found in the *ALDH2*2* homozygotes that was comparable to that of the heterozygotes. It is noteworthy that elevation in heart rate as well as reduction in diastolic blood pressure can account for most of the observed alterations of cardiac output and systemic vascular resistance induced by acetaldehyde.

Plasma levels of epinephrine and norepinephrine are significantly increased in Asian persons deficient in ALDH2 activity after alcohol consumption (36). This seems to be due to acetaldehyde-induced release of catecholamines from the chromaffin cells of the adrenal medulla and from the adrenergic nerve terminals. It has been proposed that the elevation of plasma catecholamines is a compensatory reaction to the acetaldehyde-induced decreases in diastolic blood pressure and total peripheral resistance (36). The mechanisms underlying the acetaldehyde-induced vasodilation are not precisely known. Both central (neurally mediated vasodilation) and peripheral (local vasodilation due to circulating substances) mechanisms may be involved (5). Biogenic amines, adenosine, opioids, prostaglandins, or histamine could play important roles downstream of the metabolic event because they could produce or attenuate flushing without affecting the acetaldehyde formation (38,39). The endogenous vasodilator nitric oxide may be released in response to receptor-operated agonists such as bradykinin, serotonin, or histamine, among others. Further studies are required to elucidate mechanisms underlying the cardiovascular hemodynamic effects induced by acetaldehyde.

Elevation of blood acetaldehyde causes dysphoric subjective symptoms. It is especially intense for persons homozygous for *ALDH2*2* because of the persistent accumulation of high levels of acetaldehyde following challenge with a quite small amount of alcohol (30). The *ALDH2*2* homozygotes self-reported significant perceptions of palpitation, facial warming, effect of alcohol, and overall terrible feelings throughout 2 hr following ingestion. This is compatible with the pharmacokinetic and cardiovascular findings from the same persons (Figs. 1 and 2). Interestingly, the heterozygotes, who regularly consume small amounts of alcohol, may have a more intense, although not necessarily a more negative, subjective feeling response to alcohol than the *ALDH2*1* homozygotes (40). This suggests that there may exist a complex interaction between the alcohol rewarding system and acetaldehyde in the brain. The neurochemical mechanism by which elevated acetaldehyde discourages excessive drinking remains unclear.

Alcohol-flushing reaction was found in approximately 50% female and 8% male British subjects but of much shorter duration and intensity than that of Asian alcohol-induced flushers (41). Twenty-two percent of the Native American subjects self-reported that they almost always or always flushed after one or two drinks (42). Genotype and pharmacokinetic data indicate that the non-Asian alco-

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hol flush reaction is not associated with high circulating concentrations of acetaldehyde (41,42). Although low ALDH1 activity in erythrocyte was found in the Caucasian flushers (41), the exact mechanism of the reduced ALDH1 activity and the biochemical basis for the flushing response in the homozygous *ALDH2*1* persons are presently unknown (43). The alcohol sensitivity reaction and its influence on alcohol use and alcoholism among Caucasian populations will be reviewed by J. B. Whitfield (this volume).

IV. DRINKING BEHAVIOR AND ALCOHOLISM

A. Functional Polymorphism and Drinking Behavior

Alcohol-drinking behavior can be affected by rewarding or aversive effects of alcohol/acetaldehyde on the brain and the body. The allelic variations of *ALDH2* significantly influence drinking pattern in normal individuals. It is well documented that both drinking frequency and the amount of consumption per occasion are significantly decreased in the following order: *ALDH2*1/*1* > *ALDH2*1/*2* > *ALDH2*2/*2* among Japanese (44–48) and Han Chinese (49). Similar findings are reported for East Asians born and living in North America (50), suggesting that *ALDH2* polymorphism is a significant biological factor in determining drinking behavior in spite of different sociocultural backgrounds. It is noteworthy that homozygous *ALDH2*2* persons consume strikingly less alcohol than do *ALDH2*1* homozygotes and the heterozygotes regardless of gender and ethnicity (44,46,49). In fact, no single *ALDH2*2* homozygote was found having binge drinking behavior (48). This is in agreement with the potent aversive symptoms of alcohol sensitivity observed in persons carrying homozygous *ALDH2*2* alleles (30,40,45). The functional polymorphism of *ADH2*, however, does not seem to exert a significant effect on drinking behavior in normal Han Chinese and Japanese (46,49,51), although *ADH2*2* allele appeared to be associated with alcohol flushing in the Japanese (51).

B. Functional Polymorphism and Alcoholism

Alcohol metabolic genes can influence susceptibility to the development of alcoholism. It has been documented that the allele frequencies of *ADH2*2*, *ADH3*1*, and *ALDH2*2* are significantly decreased in alcoholics as compared with the general population of East Asians, including the ethnic Han Chinese (52,53), Koreans (54), and Japanese (55–61). Association studies of functional polymorphism involving the other alcohol-metabolizing enzymes, such as cytochrome P450 2E1 (*CYP2E1*), with alcoholism have thus far been negative (58,59,61–63). Therefore, the previous molecular epidemiological evidence appeared to be in support of the long-standing hypothesis that the *ADH2*2* and *ADH3*1* alleles,

which encode the high-activity β_2 and γ_1 subunits, respectively, and the *ALDH2*2* allele, which encodes the inactive subunit K, protect individuals from developing alcoholism through either faster production or slower removal of acetaldehyde, a metabolite that triggers aversive reactions (52,64,65). The hypothesis also implies that the three alcohol metabolic genes may act synergistically in ethanol metabolism to produce more acetaldehyde and hence more protection. The latter implication is a complex issue that involves potential interaction among the *ADH2*, *ADH3*, and *ALDH2* genes that is further complicated by the existence of dominant nature of the *ALDH2*2* variant allele in protection against alcoholism.

1. Linkage of ADH2 and ADH3

Involvement of the functional polymorphism at the *ADH3* locus in susceptibility to alcoholism has been an intriguing and controversial subject. In theory, Caucasian subjects would be best for testing this hypothesis; the power to detect differences between genotypes is greater in this racial group, because of nearly equal distribution of *ADH3*1* and *ADH3*2*, nearly homogenous distribution of *ADH2*1*, and the absence of confounding effects by *ALDH2*2*. Results of studies of association between the *ADH3* allelic variations and alcoholism in various European populations have so far been negative (66–68). This implies the effect of *ADH3* polymorphism on propensity to alcoholism is neutral or very small. However, a positive association when *ALDH2* genotype is controlled for has been consistently found among East Asians, including the Han Chinese (52,53) and Japanese (60). These contradictory findings have been clarified by the multiple logistic regression analysis of *ADH3*, *ADH2*, and *ALDH2* in the recent study of a large number of alcoholics and controls ($n = 885$) of Han Chinese descent in Taiwan (69). As the *ADH2* genotype is adjusted, allelic variations at *ADH3* did not exhibit a significant effect on risk of alcoholism, irrespective of which model of *ALDH2* dominance was applied. This is in agreement with the negative finding among Caucasian alcoholics (66–68). Nullification of the influence of *ADH3* on the susceptibility of East Asians to alcoholism can be fully ascribed to the existence of linkage disequilibrium between *ADH3* and *ADH2* (69,70). The observed significant reduction in the frequency of *ADH3*1* in alcoholics as compared to controls is caused by its linkage to *ADH2*2*. Therefore, the *ADH2* allelic variation is sufficient to explain the different levels of susceptibility to alcoholism.

In view of the close vicinity of the *ADH2* and *ADH3* loci, which are only 15 kb apart on the long arm of chromosome 4 (10), it is reasonable to see linkage disequilibrium between the functional and neutral polymorphisms of the two loci among various populations (69–71). Possible explanations for the lack of influence of *ADH3* on susceptibility to alcoholism include: (a) a much smaller difference in V_{max} values for ethanol oxidation of the $\gamma\gamma$ allozymes compared with $\beta\beta$ allozymes (6,8), (b) low expression of the γ subunits in liver ($\sim$20% that of the

β subunits in terms of protein content; S.-J. Yin et al., unpublished data), and (c) large interindividual variation in alcohol elimination (72).

2. Dominance of ALDH2*2

Strikingly, a recent survey demonstrated that none of 1300 Japanese alcoholics studied were homozygous for *ALDH2*2* (56). On the basis of genotype frequencies in the Japanese, it is expected 118 of these alcoholic individuals would be *ALDH2*2* homozygotes. In fact, no single alcoholic patient who carries the *ALDH2*2/*2* genotype was found in the earlier East Asian studies (52–61,69). The seemingly full protection by the homozygosity of *ALDH2*2* can be attributed to a total loss of the ALDH2 activity, resulting from two copies of the missense mutation, which causes the subjects either to abstain or deliberately moderate alcohol consumption because of prior experience of an unpleasant reaction following drinking (30).

The *ALDH2*1/*2* heterozygosity displays partial protection as it was found in only 2.5–18% of the Han Chinese and Japanese alcoholics (cf. ~40% in controls) (52,56,69). Interestingly, the frequency of the heterozygotes in the Han Chinese alcoholic population appeared to be rising, i.e., 10% up to 18%, in the period between 1989 and 1997 (69). A similar observation was noticed among the Japanese alcoholics, 2.5% up to 13% between 1979 and 1992 (56). These findings indicate that among *ALDH2*1/*2* alcoholics, the other biological determinants, such as functional polymorphism of the *ADH* genes, as well as sociocultural factors, are contributing increasingly to development of the disease (19–21). It is worth noting that no significant difference was found in the sociofamilial backgrounds and comorbid psychiatric disorders between the *ALDH2*1/*2* and *ALDH2*1/*1* Japanese alcoholics (73). The heterozygosity of *ALDH2*1/*2* can explain at least part of the reason why, in combination with the other possible biological and environmental factors, Han Chinese (among whom the allele frequency of *ALDH2*2* is 0.24) living in Taiwan exhibited an ~eightfold lower lifetime prevalence of alcohol dependence than did the Atayal natives (among whom the allele frequency of *ALDH2*2* is 0.05) (74,75).

Both complete dominance (23,24) and partial dominance (19–21) of the variant ALDH2 K subunits over activity loss of the tetrameric enzymes have been described. Surgical liver samples with the *ALDH2*1*2* genotype exhibited ~20% of the specific activity in the *ALDH2*1/*1* liver samples, as measured with 3 μM acetaldehyde, whereas the activity in the *ALDH2*2/*2* livers was undetectable (S.-J. Yin et al., unpublished data). These levels of specific activity are close to those predicted from a model study using transduced cell lines (21). The partial-dominance model has been substantiated by the strikingly different blood acetaldehyde profiles found in subjects with the different *ALDH2* allelo-

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types, but carrying the identical *ADH2* and *ADH3* genotypes, following a low dose of alcohol (Fig. 1). It seems clear that the mitochondrial ALDH2 and the cytosolic ALDH1 are mainly responsible for oxidation of acetaldehyde in the homozygous *ALDH2*1/*1* and *ALDH2*2/*2* individuals, respectively, and that the residual ALDH2 activity, plus that of ALDH1, contributes to removal of acetaldehyde in the heterozygotes during alcohol consumption.

Surprisingly, a single unique case of an alcoholic who was identified to be *ALDH2*2/*2* out of the survey of 420 Han Chinese alcoholics has recently been documented (76). This suggests that the protection against the development of alcoholism by homozygosity of the *ALDH2*2* is indeed powerful, but may not be as complete as previously thought (56,59). To accommodate this inborn impairment of acetaldehyde metabolism, this patient had adopted a drinking pattern characterized by slow and prolonged consumption of alcohol, but low in total overall quantity. The unique drinking pattern of the homozygous *ALDH2*2* patient consists of (a) beer, instead of wine or spirit that contains high alcohol content as a favorite beverage type; (b) sipping of the beverage almost continuously throughout the day rather than fast, binge drinking; (c) consuming a relatively low amount of alcohol with 3–5 bottles (i.e., 350 ml of 4.5% by volume of ethanol or 12.4 g ethanol per bottle) of beer per day (76). Even though the patient satisfied diagnostic criteria for alcohol dependence, he never became heavily intoxicated. Interestingly, a pattern of low-quantity consumption of alcohol has also been found in normal individuals with the *ALDH2*2/*2* genotype (44,46,48,49). It has been proposed that physiological tolerance or innate insensitivity to acetaldehyde may be crucial in developing alcoholism among *ALDH2*2* homozygotes (76).

3. Functional Polymorphism of ADH2

Contrary to the well-received notion that *ADH2*2* and *ALDH2*2* may act synergistically to protect against development of alcoholism by means of producing more acetaldehyde during alcohol metabolism, recent studies find that the functional polymorphisms at these two loci independently influenced susceptibility to the disease (69). This is evidenced by the near-identical distributions of the genotype and allele frequencies of *ADH2* in the alcoholics with stratification of the genotype *ALDH2*1/*1* and *ALDH2*1/*2* (Table 2). Results of the multiple logistic regression analysis further demonstrate that there are no significant interactions between *ADH2* and *ALDH2* in risk for alcoholism (69; Y.-C, Chen and S.-J. Yin., unpublished data).

Independent effects of the functional polymorphisms of *ADH2* and *ALDH2* on alcoholism would imply that the molecular protection mechanism of *ADH2* may not be mainly through the pathway of blood acetaldehyde accumulation after

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Table 2 Genotype and Allele Distribution of *ADH2* with Stratification of *ALDH2* Genotypes in Asian Alcoholics

ALDH2 genotype and group	Subject number	*ADH2* Genotype number (Frequency)				*ADH2* Allele number (Frequency)		
		*1/*1	*1/*2	*2/*2	*p* value	*1	*2	*p* value
*ALDH2*1/*1*								
Controls[a]	383	34 (0.09)	136 (0.36)	213 (0.56)		204 (0.27)	562 (0.73)	
Alcoholics[b]	341	123 (0.36)	108 (0.32)	110 (0.32)	$<10^{-6}$	354 (0.52)	328 (0.48)	$<10^{-6}$
*ALDH2*1/*2*								
Controls[a]	274	20 (0.07)	106 (0.39)	148 (0.54)		146 (0.27)	402 (0.73)	
Alcoholics[b]	78	29 (0.37)	22 (0.28)	27 (0.35)	$<10^{-6}$	80 (0.51)	76 (0.49)	$<10^{-6}$
*ALDH2*2/*2*								
Controls[a]	32	1 (0.03)	10 (0.31)	21 (0.66)		12 (0.19)	52 (0.81)	
Alcoholics	1	0	0	1 (1.00)		0	2 (1.00)	

Han Chinese alcoholics, $n = 420$; controls, $n = 689$. From Y.-C. Chen and S.-J. Yin (unpublished results).

[a] No significant difference was found in the distribution of the genotype number ($p = 0.57$) and allele number ($p = 0.38$) of *ADH2* among controls with different *ALDH2* genotypes.

[b] No significant difference was found in the distribution of the genotype number ($p = 0.83$) and allele number ($p = 0.95$) of *ADH2* between alcoholics with *ALDH2*1/*1* and *ALDH2*1/*2* genotypes.

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alcohol ingestion, as has been firmly established with *ALDH2* (30,35,36). Indeed, during alcohol consumption allelic variations at *ADH2* did not cause significant elevation of blood acetaldehyde levels, which were actually near zero, in the homozygous *ALDH2*1/*1* Japanese (28) and Han Chinese (S.-J. Yin et al., unpublished data). The rates of elimination from blood at saturating ethanol concentrations for class I ADHs also did not show a significant difference among the three *ADH2* genotypes (28). The alcohol-induced facial flushing appeared to be associated solely with the *ALDH2* polymorphism, and not with *ADH2*, following a low dose of 0.3 g/kg ethanol (S.-J. Yin et al., unpublished data). Therefore, the recent new findings reviewed herein seem not to be supportive of the long-standing hypothesis that *ADH2*2*, which encodes the high-activity β_2 subunits, produces facial flushing (64) and other dysphoric reactions through the accumulation of acetaldehyde in blood, thereby influencing drinking behavior (52).

Association between reduced alcohol consumption or reduced risk for alcoholism and the variant *ADH2*2* allele has recently been found in other ethnic groups that predominantly carry *ALDH2*1/*1*, including Europeans (68,77), the Jews in Israel (78), the Mongolians in China (54), and Atayal natives of Taiwan (75). This is consistent with the recent findings that *ADH2* may affect vulnerability to alcoholism independent of *ALDH2* (69). The molecular mechanism of the *ADH2* effect remains unclear. There are a few possible explanations. (a) Target organs of class I ADH other than the liver may be involved, such as the brain (79) and the heart, in which only ββ allozymes are expressed (6). (b) Target substrates of class I ADH may be unrelated to the conventional ethanol/acetaldehyde, for instance, alcohol/aldehyde metabolites of the neurotransmitter dopamine (80), serotonin (81,82), and norepinephrine (83). The ββ allozymes may display strikingly different kinetic properties with metabolites of the biogenic amines. (c) Potential functional polymorphism of the high-K_m class II ππ ADH may interact with *ADH2* to influence ethanol metabolism in the liver (84) and/or biogenic amine metabolism in the brain (81,82,85), although functional polymorphism of ππ (86), as well as its regional localization in the brain, remains to be established. (d) Other candidate genes for alcoholism and *ADH2* may have functional interaction because of the presence of allelic variations, like the tryptophan hydroxylase gene (87).

Recently, genome-wide surveys of the families of alcoholic probands have provided evidence suggestive of a protective locus on chromosome 4, affecting the risk for alcohol dependence, which includes the *ADH* gene cluster in both white and American Indian populations (88,89). Association between *ADH2*3* and alcoholism in black populations has not been reported, although association between *ADH2*2* and alcoholism in other racial groups has. Further studies of various ethnic groups are needed to elucidate the underlying mechanisms by which the allelic variations at *ADH2* affect predisposition to alcoholism.

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4. Combinatorial Genotype of ADH2 and ALDH2

Interactions between the functional polymorphisms of *ADH2* and *ALDH2* have recently been evaluated by logistic regression of the six combinatorial genotypes between 420 Han Chinese alcoholics and 689 controls (76). The relative risk for alcoholism (odds ratio) for the combinatorial genotypes are in following order: *ADH2*1/*1-ALDH2*1/*2* > *ADH2*1/*2-ALDH2*1/*1* > *ADH2*2/*2-ALDH2*1/*1* > *ADH2*1/*2-ALDH2*1/*2* > *ADH2*2/*2-ALDH2*1/*2* > *ADH2*2/*2-ALDH2*2/*2* when employing *ADH2*1/*1-ALDH2*1/*1* as the reference group (Table 3). The *ADH2*1/*1-ALDH2*1/*2* individuals had an odds ratio of 1.83 (95% confidence interval 0.98–3.41) for alcoholism compared with those of the *ADH2*1/*2-ALDH2*1/*1* at a marginal significance level ($p = 0.06$). It is interesting to note that the risk for alcoholism in the *ADH2*2/*2-ALDH2*2/*2* individuals is 100-fold lower than that in the *ADH2*1/*1-ALDH2*1/*1* individuals. The combinatorial genotype of the latter is predominant among Caucasian populations (4,10). East Asians homozygous for both *ADH2*2* and *ALDH2*2* appear to be at the least risk for developing alcoholism. Interestingly, *ADH2*1/*1-ALDH2*1/*2* individuals may have 1.83-fold greater risk than do *ADH2*1/*2-ALDH2*1/*1* individuals. Therefore, the current findings suggest that a single copy of *ALDH2*2* allele may protect less strongly than

Table 3 Logistic Regression Analysis of Combinatorial Genotypes of *ADH2* and *ALDH2* for Risk of Alcoholism

Reference group	Variable	Odds ratio	95% Confidence interval	*p* value
*ADH2*1/*1–ALDH2*1/*1*	*ADH2*2/*2-ALDH2*2/*2*	0.01	0.002–0.10	$<10^{-4}$
	*ADH2*2/*2-ALDH2*1/*2*	0.05	0.03–0.09	$<10^{-6}$
	*ADH2*1/*2-ALDH2*1/*2*	0.06	0.03–0.10	$<10^{-6}$
	*ADH2*2/*2-ALDH2*1/*1*	0.14	0.09–0.22	$<10^{-6}$
	*ADH2*1/*2-ALDH2*1/*1*	0.22	0.14–0.34	$<10^{-6}$
	*ADH2*1/*1-ALDH2*1/*2*	0.40	0.20–0.79	0.008
*ADH2*1/*2–ALDH2*1/*1*	*ADH2*2/*2-ALDH2*2/*2*	0.06	0.008–0.45	0.006
	*ADH2*2/*2-ALDH2*1/*2*	0.23	0.14–0.38	$<10^{-6}$
	*ADH2*1/*2-ALDH2*1/*2*	0.26	0.16–0.44	$<10^{-6}$
	*ADH2*2/*2-ALDH2*1/*1*	0.64	0.46–0.90	0.01
	*ADH2*1/*1-ALDH2*1/*2*	1.83	0.98–3.41	0.06
	*ADH2*1/*1-ALDH2*1/*1*	4.60	2.92–7.26	$<10^{-6}$

Han Chinese alcoholics, $n = 420$; controls, $n = 689$. No single individual with the combinatorial genotypes of *ADH2*1/*1-ALDH2*2/*2* or *ADH2*1/*2-ALDH2*2/*2* was found in alcoholic group. *Source*: Adapted from Ref. 76.

a single copy of *ADH2*2* in individuals carrying only one copy of either *ADH2*2* or *ALDH2*2* (76). Very large numbers of alcoholics and the corresponding controls are required to substantiate this conclusion.

V. ALCOHOL-INDUCED END-ORGAN DAMAGE

Excessive consumption of alcohol causes a variety of medical complications (90). Primary target organs and tissues of alcohol-induced damage include liver, gastrointestinal tract, pancreas, brain, peripheral nerve, heart, skeletal muscle, testes, blood cells, and skin. Functional polymorphisms of the *ADH2* and *ALDH2* genes have been reported to be in association with certain types of alcohol-induced organ injury.

The frequencies of *ADH2*2* and *ALDH2*2* alleles were significantly decreased in the Han Chinese and Japanese liver cirrhotic patients with heavy drinking, whereas the distribution was not different from that of the alcoholics without severe liver disease (91,92). This would imply that highly reactive, toxic acetaldehyde may not be a major causative agent in the development of alcoholic liver disease as previously thought (90). Significant rise in blood acetaldehyde levels have been described in the *ALDH2* heterozygotes following alcohol consumption (27–30).

The *ADH2*2* allele, not the *ALDH2*2*, appeared to be associated with alcoholic pancreatitis in the Japanese and Han Chinese (93,94). *ADH2*1*, the usual allele, has recently been associated with susceptibility to alcoholic brain atrophy in Japanese (95) and to alcohol-related birth defects in African Americans (96). The *ALDH2*1* has been proposed to link to alcohol-related gout and hyperuricemia in Japanese (97). The *ALDH2*2* appeared to be associated with alcohol-induced asthma in Japanese (98) and also to be a risk factor for esophageal cancer in Japanese alcoholics (99). The homozygous *ADH3*1/*1* genotype, in combination with alcohol consumption, appeared to increase the risk for breast cancer in American white women (100). Acetaldehyde, a potential carcinogen, as well as disturbance of the homeostasis of retinoic acid by ethanol, a ligand for the transcription factors responsible for cellular differentiation, through the ADH/ALDH pathway, have been implicated in the pathogenesis of the alcohol-related cancer and fetal alcohol defect (16,90,96).

It should be stressed that most of the previous studies of the association between functional polymorphisms of the *ADH/ALDH* genes and alcohol-induced organ damage remain unconclusive or controversial. To answer these questions, it will certainly require a sufficiently large number of subject samples to avoid the possible type I or type II error in statistical analysis, and will require necessary controls of the disease group, i.e., heavy drinkers without organ damage as well as patients with organ damage but without heavy drinking, who will

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match for age, gender, and the drinking patterns in frequency, amount, and duration. Possible interactions among ethanol and the other causative agents/factors involved in development of the disease should also be taken into account.

VI. SUMMARY AND FUTURE PERSPECTIVES

The functional polymorphisms at the *ADH2*, *ADH3*, and *ALDH2* gene loci exhibit a complex pattern of influences on susceptibility to alcoholism in East Asians. The observed differences in the frequency of *ADH3*1* between alcoholics and controls can be accounted for by the linkage disequilibrium with *ADH2*2*, indicating that the *ADH3* polymorphism contributes negligibly to the vulnerability of alcoholism. The *ALDH2*2* variant alleles protect against development of alcoholism in a partially dominant fashion. Gene status of the *ALDH2*2* homozygosity can greatly but not completely, as thought previously, protect against alcoholism. Individuals carrying the combinatorial genotype of *ADH2*2/*2-ALDH2*2/*2* appear to be at the least risk for alcoholism, i.e., 100-fold lower than that in the *ADH2*1/*1-ALDH2*1/*1* individuals. The *ADH2*1/*1-ALDH2*1/*2* individuals with homozygosity at the *ADH* gene and heterozygosity at the *ALDH* gene may have 1.8-fold greater risk than do the *ADH2*1/*2-ALDH2*1/*1* individuals, suggesting that a single copy of *ALDH2*2* allele may protect less strongly than a single copy of *ADH2*2* in individuals carrying only one copy of either *ADH2*2* or *ALDH2*2*. The functional polymorphisms at *ADH2* and *ALDH2* appear to affect independently the susceptibility to alcoholism.

Studies of direct correlation of physiological and psychological responses after challenge with a low dose of alcohol in healthy men with the different *ALDH2* genotypes support the theory that elevated blood acetaldehyde causes persistent discomfort in the homozygous *ALDH2*2* individuals. Therefore, nearly full protection against alcoholism in these individuals will derive from either abstinence or deliberate moderation in alcohol consumption due to prior experience with an unpleasant reaction. The pharmacokinetic parameters of blood ethanol and acetaldehyde appear not to be significantly different between alcoholics and controls with stratification of the *ALDH2* genotypes. Thus current evidence suggests that physiological tolerance or innate insensitivity to the accumulation of blood acetaldehyde following alcohol ingestion may be crucial for development of alcoholism in individuals carrying the *ALDH2*2* variant alleles. Association of functional polymorphisms of the *ADH2* and/or *ALDH2* with certain types of alcohol-induced organ damage may exist but still need to be established.

To date, *ADH* and *ALDH* are the only so-called alcoholism genes that have been firmly established to influence vulnerability to the disease. One of the major reasons is that both the genotypes and phenotypes of allelic variations at these loci have been well defined. Complex interrelationships between functional poly-

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morphisms of the alcohol metabolic genes partly illustrate the current concept that alcoholism is a complex behavioral trait that is influenced by multiple genes as well as by sociocultural factors. Several intriguing questions remain to be answered. (a) The protection against developing alcoholism afforded by the *ADH2*2* allele appeared to be independent of that afforded by *ALDH2*2* (69), a finding that requires further studies to define the underlying biochemical mechanism. (b) Potential common functional polymorphisms of the alcohol metabolic genes, for instance, SNPs at the promoter region of *ADH4* (86) and *ALDH2* (101,102), and their possible influences on the ethanol metabolism and alcoholism, particularly in the context of interactions with the other polymorphic *ADH/ALDH* genes, will be an important direction in the future research. (c) Since the *ALDH2*2* alleles showed partial dominance in protection against alcoholism (56,76), the heterozygous individuals will be an interesting study group for illustrating, apart from the influence of sociocultural factors, interactions between the genes that are predominantly expressed in the liver and are involved in ethanol metabolism, and the other candidate genes that may be predominantly expressed in the brain and are involved in the rewarding, craving, tolerance, or withdrawal effects during the development of alcoholism.

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2

Genes for Alcohol Metabolism and Alcohol Sensitivity

Their Role in the Genetics of Alcohol Dependence

John B. Whitfield
Royal Prince Alfred Hospital, Sydney, Australia

I. BACKGROUND

As a result of multiple family, adoption, and twin studies over the past 20–30 years, it is generally accepted that there is an inherited component to the risk of alcohol dependence, and that this is due to the additive or interactive effects of multiple genes (1–6). The challenge is to identify these genes, to determine the magnitude of the risk that each variant confers, and to understand the mechanisms by which they exert their effects.

The building blocks for understanding the genetics of alcohol dependence are illustrated in Figure 1. The phenotype of alcohol dependence is determined by contributions from both environmental and genetic sources. In the genetic area, we need to understand how the effects of individual polymorphisms in genes, and the differing risks associated with allelic variants, contribute to the observed heritability.

In searching for genes affecting a complex condition such as alcohol dependence, one can start from physiological/biochemical processes known to affect risk (intermediate phenotypes) and study variations in genes that are likely to be relevant to them; or one can start without preconceptions by conducting a genome-wide search for genes that affect alcohol dependence risk and thereby discover novel and relevant metabolic or neurochemical systems. Each approach has its place, although the second requires a much larger investment in recruiting

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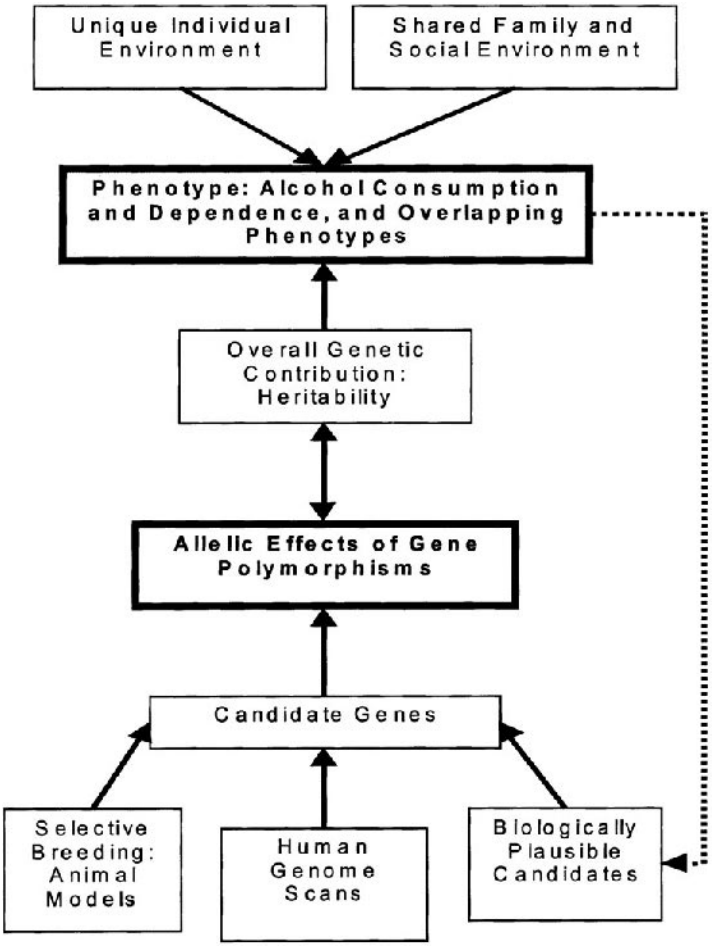

Figure 1 Sources of variation in alcohol dependence risk, and routes to understanding of the contributions of individual genes to the overall genetic component of risk. (Top) Partition of phenotypic variance into genetic and environmental sources, usually by twin studies. (Bottom) Ways in which genes affecting the phenotype may be located and characterized. Knowledge of the phenotype and its biochemistry or pathophysiology will often suggest candidate genes. Overall, the fullest picture is obtained when the allelic effects of multiple genes are measured in family or twin studies, so the heritability can be compared against individual genes' effects.

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large numbers of related individuals; and each can be applied in both human and animal studies. Ultimately an integration of knowledge from both will be needed.

Genes that affect alcohol dependence risk may be considered in two classes: those that produce some measurable difference between low-risk and high-risk people in the absence of alcohol, and those whose effects are apparent only after consumption of alcohol. Examples of the first class (Fig. 2, left) may include genes that affect personality characteristics, such as novelty seeking and components of the Eysenck Personality Questionnaire (6); behavior problems such as conduct disorder (7); or addiction to other drugs including nicotine (8). The existence of comorbid psychiatric conditions in many patients with alcohol depen-

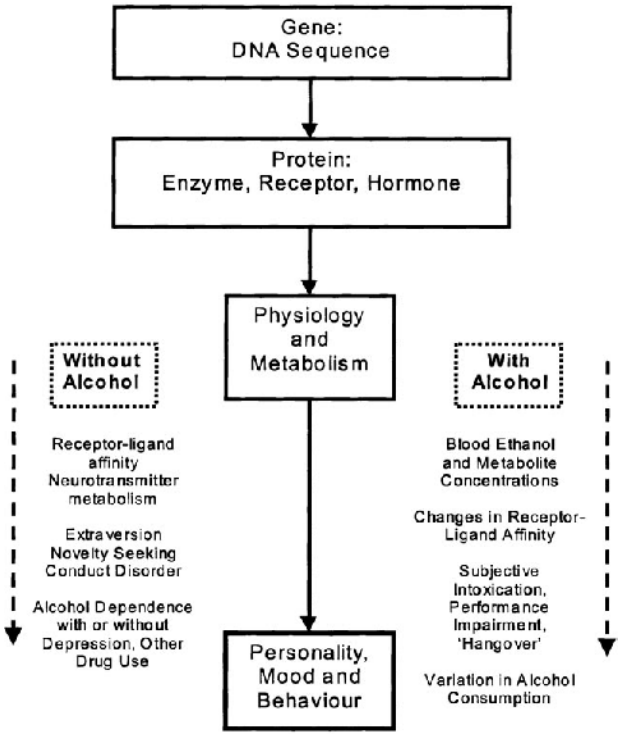

Figure 2 Behavior is influenced by genes through proteins and through the metabolic or physiological processes that the enzymes or other proteins control. Genes that contribute to differences in alcohol dependence risk may do so by affecting personality or psychopathology (left), or by affecting alcohol metabolism or susceptibility to alcohol's effects (right). Together, these classes of genes determine genetic risk of alcohol dependence and associated conditions.

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dence (9) also suggests that an element of common psychiatric vulnerability—possibly with a genetic component—may exist. However, this class of genes, which are so far uncharacterized, is not considered in this chapter.

The second class (Fig. 2, right) is inherently more time-consuming and expensive to study, because observations made under controlled conditions before and after a standardized dose of alcohol are required. The alcohol-related group of risk factors may be affected by genes whose products bring about the metabolism of ethanol, or determine the degree of intoxication. These phenotypes and genes are the subject of this chapter.

This survey of progress up to the end of 1999 will cover four areas. Two relate phenotype to risk, and two relate genotype to the intermediate phenotypes and thence to dependence risk: (1) alcohol sensitivity and dependence risk; (2) alcohol metabolism and dependence risk; (3) genetic polymorphisms in alcohol sensitivity genes, and their effects on dependence risk; and (4) genetic polymorphisms in alcohol metabolism genes, and their effects on dependence risk.

II. ALCOHOL SENSITIVITY AND DEPENDENCE RISK

The concept of an inverse relationship between sensitivity to the effects of alcohol and the amount of alcohol consumed has intuitive appeal and has been supported by evidence from three areas:

1. The physiological sensitivity to alcohol manifested through facial flushing and other vascular symptoms, mainly seen in Asians but also in some people from other racial groups;
2. Studies of the relationship between psychomotor and subjective alcohol sensitivity, family history of alcohol dependence, and development of dependence in humans; and
3. Selective breeding of animals for alcohol consumption or alcohol sensitivity, and genetic analysis of the resulting inbred strains.

Sensitivity to alcohol may be defined in a number of ways. Many psychomotor tests such as reaction time or body sway are altered by alcohol and people vary in the degree of impairment. Subjectively, people can be asked to assess their overall degree of intoxication (although there are difficulties in comparing responses between individuals) or they can assess a number of separate aspects of intoxication such as euphoria or perceived loss of coordination. Physiological responses to alcohol can be assessed from changes in blood pressure or skin temperature, or in superficial blood flow in the skin using Doppler ultrasound. Hormonal or metabolic responses to acute alcohol intoxication, such as the function of the hypothalamic/pituitary/adrenal (HPA) axis, may be measured by tak-

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ing blood or, possibly, saliva samples. All of these have been used in past studies of susceptibility to alcohol's acute effects in humans.

Before going on to consider human intoxication, and the consequences of variation in susceptibility to intoxication, a number of difficulties should be mentioned. One of these is the phenomenon of tolerance; people who consume comparatively large amounts of alcohol become less sensitive to its effects, and this tolerance can be reversed by abstinence. It is therefore a consequence of alcohol use, rather than a cause, but cause and effect can be difficult to resolve. If people who are insensitive to alcohol's effects are later found to be more likely to develop alcohol dependence, one cannot be certain that the insensitivity leads to dependence unless the subjects are studied before excessive drinking has commenced. Some aspects of causality can be addressed if the study design includes monozygotic twin pairs discordant for the proposed cause—in this case, pairs discordant for excessive alcohol consumption would allow us to decide whether insensitivity to alcohol is caused by alcohol consumption, or is an innate feature of the subjects' genetic makeup.

Second, large numbers of subjects are needed for a prospective population-based study, because only around 20% of men and 5% of for women will become alcohol dependent by commonly accepted criteria. As always, the size of the study (and the proportion of affected subjects) will determine the power to detect risk factors; there may well be several independent risk factors with small but additive effects. For this reason some investigators have chosen to use a combination of high- and low-risk groups such as family-history-positive and (FHP) family-history-negative (FHN) subjects. Others have studied unselected groups and relied on the natural frequency of alcohol dependence (or other conditions of interest) in the population.

A. Alcohol-Induced Flushing

The alcohol flush reaction in Asians is well characterized. The molecular basis is a mutation in mitochondrial aldehyde dehydrogenase (*ALDH2*), which leads to low enzymatic activity in both the homozygous and heterozygous states (10) and high acetaldehyde levels during alcohol metabolism. It has been shown in many studies to reduce the risk of alcohol dependence (e.g., 11,12). The reactions to alcohol that occur in Europeans are generally less severe and are probably heterogeneous in their causes (13), but they can decrease alcohol use and seem to have the paradoxical effect of increasing dependence risk (14).

B. Sensitivity to Intoxication: San Diego, 1978 Onward

A series of studies on the effects of alcohol in young adult men with or without a family history of alcoholism was commenced by Schuckit and colleagues in

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the 1970s. Initially, groups of around 30 FHP subjects and equal numbers of FHN controls were tested with two different doses of alcohol, or placebo, and a wide range of metabolic, endocrine, psychomotor, and subjective responses were investigated. A number of significant differences between the groups were found, and a discriminant function was constructed (15) that had reasonable success in distinguishing the groups (see Fig. 3). The items that made up the discriminant function were the maximum self-rated ''terrible feeling,'' the maximum and 210-min plasma cortisol after 1.1 ml/kg of 95% ethanol (0.87 g/kg), and the maximum plasma prolactin after 0.75 ml/kg (0.59 g/kg).

The number of subjects tested was later increased to 453 and these have been followed up at intervals to assess the impact of family history and alcohol sensitivity on the development of alcohol dependence. The success of the follow-up at 8 years was a remarkable 100%, but variation in the testing protocol over the period 1978–1988, death of some subjects, and questions about paternity or the familial alcoholism status in others reduced the number for inclusion in the analysis of results to 335 (16). For the follow-up study, the sensitivity to alcohol (level of response) was defined slightly differently, being based on the prealcohol to 1-hour post 0.75 ml/kg change in subjective scores, body sway, and plasma cortisol. Family history had significant effects on maximum quantity (but not

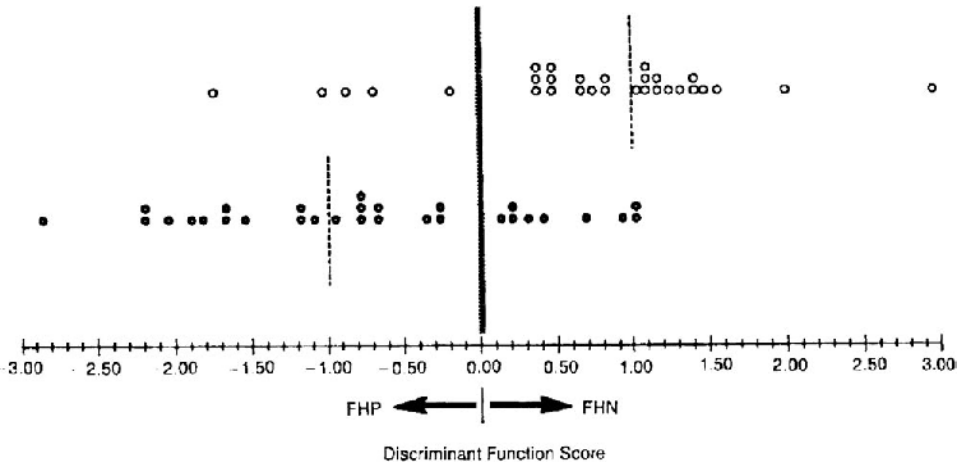

Figure 3 Discriminant function scores, based on subjective feelings and cortisol and prolactin results, for 30 family-history-positive (FHP) and 30 family-history-negative (FHN) male subjects. Although the groups of FHP and FHN subjects show different means, classification of individual subjects with this discriminant function is only partially achieved. (Reproduced with permission: From Schuckit and Gold, Archives of General Psychiatry 1988; 45:211–316. Copyrighted 1988, American Medical Association.)

frequency) of alcohol use, and on abuse and dependence on alcohol but not on abuse/dependence on cannabinoids or stimulants. This is in accordance with expectation, showing familial transmission of alcohol-specific dependence risk. Data from the full cohort of subjects suggested that both family history and the level of response to alcohol had independent effects on alcohol dependence risk. Restriction of the analysis to subjects with extremely high or low levels of response suggested that the effect of family history was mediated by differences in the level of response.

Studies in these subjects are continuing with the aims of achieving 20-year follow-up, recruitment of children of the original subjects, and progressive inclusion of genotyping for candidate genes in the evolution of the project.

C. Sensitivity to Intoxication: Australia, 1979 Onward

The second large study of sensitivity to intoxication commenced in 1979 with the work of Martin and colleagues in Canberra and Sydney, Australia. They tested pairs of twins with alcohol to determine the relative importance of genetic and nongenetic factors as causes of variation in alcohol pharmacokinetics and in alcohol's effects. Results on alcohol metabolism are discussed below. There were substantial genetic effects on intoxication (17), shown as genetic effects on test performance that were found only in the presence of alcohol. Initial analysis (18) of the relationship between alcohol consumption at the time of testing and susceptibility to intoxication suggested that the direction of causation was from consumption to sensitivity, but long-term follow-up now suggests otherwise (see below).

A total of 412 subjects were included in this Alcohol Challenge Twin Study (ACTS), and many of them also participated in postal surveys of alcohol use over the following decade. In 1990–92 a systematic program of follow-up of the ACTS subjects was initiated with the aim of obtaining blood samples for genotyping. In 1992–93 they were invited to participate in telephone interviews using the SSAGA questionnaire, which provides information on (among other conditions) alcohol dependence. Information on alcohol sensitivity and pharmacokinetics, subsequent alcohol dependence, and genotypes at selected loci could therefore be integrated for 334 of the original 412 subjects.

Examination of the results showed that many of the variables measured after alcohol challenge differed significantly between subjects who did and did not subsequently show alcohol dependence by the DSM-III-R criteria. Among the alcohol sensitivity measures, both body sway (Fig. 4) and self-report intoxication (Fig. 5) were less in the subsequently alcohol-dependent group. These variables were integrated into a composite alcohol sensitivity measure by Heath et al. (19). The difference in sensitivity between groups (alcohol dependence positive and negative) was significant in men but not in women.

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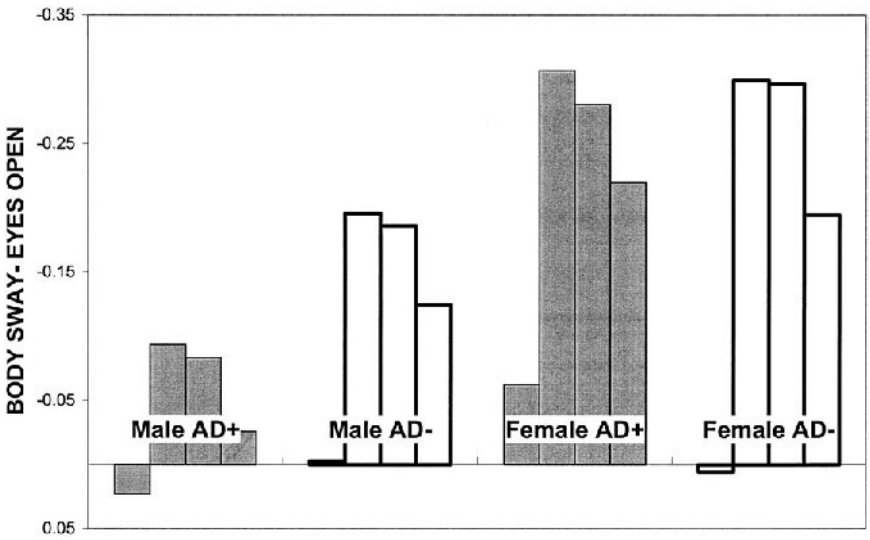

Figure 4 Change in body sway by subsequent alcohol dependence status. AD+: alcohol dependent by DSM-III-R criteria, AD−: no alcohol dependence. The four columns in each group represent values for prealcohol and three postalcohol times in the ACTS study where subjects received 0.75 g/kg of ethanol. Sway data have been adjusted for height and weight and increasing (negative) values on the y-axis represent greater body sway, i.e., increased sensitivity to alcohol's effects. Note the greater sway at all postalcohol times for the male AD−subjects.

This question of whether the alcohol insensitivity was caused by, or a consequence of, alcohol intake was reexamined by Heath et al (19). The intoxication/dependence association could be due to neurological tolerance to alcohol's effects (as discussed above) if the subsequently alcohol-dependent subjects had been drinking more heavily at the time of original testing. Therefore, adjustment for the alcohol intake, and an alcohol problem score, at the time of alcohol challenge was incorporated into the analysis; the sensitivity score remained a significant predictor of dependence risk in men. Moreover, there was a substantial genetic correlation (0.72) and negligible environmental correlation (0.04) between alcohol dependence symptom count and alcohol sensitivity score; this means that some genes affect both sensitivity and dependence. Looking at the results in another way, men who were not themselves alcohol dependent but who had a monozygotic (''identical'') cotwin with alcohol dependence had lower alcohol sensitivity scores than subjects from twin pairs where both twins were unaffected.

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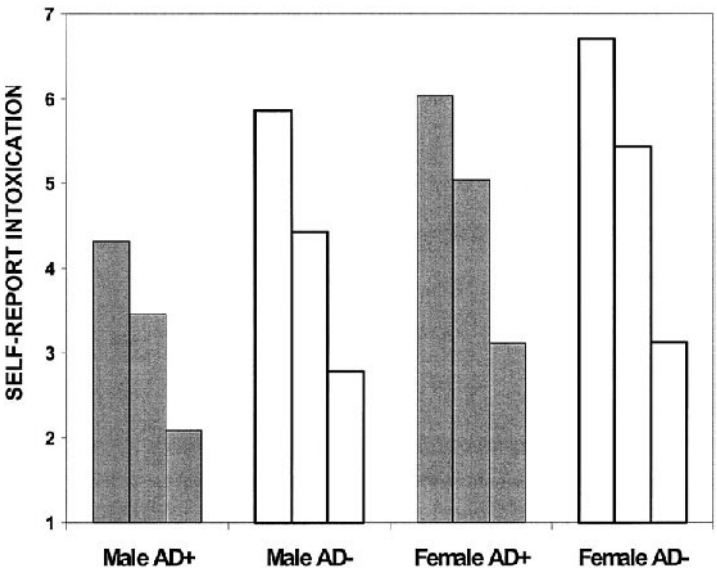

Figure 5 Self-report intoxication by subsequent alcohol dependence status. AD+: alcohol dependent by DSM-III-R criteria, AD−: no alcohol dependence. The three columns in each group represent three postalcohol times in the ACTS study where subjects received 0.75 g/kg of ethanol. Increasing values on the y-axis represent greater perceived intoxication, i.e., increased sensitivity to alcohol's effects. Note the greater self-reported intoxication at all postalcohol times for the male AD− subjects.

Despite some limitations discussed in the paper, and the absence of significant effects in women, this twin study and Schuckit's FHP/FHN study lead to similar conclusions. They support the concept that innate resistance to intoxication increases dependence risk and sensitivity to alcohol's effects decreases it. It follows that the subjects who are resistant to alcohol's intoxicating effects are not thereby resistant to its addictive properties; they are more likely to proceed to alcohol dependence.

D. Other Human Studies of Intoxication

A number of other studies have attempted to find a link between susceptibility to intoxication by alcohol and risk of alcohol dependence, usually by comparing high-risk and low-risk groups of subjects. Results have been mixed, but a meta-analysis of FHP/FHN studies (20) showed a significant effect. However, it may be relevant that some difference (nonsignificant) between FHP and FHN groups was found to occur with placebo.

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A 10-year follow-up study of 43 sons of alcoholics and 28 control subjects was carried out by Volavka et al. (21). The indicator of alcohol sensitivity was changes in the EEG, and subjects with DSM-III-R alcohol dependence (but not alcohol abuse) had significantly less alpha wave changes in response to alcohol than subjects without abuse or dependence. It is unclear whether there is any association between EEG changes and other measures such as body sway or self-rating of intoxication, but the results are consistent with the general hypothesis of a reduced response to alcohol being associated with higher risk of dependence.

Genetic influences on sensitivity to intoxication in humans have also been studied. Genetic (father/son) transmission of sensitivity can be inferred from Schuckit's studies on sons of alcoholics, and is explicit in a number of twin studies that measured either multiple end-points (17), changes in EEG (22,23), or acute tolerance to alcohol as shown in changes in EEG with time after alcohol (24). All these studies found evidence of significant genetic effects on sensitivity to alcohol.

E. Assessment of Susceptibility to Intoxication

If we accept that sensitivity or resistance to intoxication is an important predictor of alcohol dependence, two technical questions arise. First, which variables should be measured after alcohol consumption to obtain the best prediction? Second, is there any reliable way to obtain this information with a simpler and cheaper method?

In both of the prospective studies discussed above, a large number of variables were measured and only some have been used in the follow-up studies. The twin study of Martin et al. (17) showed that the various measures of intoxication are largely independent and no general intoxication factor can be extracted. Self-report of intoxication after a standard dose of alcohol, and change in body sway, are common to both groups while Schuckit also included plasma cortisol. The amounts of alcohol used in the two studies were similar, either 0.75 g/kg of ethanol or 0.75 ml/kg of 95% ethanol. Because of the alcohol challenge studies needed, the large number of subjects, and the substantial period of follow-up, it seems unlikely that we shall obtain a definitive answer to the question of the best predictor of dependence risk.

Alternative approaches may offer a way of assessing susceptibility to intoxication without experimental administration of alcohol. Schuckit has developed a brief questionnaire (Self-Rating of the Effects of alcohol, SRE) designed to determine the number of drinks required to attain intoxication in the ''real-life'' situation (25). This covers the subjects' experiences when they first began to use alcohol, during their period of heaviest alcohol use, and currently. The questionnaire results have been compared retrospectively with data collected at the time of alcohol testing (15 years previously) in 94 of the men in the FHP/FHN studies,

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and with alcohol dependence diagnosis in 551 subjects interviewed as part of the COGA study (26). There were highly significant associations between SRE scores and subjective ''high'' results after alcohol, and between SRE scores and alcohol dependence diagnosis, but the sensitivity and specificity of SRE in predicting response or dependence for any individual were only moderate. Nevertheless this questionnaire is likely to be useful in tracing the pathways from genotype to sensitivity to dependence in large studies where alcohol challenge is impractical, and in identifying subgroups of subjects in whom alcohol insensitivity is, or is not, an important factor in the development of dependence.

F. Animal Breeding: Selection for Consumption and Sensitivity

In addition to these human studies, many strains of rats and mice have been developed by selection for extremes of alcohol sensitivity or alcohol preference. It was found that various strains of rats that differed in their alcohol preference differed, in the opposite direction, in their sensitivity to alcohol's effects (27). More recent studies have used inbred animals, initially produced as model organisms in which to investigate neurobiological mechanisms of dependence. After 25–30 generations of selection and inbreeding, animals with predictably high or low preference or sensitivity can be produced (28,29).

Initial studies concentrated on differences in neurotransmitter systems between the contrasting strains, while more recent work has identified quantitative trait loci (QTLs) through linkage studies in crosses between the strains. The QTLs are discussed below, but it is relevant to note that strains selected on preference tend to differ in sensitivity, and vice versa, reinforcing the concept that sensitivity to alcohol's effects reduces intake and the probability of dependence. Sensitivity to alcohol withdrawal in mice has also been analyzed (30).

III. ALCOHOL METABOLISM AND DEPENDENCE RISK

Some of the practical issues with prospective studies of alcohol metabolism or alcohol pharmacokinetics are similar to those mentioned above for alcohol sensitivity. Metabolic tolerance, in which the rate of alcohol metabolism is increased as a reversible response to regular alcohol use, can confound any association between blood alcohol results and risk of dependence. Study of subjects before they adopt hazardous drinking habits is necessary. Again as discussed above, substantial numbers of subjects are required to give the study adequate power; and the costs associated with initial testing of subjects with alcohol and of maintaining contact for follow-up are a consideration.

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Probably because of these reasons, the Alcohol Challenge Twin Study and related work seem to be the only source of published information about alcohol pharmacokinetics and prospective dependence risk in humans. In the original ACTS (31), data were collected on both blood and breath alcohol concentrations at times between 40 and 210 min after the subjects' consumption of alcohol. From these data it was possible to calculate the peak concentration and the rate of decrease in the postabsorptive phase, or to work with the individual readings. Both the peak and the rate showed significant genetic effects. Studies of the relationships between either peak or rate and reported usual alcohol consumption at the time of testing showed that both peak and rate were significantly and positively correlated with consumption, even at quite low levels of alcohol use. These associations seemed to depend on genes that affected both alcohol consumption and the alcohol pharmacokinetics (32).

Extension of this work became possible when alcohol dependence diagnoses were derived from the SSAGA interview results for the ACTS subjects. Initial

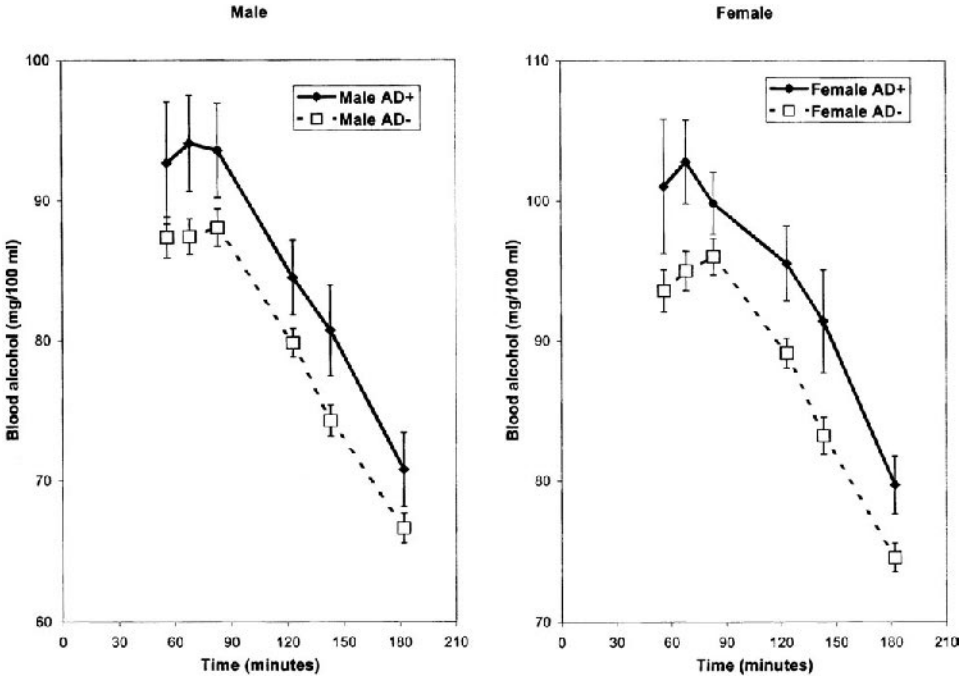

Figure 6 Blood alcohol concentrations in men and women after 0.75 g/kg of ethanol. AD+ (continuous lines): subsequently alcohol dependent by DSM-III-R criteria, AD− (dashed lines): no alcohol dependence. Error bars show standard errors.

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division of subjects into dependence-positive (AD+) and -negative (AD−) groups showed a difference in blood alcohol values between these groups for both men and women (Fig. 6). Differences between groups were also found for breath alcohol readings. Correction for usual alcohol consumption at the time of alcohol challenge did not abolish the difference between the AD+ and AD− groups, which appears to be based on a relationship between the early alcohol metabolism and risk of future dependence. Further analysis of these data is in progress, with the aims of correcting the results for gene sharing between members of twin pairs and clarifying the relationships between alcohol metabolism in vivo, alcohol dehydrogenase genotypes, and alcohol dependence risk. So far, it seems that ADH2 and ADH3 polymorphisms do not fully explain the link between alcohol metabolism and dependence.

Comparison of the separation between AD+ and AD− subjects, by calculating Z-scores to put the variables on a common scale (Fig. 7), suggests that the

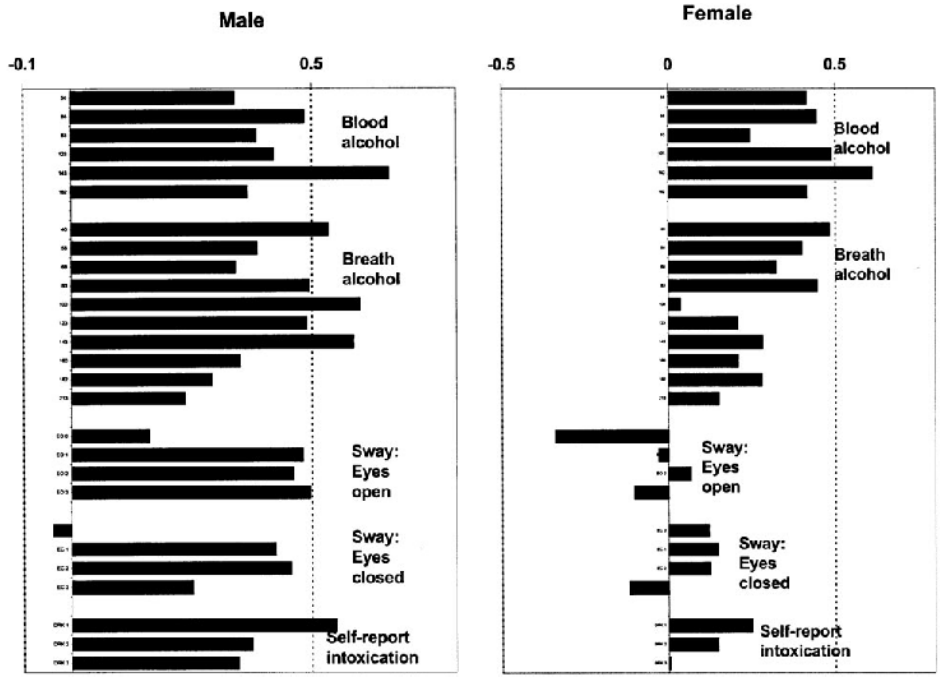

Figure 7 Summary of differences in blood and breath alcohol values, body sway, and self-report intoxication after alcohol challenge, between subjects with and without subsequent alcohol dependence. All variables have been transformed to Z-scores by dividing the mean difference between AD+ and AD− groups by the standard deviations.

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association between alcohol dependence and blood or breath alcohol concentrations is at least as strong as those between alcohol dependence and body sway or self-report intoxication. Moreover, the blood and breath alcohol values are associated with subsequent dependence risk in women as well as men. There is little relationship between blood alcohol results and sensitivity to alcohol effects (17), and in particular the sensitivity to alcohol is not associated with ADH type (19), so there appear to be two distinct groups of genes involved in the pathways from alcohol metabolism, and from intoxication, to the common end-point of dependence risk.

IV. POLYMORPHISMS IN ALCOHOL SENSITIVITY GENES

Phenotypes have been defined that lead to increased alcohol dependence risk, and these phenotypes show significant heritability. Therefore, the next step is to determine what genes may affect these phenotypes, and to test whether they also show significant effects on alcohol dependence. At the same time, genes found by other routes to influence alcohol dependence can be tested for effects on the postulated intermediate phenotypes of alcohol sensitivity and alcohol metabolism.

Animal work has been quite productive in defining the neurochemical differences between animal lines that differ in their sensitivity to alcohol's effects. Multiple receptor systems, including serotonin, dopamine, GABA, and opioid, have been shown to differ between the contrasting inbred lines in one or more of the animal models (33). This is a helpful initial step in defining candidate genes. Crossing of the inbred lines and use of genetic linkage markers to detect quantitative trait loci (QTLs) has located one highly significant locus in the P/NP rats, which includes the neuropeptide Y (NPY) gene (34,35). QTLs have also been located in mice (36) and ways of moving forward from QTL location have been discussed (37).

Testing of genetically engineered mice (knockout or overexpressing for the gene of interest) for alcohol's effects has shown a number of instances where genes affect alcohol preference and/or sensitivity to its effects. Such studies confirm and extend the results from the more pharmacologically oriented studies on the inbred rats and show which genes and gene products deserve further study.

Some of the results of these animal studies are summarized in Table 1. The existence of a reciprocal relationship between alcohol sensitivity and alcohol preference has been shown in several cases. The genes involved are mainly neurotransmitters and their receptors, or protein kinases that may modify receptors and their binding properties. The modification of receptors by phosphorylation may be a mechanism of acute tolerance to alcohol, and such tolerance may well be a relevant aspect of alcohol sensitivity. The role of insulin-like growth factor 1

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Table 1 Genes Shown to Affect Alcohol Sensitivity in Studies of Recombinant Mice

Gene	Knockout	Overexpressed	Ref.
Dopamine receptor D2	Intake − Sensitivity −		38
Dopamine receptor D4	Sensitivity +		39
FYN tyrosine kinase	Sensitivity +		40
Insulin-like growth factor 1		Sensitivity: Sleep time − Tolerance −	41
IGF binding protein 1		Sensitivity: Sleep time + Tolerance +	41
Neuropeptide Y	Intake + Sensitivity −	Intake − Sensitivity +	42
Protein kinase C epsilon	Intake − Sensitivity +		43
Serotonin receptor 5HT1B	Intake + Sensitivity −		44 45
Serotonin receptor 5HT3		Intake − Sensitivity +	46 47

(IGF-1), however, is unexpected and so far unexplained; it illustrates the potential of gene studies to provide a starting point for further physiological studies.

However, the fact that a gene produces a protein that is involved in the chain of events leading to intoxication does not prove that the equivalent gene in humans is responsible for the heritability of susceptibility to intoxication or dependence. The gene may not be polymorphic in humans, or the variants may be rare. With this reservation in mind, genes found to be relevant in experimental animals provide prime candidates for human linkage or association studies. Some results from such studies are starting to appear. However, we do not yet know whether variation in the syntenic genes affects alcohol sensitivity in humans. Studies on dopamine receptor genes (probably not related to alcohol sensitivity) have given contradictory results, possibly because of variation between and within populations and possibly because the effects are small and the power of some studies has been insufficient. These association studies illustrate some of the difficulties that must be overcome.

Although large-scale studies with linkage techniques and microsatellite markers have yielded QTLs in rats and mice, this has not yet been a route to discovery of alcohol sensitivity genes in humans. Investigation of the serotonin transporter gene (5-HTTLPR) appears to have arisen from the probable involve-

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ment of serotonin systems in impulsive or violent behavior, and from the existence of a deletion/insertion variant that affects gene transcription and transporter expression. Turker et al. (48) reported an association between the short form of the 5-HTTLPR and high ethanol tolerance in young adults, but the association was significant only in this subgroup of subjects. Schuckit et al. (49) found an association between serotonin transporter genotype and response to alcohol, and development of alcoholism, in a small number of subjects from their long-term study but Edenberg et al. (50) were unable to find any linkage or association between this polymorphism and alcohol dependence.

V. POLYMORPHISMS IN ALCOHOL METABOLISM GENES

Animal breeding and human genome-scan methods have not been applied to discovery of alcohol metabolism genes per se, and animals selected for alcohol preference or sensitivity have not been found to have major differences in their alcohol metabolism. Nevertheless, enzyme and protein techniques revealed a number of polymorphisms in human alcohol-metabolizing enzymes and the molecular basis of these has now been clarified.

The impact of variation in *ALDH2* is restricted to subjects from Northeast Asia and their descendants. No common polymorphisms in *ALDH2* outside Asia have been found despite searches (51–53), and the frequency and significance of any variation in *ALDH1* (54) is uncertain. A polymorphism in *ALDH2* in rats bred for alcohol preference or nonpreference (P/NP) offers a cautionary example as it causes a significant amino acid substitution and was found at higher frequency in the nonpreferring line (55), but it was later shown to be unrelated to alcohol preference (56). However, it is possible that a recently described promotor polymorphism in human *ALDH2* (57,58) affects expression and hence enzyme activity.

ADH2 variation also has significant effects on alcohol dependence risk, both within Asia (59,60) and beyond (61,62). This is independent of *ALDH2* status, as the risk varies by *ADH2* genotype even among subjects with the active form of *ALDH2*. Associations between *ADH3* genotype and alcohol dependence risk have also been reported, but it has recently been shown that this effect is due to linkage disequilibrium with *ADH2* at least in Asian populations (60,63). Variation in other ADHs is unlikely to affect alcohol metabolism, although a recently reported *ADH4* polymorphism (64) may affect expression of this comparatively low-affinity isoenzyme.

The effects of ADH variation on dependence risk are usually ascribed to faster alcohol metabolism and higher acetaldehyde concentrations in subjects with the more active forms of the enzymes. *ADH2* genotype has strong effects on alcohol dehydrogenase activity in vitro (65), but the in vivo effects are not

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as predicted (66,67). It is possible that other mechanisms for the *ADH2* effect, such as variation in competitive inhibition of the metabolism of other ADH substrates by ethanol, must be sought.

It was shown above that variation in blood and breath alcohol levels after a test dose was associated with variation in subsequent alcohol dependence. Since *ADH2* genotype determines the in vitro enzyme activity and affinity for substrate and coenzyme, and affects dependence risk, we need to investigate whether *ADH2* variation accounts for all the association between alcohol pharmacokinetics and alcohol dependence. Preliminary results suggest that *ADH2* type does not offer a full explanation, and other genes that affect alcohol metabolism (31) need to be sought and tested for their effects on dependence risk.

VI. CONCLUSIONS

It seems that some, but not all, of the genetic effects on alcohol dependence risk are mediated through genes that affect alcohol metabolism or sensitivity to intoxication. Animal studies suggest that around a half-dozen genes are involved in alcohol sensitivity. Presumably other genes will be located that will account for genetic effects on other pathways to alcohol dependence, including the influence of personality variation and susceptibility to addiction to other drugs including nicotine. Not all genes whose products are involved in alcohol metabolism or intoxication will prove to be polymorphic in humans.

Both candidate gene and genome-scan approaches have been widely and successfully used in the investigation of inborn errors of metabolism and other single-locus diseases. The so-called ''reverse genetics'' has been successful with conditions such as cystic fibrosis, muscular dystrophy, or Huntington disease where the biochemical defect was not understood until the gene was discovered. However, this approach may be less successful for complex diseases where the additive effects of many genes are important, because the power of linkage techniques to detect small effects is limited. For alcoholism, as for other multifactorial conditions such as diabetes or heart disease, an understanding of risk factors and the investigation of candidate genes suggested by this understanding will continue to be important.

If and when genes affecting alcohol dependence risk in humans are identified, what are the likely consequences? The number of loci that would have to be genotyped to obtain a reasonable estimate of dependence risk for an individual will depend on the number of relevant polymorphic genes, the frequencies of the alleles, and the risk associated with each allele. There may be many such genes but this presents little technical difficulty; the problems lie in establishing data for the conversion of genotypic information to risk assessment and in developing useful risk-based counseling approaches. However, even those genes and their

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products that are not polymorphic in humans will be potential targets for therapeutic agents; and in this respect animal studies offer a useful complement to purely human ones because the details of the relevant polymorphisms will differ across species.

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Pharmacogenetic Relevance of Aldehyde Dehydrogenases

Dharam P. Agarwal
University of Hamburg, Hamburg, Germany

I. INTRODUCTION

Inherited differences in metabolic capacity of drugs and chemicals may play a primary role in susceptibility to environmentally induced diseases. Genetically determined variations in drug-metabolizing enzymes are of obvious importance in the presence of drugs that are substrates for these enzymes. It is not always evident, however, whether or not such variation has important biological consequences in the absence of these drugs. Examples of some of the best-studied pharmacogenetic variations in alcohol metabolizing enzymes are CYP2E1 (cytochrome P-450), ADH2, ADH3 (alcohol dehydrogenases), and ALDH2 (aldehyde dehydrogenase). Studies in humans have reliably shown that the genes for the principal enzymes of alcohol metabolism influence drinking behavior and alcoholism risk. Notably, the functional genetic variants of ADH that exhibit high alcohol-oxidizing activity, and the genetic variant of ALDH that exhibits low acetaldehyde-oxidizing activity, protect against heavy drinking and alcoholism (1). Moreover, neuroanatomical, neurochemical, and genetic studies have shown the involvement of serotonin, dopamine, glutamate, gamma-aminobutyric acid, and opioid pathways in these actions of ethanol. In Chapter 1, we have reviewed recent advances in the understanding of the functional polymorphisms of ADH and ALDH as well as their metabolic, physiological, and clinical correlations. The present chapter focuses attention on the pharmacogenetic relevance of various human ALDHs in detoxification of endogeneous and exogeneous aldehydes as well as aldehydes produced from xenobiotic drugs.

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II. PHARMACOGENETIC BASIS OF VARIABILITY IN DRUG METABOLISM

Pharmacogenetic phenomena are the expression of preexisting inborn differences among individuals that become apparent upon exposure of the body to drugs or chemicals (2). Pharmacogenetics is usually thought of as the study of a situation in which a single gene product exerts control over a given drug response so that a failure to respond, or an excessive response, may result. Often, such individual variation is the result of genetic polymorphisms in enzymes that are involved in the metabolism of drugs. The role of polymorphisms in specific enzymes, such as thiopurine S-methyltransferases (TPMT), dihydropyrimidine dehydrogenase (DPD), aldehyde dehydrogenases (ALDH), glutathione S-transferases (GST), uridine diphosphate glucuronosyl-transferases (UGTs), and cytochrome P-450 (CYP-450) enzymes in cancer development and therapy is well documented. Such variations are often due to genetic alterations in drug-metabolizing enzymes (pharmacokinetic polymorphisms) or receptor expression (pharmacodynamic polymorphisms).

Pharmacogenetics is a field of growing interest in medicine and within the pharmaceutical industry. Pharmacogenetic variation is truly important with respect to the adaptability and survival of populations. The availability of molecular techniques has dramatically expanded this field, which is now often referred to as ''pharmacogenomics.'' In modern life, however, the meaning of the prefix ''pharmaco-'' is often equated in a narrow sense with medicine or drug. Hence, geneticists and other scientists sometimes referred to ''ecogenetics'' when concerned with variable response to environmental chemicals. Other terms used are ''toxicogenetics'' and ''environmental genetics.'' In the present context, pharmacogenetics refers to any kind of inborn variation in any group of creatures in response to xenobiotics. The frequencies of variant genes are usually not the same in different populations. Also the nucleotide substitutions in a variable gene often differ between populations. In other words, pharmacogenetic differences between populations are typical events. Pharmacogenetic variants are preadaptive in the sense that they occur before there has been any exposure to the drug. Because there are innumerable xenobiotics, pharmacogenetic variability must represent a multiplicity of variants (2).

III. PHARMACOGENETICS OF ETHANOL ELIMINATION

In humans, more than 90% of the ingested ethanol is degraded in the liver by oxidative and nonoxidative metabolic pathways. The major enzymes involved in the metabolism of ethanol are ADH, ALDH, CYP2E1. In addition, catalase and fatty acid ethyl ester synthase (FAEES) are also considered to be involved in

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ethanol degradation. Ethanol enters rapidly into the circulation by diffusion mainly across the lining or membrane of the duodenum and jejunum, and to a lesser extent from the stomach and large intestine. The absorption of ethanol is normally over in 2 hr and overlaps with the diffusion phase during which it is distributed throughout the body water. Variation in hormonal status, e.g., stage of the menstrual cycle, affects ethanol absorption. Interplay between the kinetics of absorption, distribution, and elimination of the ingested alcohol is an important determinant of the blood alcohol concentration. Only 5–15% is excreted directly through the lungs, sweat, and urine, the remainder being metabolized by the liver, via oxidation to carbon dioxide and water. The total alcohol eliminated by the human body per hour is usually in the range of 100–300 mg/liter/hr, which is equivalent to about 6–9 g alcohol per hour for a healthy subject with an average body weight. Comparison of the blood alcohol concentration (BAC) after oral ingestion versus intravenous administration of alcohol indicates that a fraction of ingested alcohol never reaches the peripheral circulation. Recent studies have shown that indeed a significant fraction of ingested ethanol is metabolized in the gastrointestinal tract by the so-called first-pass metabolism (3).

A. Alcohol Dehydrogenases

At least seven different genetic loci code for human ADH arising from the association of different types of subunits. Over 20 ADH isoenzymes are known, which vary in their pharmacokinetic properties: (i) the types of alcohols they preferentially oxidize; (ii) the amount of alcohol that has to accumulate before appreciable degradation occurs; and (iii) the maximal rate at which they oxidize alcohol. Various human ADH forms can be divided into five major classes or distinct groups (I–V) according to their subunit and isoenzyme composition as well as their physicochemical properties.

IV. PRODUCTION AND DETOXIFICATION OF ALDEHYDES

Aldeydes, per se, are widespread in the environment. Biotransformation of drugs and chemicals that are not aldehydes frequently gives rise to aldehyde metabolites with a significant toxic potential. Besides through the oxidation of primary alcohols, aldehydes are also generated directly via oxidative dealkylation, decyanation, and deamination of many drugs and xenobiotics. Acetaldehyde is the first metabolic product of enzymatic ethanol oxidation in human liver, and is far more toxic than the parent compound ethanol. It is a highly reactive molecule and is involved in a number of physiological, biological, and pharmacological processes.

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A. Aldehyde Dehydrogenases

Though various metabolic pathways exist for the detoxification of acetaldehyde, the major oxidation of acetaldehyde to acetate in the liver and other organs is catalyzed by the NAD+-dependent aldehyde dehydrogenase (ALDH, aldehyde: NAD+ oxidoreductase, EC 1.2.1.3). ALDH is an important enzyme for detoxifying a wide variety of aldehydes, toxins, and pollutants and is found in the mitochondria, endoplasmic reticulum, and/or cytosol of mammalian tissues. A number of isoenzymes of ALDH coded by different gene loci have been detected in human organs and tissues, which differ in their electrophoretic mobility, kinetic properties, as well as in their cellular and tissue distribution. Human ALDHs consist of at least 13 genes mapped to various chromosomes. In Table 1, all known human ALDH genes including their trivial names and chromosomal location are listed. Broadly speaking, the major ALDH genes can be grouped into three larger families; members of family 1 are cytoplasmic ALDHs (*ALDH1*), members of family 2 are mitochondrial ALDHs (*ALDH2*), and members of family 3 are the major constitutive and inducible high K_m ALDH forms (*ALDH3*) found in rat and mouse tissue such as stomach, lung, liver, cornea as well as in human stomach, saliva, and hepatocarcinoma (4).

The various ALDH isoenzymes show a broad range of substrate specificity for aliphatic and aromatic aldehydes. Whereas *ALDH1* and *ALDH2* both show K_m values in the micromolar range with acetaldehyde and propionaldehyde, the Michaelis constants for *ALDH3* and *ALDH4* are in the millimolar range for these

Table 1 Human ALDH Genes

Gene	Trivial name	Chromosomal localization
ALDH1A1	*ALDH1*	9q21
ALDH1B1	*ALDH5*	9q13
ALDH1A6	*ALDH6*	15q26
ALDH2	*ALDH2*	12q24
ALDH3A1	*ALDH3*	17p11.2
ALDH3A2	*ALDH10*	17p11.2
ALDH3B1	*ALDH7*	11q13
ALDH3B2	*ALDH8*	11q13
ALDH4A1	*ALDH4*	1
ALDH5A1	*SSDH*	6p22
ALDH7A1	*ATQ1*	5q31
ALDH8A1	*ALDH12*	6q241.1–25.1
ALDH9A1	*ALDH9*	1q22–q23

SSDH: succinic semialdehyde dehydrogenase; MMSDH: methylmalonic acid semialdehyde dehydrogenase; ATQ1: Antiquitin. *Source*: Adapted from Ref. 4.

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substrates. NAD+ is the preferred coenzyme for the low-K_m isoenzymes (ALDH1 and ALDH2), whereas the high-K_m isoenzynmes (*ALDH3* and *ALDH4*) can use either NAD+ or NADP+. The major human liver *ALDH1* and *ALDH2* isoenzymes are homotetramers consisting of equal but isoenzyme-specific subunits with a MW of about 54 kD each (5). The putative physiological role of the major ALDH isoenzymes is summarized in Table 2.

B. Polymorphisms of ALDH Genes

Genetic polymorphisms have been reported in a number of ALDH genes. In Table 3, nucleotide changes and effect on encoded proteins are compiled. Naming of the human alleles is based on the new nomenclature (4).

C. Polymorphism of ALDH2

The single genetic factor most strongly correlated with reduced alcohol consumption and incidence of alcoholism is a naturally occurring variant of mitochondrial

Table 2 Major ALDH Isozymes and Their Putative Physiological Role

ALDH1 (cytosolic)
The *ALDH1* gene encodes the cytosolic isoenzyme, which has a high K_m for the oxidation of acetaldehyde; present in liver and red cells
Major physiological substrate is retinal resulting in modulation of cell differentiation by retinoic acid
May be involved in the oxidation of oxazaphosphorines
Plays a possible role in alcohol-related Caucasian flushing
ALDH2 (mitochondrial)
The *ALDH2* gene encodes the major liver mitochondrial *ALDH2*, which has a very low K_m for acetaldehyde
The atypical *ALDH2*2* allele is common (about 30%) in Orientals
Subjects with *ALDH2*2* allele, both homozygous and heterozygous status, lack functional ALDH activity
The *ALDH2*2* homozygotes experience intense alcohol sensitivity symptoms after drinking a small dose of alcohol
A low frequency of the mutant alllele is found in Asian alcoholics
ALDH3 (stomach-specific)
Constitutively expressed in stomach, lung, esophagus, and cornea, but hardly detectable in the normal liver
May play a role in the oxidation of toxic aldehydes (e.g., oxazaphosphorines)
Highly induced in the hepatocellular carcinoma tissues
The *ALDH3* locus is polymorphic in Orientals and presumably in other populations

Table 3 Frequency of *ALDH2* Alleles in Differen Populations

Population	Allele frequency	
	ALDH2[1]	*ALDH2*[2]
Caucasians		
Germans	1	0
Swedes	1	0
Finns	1	0
Turks	1	0
Asian Indians	0.98	0.02
Hungarians	0.987	0.013
Orientals		
Chinese	0.841	0.159
Japanese	0.764	0.236
Koreans	0.849	0.151
Fillipinos	0.994	0.006
Thais	0.950	0.050
Malayans	0.966	0.034
American Indians		
Caboclos (Brazil)	0.826	0.174
Aboriginals		
Papua New Guineans	0.996	0.004

Source: Data from Ref. 9.

aldehyde dehydrogenase (*ALDH2*). This variant contains a glutamate to lysine substitution at position 487 (E487K) (6). The E487K variant of *ALDH2* is found in approximately 50% of the Asian population, and is associated with a phenotypic loss of *ALDH2* activity in both heterozygotes and homozygotes (5,7). Functionally, a single base mutation at this position, resulting in loss of the catalytic activity, is compatible with the proximity in the primary structure between this region and the segment that contains cysteine residues. *ALDH2*-deficient individuals exhibit an aversive response to ethanol consumption, which is probably caused by elevated levels of blood acetaldehyde (8).

D. Population Distribution of ALDH2 Variants

As shown in Table 4, Asian populations of Mongoloid origin widely show the presence of the inactive *ALDH2* isoenzyme phenotype whereas none of the Caucasian or Negroid populations have this isoenzyme abnormality (9). More recent genotyping data hint to a considerable genetic heterogeneity in the

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Table 4 Polymorphism of ALDH Genes

Allele symbol	Nucleotide change	Effect on protein	Exon
*ALDH1B1*1*		Wild type	
*ALDH1B1*2*	257C>T	V69A	2
*ALDH1B1*3*	320T>G	R90L	2
*ALDH1B1*4*	183C>T	Silent	2
*ALDH2*1*		Wild type	
*ALDH2*2*	1510G>A	E487K	12
*ALDH2*3*	1486G>A	E479K	12
*ALDH2*4*	1464G>A	Silent	12
*ALDH3A1*1*		Wild type	
*ALDH3A1*2*	985C>G	P329A	9
*ALDH3A2*1*		Wild type	
*ALDH3A2*2*	521delT	Frameshift	4
*ALDH3A2*3*	808delG	Frameshift	6
*ALDH3A2*4*	941del3,	A314G, P315A,	7
	94lins21	AKSTVG313ins	7
*ALDH3A2*5*	641G>A	G214Y	4
*ALDH3A2*6*	1297delGA	Frameshift	9
*ALDH3A2*7*	1311linsACAAA	Frameshift	9
*ALDH3A2*8*	1297delGA	Frameshift	9
*ALDH4A1*1*		Wild type	
*ALDH4A1*2*	21delG	Frameshift	[a]
*ALDH4A1*3*	1055C>T	S352L	[a]
*ALDH4A1*4*	47C>T	P16L	[a]
*ALDH4A1*5*	1563insT	Frameshift	[a]
*ALDH9A1*1*		Wild type	
*ALDH9A1*2*	344G>C	C115S	2
*ALDH9A1*3*	327C>T	Silent	2

[a] Gene structure not known yet.
Source: Adapted from Ref. 4.

distribution of the *ALDH2*2* gene in American Indian and Central Asian populations (10–14).

V. PHARMACOGENETIC RELEVANCE OF ALDHs

The pharmacogenetic significance of ALDH relates mainly to its role in the detoxification of acetaldehyde and other aldehydes that show a variety of toxic effects in human organs and tissues. Many biogenic amines are converted to their corre-

sponding aldehydes via monoamine and diamine oxidase systems. Moreover, in vivo biotransformation of many drugs and xenobiotics that are not aldehydes gives rise to aldehyde metabolites. Acetaldehyde is a critical intermediate of ethanol metabolism, and its effect on the organs is thought to be important in the etiology of alcoholism.

A. Acute Reactions to Alcohol and Its Metabolites

Alcohol is a known vasodilator, and this property is not the direct effect of alcohol on the blood vessel but is a consequence of its actions on the central nervous system. The effects of ethanol per se are influenced by its sympathomimetic activity and also by its metabolites, acetaldehyde and acetate. Acetaldehyde shows stronger sympathomimetic action than alcohol, and facilitates the release of catecholamines from the chromaffin cells of the adrenal medulla and from the sympathetic nerve endings. Increase of plasma catecholamines apparently leads to an elevation of heart rate, dilation of peripheral vessels accompanied with the rise of blood flow in carotid arteries, and increased cardiac output (15).

In some individuals, ingestion of moderate amounts of alcohol exerts the so-called alcohol sensitivity symptoms (facial flushing, increase in heart rate, enhancement of left ventricular function, hot feeling in stomach, palpitation, tachycardia, muscle weakness, etc.). Wolff (16,17) reported significant differences among the Caucasian group, on one hand, with a very low percentage (5%) of subjects showing a flush response to alcohol, and the Mongoloids and American Indians, on the other hand, with over 80% who showed flushing reactions. The apparent individual and racial differences in euphoric and dysphoric response to alcohol were replicated and extended in various ethnic and racial groups (5,18).

American Indians are also sensitive to alcohol and exhibit facial flushing associated with various subjective and objective vasomotor symptoms after drinking moderate amounts of alcohol. Wolff (17) reported that Eastern Cree Indians who consumed no alcohol or less than 5 bottles of beer per week responded more intensely than those who reported drinking more than 10 bottles of beer per week or an equivalent amount of alcohol in other forms.

B. Mechanism of Biological Sensitivity to Alcohol

As stated earlier, acetaldehyde and not ethanol per se seems to be mainly responsible for most of the severe symptoms of alcohol-related cardiovascular sensitivity. Indeed, higher steady-state blood and breath acetaldehyde levels have been noted postdrink in those Japanese and Chinese subjects who show flushing after drinking mild doses of alcohol (5,19). The maximum alcohol absorption takes place in the small intestine. Anatomical variations in the internal organ size may be important in this respect; Orientals and American Indians have longer intes-

tines. Since alcohol diffuses through the lining of the stomach and the small intestine, any variation in the surface areas will lead to a more rapid absorption rate (20). Individual and ethnic differences in the alcohol metabolism rate (mg ethanol/kg total weight/hr) and alcohol clearence rate (mg ethanol/100 ml blood/min) also vary considerably between and within various racial and ethnic groups (21). Thus, any genetically determined variation in the ethanol metabolism rate could also influence the steady-state blood acetaldehyde levels. According to Ijiri (15), the cardiovascular symptoms are caused by acetaldehyde itself. Urinary excretions of both norepinephrine and epinephrine increased in the flushing cases after drinking sake in comparison with those who drank the same volume of water. The catecholamines released from the sympathetic nerve end or the adrenal medulla by acetaldehyde cause an increase in the pulse rate. Bradykinin is released from high-molecular-weight kininogen by activated kallikrein and acts to dilate distal blood vessels and raises permeability in tissues.

The atypical ADH, quite frequent in the Japanese, was initially thought to be responsible for a rapid oxidation of ethanol to acetaldehyde thereby producing alcohol sensitivity symptoms. More than 90% of the Japanese and other Mongoloids possess the atypical ADH with several times higher catalytic activity, whereas the incidence of flushing accompanied by higher blood acetaldehyde levels is only about 50%. Hence, rapid or higher-than-normal production of acetaldehyde via an atypical ADH alone cannot be the major cause of intense adverse reactions to alcohol. A positive correlation between alcohol sensitivity and elevated blood acetaldehyde level in conjunction with *ALDH2* isoenzyme abnormality was noted in Japanese subjects given an acute dose of alcohol (see also Chapter 1). Apparently, slow acetaldehyde oxidation due to an *ALDH2* isoenzyme abnormality leads to elevated blood acetaldehyde levels resulting in catecholamine-mediated vasodilation associated with dysphoric symptoms.

C. Functional Polymorphisms of Aldehyde Dehydrogenases and Their Implications

In 1979 we hypothesized that the polymorphisms of both of the liver enzymes responsible for the oxidative metabolism of ethanol may modify the predisposition to development of alcoholism (7). A comparison among racial and ethnic groups has invariably shown that (i) a larger proportion of Orientals than Caucasians report no use of alcohol; (ii) Caucasians report heavier alcohol use; (iii) a large proportion of Orientals who drink alcohol experiences facial flushing and associated sensitivity symptoms after drinking alcohol. More recent studies have focused on the putative role of functional polymorphisms of alcohol-metabolizing enzymes in alcohol elimination rate, acute reactions to alcohol, alcohol drinking habits, and alcoholism across various ethnic groups. Quantity-frequency-variability distribution indicates that the percentage of heavy and moderate drinkers is higher

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among Caucasians, while the percentage of abstainers and infrequent drinkers is higher among the Chinese, Japanese, and other Orientals (5,22–27). Individuals sensitive to alcohol by virtue of their genetically controlled polymorphism of *ALDH2* may be discouraged from abuse of alcohol due to initial adverse reaction (5,28).

D. ALDH2 Polymorphism and Electrophysiological Response to Alcohol

Both EEG patterns and ERPs were measured in subjects homozygous or heterozygous for the functional *ALDH2*1* allele (29,30). Homozygous subjects with *ALDH2*1* allele showed a typical EEG response, including increased theta and slow-alpha and decreased fast-alpha activity. In the heterozygous subjects, however, the slow-alpha EEG activity decreased significantly compared with that of the homozygous subjects for *ALDH2*1*. In the same subjects the response to ERPs was studied. Compared with a placebo, alcohol consumption in all subjects significantly decreased the amplitude and increased the latency of the P300 wave. However, alcohol's effects on the P300 wave were significantly greater in heterozygous subjects than in the subjects homozygous for *ALDH2*1*. These observations support the notion that individuals heterozygous for *ALDH2* experience a more intense response to alcohol than that of people homozygous for the *ALDH2*1* allele.

E. Genetic Liability to Alcohol-Related End-Organ Damage

The fact that only a small percentage of alcohol abusers develop cirrhosis and chronic pancreatitis suggests that a possible predisposing factor is involved in alcohol-related end-organ damage. Whether there are distinct genetic factors predisposing susceptibility to tissue and organ damage from alcohol (e.g., liver cirrhosis, pancreatitis, cardiomyopathy, etc.) has been not fully elucidated. A number of biochemical and immunological mechanisms may be determinantal in susceptibility to alcohol-related organ damage. The alcoholism rate as well as alcohol-related end-organ damage is found to be lower among the Japanese, Chinese, and other Orientals as compared to Caucasian populations living in Western society (5,22,25,31–37). A significantly fewer number of patients with alcoholism and alcoholic liver disease have been found to possess the inactive *ALDH2*2* gene. Individuals heterozygous for the *ALDH2* alleles are at higher risk for the development of alcoholic liver disease when they drink more than a critical level of alcohol.

Taken together, the *ALDH2*-deficient individuals drink less, have the tendency not to become habitual drinkers, suffer less from liver disease, and are rarely alcoholics. However, acetaldehyde-induced aversion to alcohol drinking

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may represent only one aspect of the relation between acetaldehyde metabolism and human alcohol drinking.

VI. ALDEHYDE DEHYDROGENASES AND DISEASE ASSOCIATIONS

In recent years a number of studies have attempted to find out whether genotypes of *ALDH2* are associated with hepatic and extrahepatic disorders—related or unrelated to alcohol consumption. In Table 5, the findings of such studies are summarized. These studies, however, are not conclusive and need to be replicated. The inactive form of aldehyde dehydrogenase-2 (*ALDH2*), encoded by the gene *ALDH2*1/2*2*, which is prevalent in Orientals, exposes them to higher levels of acetaldehyde after drinking. Inactive *ALDH2* was found to be associated with oropharyngolaryngeal, esophageal, stomach, colon, and synchronous and

Table 5 Aldehyde Dehydrogenases and Disease Associations

Disorder	Association
Carcinoma: hepatocellular carcinoma, stomach, colon, lung, esophageal, head and neck squamous cell carcinoma	+/−
Alcoholic liver disease/chronic pancreatitis/cardiomyopathy	+/−
Cytotoxicity (sister chromatid exchange)	+
Aldehyde-adduct-related cellular damage	+
Oxidative stress-related disorders	+
Lipid peroxidation-related disorders	+
Other multifactorial disorders	
Fetal alcohol syndrome (teratognic effects)	+
MELAS; Tourette's syndrome; Weber-Christian disease	−/−/−
Hyperuricemia/primary gout	+/−
Parkinsons' disease	−
Pain threshold	−
Renal damage	+/−
Blood pressure	+/−
Leber's hereditary optic neuropathy	+
Bronchial asthma (pulmonary disorders)	+
Brain atrophy	−
Food intolerance (cheese, etc.)	+
Stress and daily hassles	+
Articular collagen disease	+
Vasospastic angina	+
Diabetes (type 1 and type 2)	+/−

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metachronous multiple esophageal cancers in Japanese alcoholics, suggesting a general role of acetaldehyde, a recognized animal carcinogen, in carcinogenesis of the human alimentary tract (38). Other authors have studied the role of *ALDH2* polymorphism in chromosomal alterations as well as in stress and daily hassles (39,40).

VII. THE ROLE OF ALDHs IN DETOXIFICATION OF OXAZAPHOSPHORINES

Oxazaphosphorines such as cyclophosphamide (CP), 4-hydroperoxycyclophosphamide (4-HC), ifosfamide, and mafosfamide (MF) are widely used as antineoplastic drugs. The cytotoxic effect is caused by alkylation reaction of these drugs with DNA and proteins inhibiting the cell proliferation. These chemotherapeutic agents are also applied as immunosuppressants during bone marrow transplantation, and in autoimmune diseases.

A. Metabolic Degradation of Cyclophosphamide

Cyclophosphamide is pharmacologically inactive, and needs to be biotransformed to its cytotoxic metabolite phosphoramide mustard via an intermediate metabolite 4-hydroxycyclophosphamide (4-HCP). The latter compound exists in equilibrium with aldophosphamide, which can get converted to a noncytotoxic compound carboxyphosphamide through irreversible oxidation of the aldehyde group catalyzed by one or more forms of aldehyde dehydrogenases (41). This enzymatic pathway leads to the detoxification of cyclophosphamide affecting its therapeutic efficiency. Therefore, induction or overexpression of one or more of the relevant form of ALDH in target cells might primarily account for the CP-specific acquired resistance shown by many neoplastic cells.

B. Role of ALDHs in Cyclophosphamide Metabolism

There are many questions related to the putative role of ALDH isoenzymes in cyclophosphamide metabolism:

1. Which specific ALDH isoenzyme is primarily responsible for the oxidation of 4-HCP or mafosfamide under in vivo conditions?
2. Which ALDH isoenzyme is specifically overexpressed during the development of resistance to 4-HCP and mafosfamide in cultured tumor cells?
3. What is the molecular basis of difference in *ALDH3* isoenzyme from

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normal tissues and from tumor cells that show different kinetic and functional properties?

4. What is the underlying mechanism of induction of ALDH expression in normal bone marrow cells by cytokines, interleukin and TNF (42)?
5. What is the diagnostic value of the detection of tumor-specific variant form of class-3 ALDH in neoplastic cells?

C. Summary of the Recent Findings

Recent experimental observations from our laboratory and those from the others have shown the following:

1. Cell lines lacking in *ALDH1* and/or *ALDH3* (constitutive and/or inducible) were more sensitive to aldophosphamide-derived toxicity than cell lines expressing either or both the isoenzymes (43,44).

2. The enzyme from neoplastic cells exhibited much greater ability to catalyze the oxidative detoxification of active CP derivatives than the enzyme isolated from the stomach tissue (42,45).

3. Interleukin and tumor necrosis factor-alpha induced cytosolic ALDH (*ALDH1*) mRNA and protein in normal bone marrow cells but not in leukemic cells and tumor cell lines (42,46).

4. Leukemic cells (K 562) transfected with *ALDH1* (electroporation) displayed resistance to 4-hydroperoxycyclophosphamide (4-HC), an active derivative to cycophosphamide, as compared to the normal cells. Addition of a specific inhibitor for *ALDH1* restored the sensitivity of the *ALDH1*-expressing K 562 cells to 4-HC (42,46).

5. *ALDH1* transduction into peripheral blood human hematopoietic progenitor cells led to significant increases (4- to 10-fold) cyclophosphamide resistance in an in vitro colony-forming assay (46). These findings indicate that ALDH1 overexpression is sufficient to induce cyclophosphamide resistance in vitro and provide a basis for testing the efficacy of *ALDH1* gene transduction to protect bone marrow cells from high-dose cyclophosphamide in vivo (47).

6. In Hodgkin's and non-Hodgkin's lymphoma patients treated with CP, a substantial decrease in enzymatic oxidation of MF by erythrocyte lysate was observed as compared to healthy controls (48).

7. After long-term culture, bone marrow progenitor cells from CML patients showed a significant increase in ALDH activity whether or not the cells were pretreated with MF (44).

8. Studies by Metzenthin et al. (48) and Dockham et al. (49) have clearly shown that aldophosphamide is metabolized by blood and suggest an important in vivo role for the erythrocyte ALDH (*ALDH1*) in systemic aldophosphamide detoxification.

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Based upon the recent findings discussed above, the following potentially clinical strategies may prove useful in cancer chemotherapy:

1. Individualizing cancer chemotherapeutic regimens based, at least in part, on the levels of *ALDH1* and *ALDH3* in the malignancy of interest.

2. Sensitizing tumor cells that express relatively large amounts of *ALDH1* and/or *ALDH3* to the oxazaphosphorines by preventing the synthesis of these enzymes, e.g., with antisense RNA, or by introducing an agent that directly inhibits the catalytic action of the operative enzyme (50).

3. Decreasing the sensitivity of hematopoietic cells to the oxazaphosphorines by selectively transferring into them the genetic information that encodes *ALDH1* or *ALDH3*.

4. Use of ALDH in the isolation of hematopoietic progenitors: *ALDH1* is expressed at high levels in hematopoietic progenitors. A fluorescent substrate for ALDH, termed BODIPY aminoacetaldehyde (BAAA), that consists of an aminoacetaldehyde moiety bonded to the BODIPY fluorochrome has been developed (51). Staining with BAAA may provide a simple and efficient strategy for isolating primitive human hematopoietic cells.

VIII. CONCLUDING REMARKS

This review has focused on the pharmacogenetic relevance of various ALDHs that play a pivotal role in the detoxification of aldehydes generated from drugs, xenobiotics, and endobiotics (ethanol, amines, etc.). Allelic variant of *ALDH2* isoenzyme is deterimental in alcohol sensitivity (flushing), alcohol drinking habits, alcohol dependence, and alcohol-induced end-organ damage in East Asians (see Chapter 1).

In addition, ALDHs play an important role with regard to the therapeutic index of, and resistance to, cyclophosphamide and other oxazaphosphorines. Thus, modulation of ALDH-catalyzed reactions can have a crucial effect on the therapeutic efficiency of anticancer drugs such as cyclophosphamide.

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4

Alcohol Metabolism by Gut Flora

Metabolic, Pathogenetic, and Clinical Implications

Mikko Salaspuro
University Central Hospital of Helsinki, Helsinki, Finland

I. INTRODUCTION

After its oral intake alcohol is absorbed to the portal blood from the stomach and upper part of the small intestine. Thereafter it is rapidly transported by blood circulation to other organs including the whole digestive tract. Ethanol is evenly distributed to the water phase of all organs, and accordingly, after the distribution phase, ethanol levels in saliva, tears, and urine as well as in the contents of terminal ileum and colon are equal to those of the blood and the liver. There is more and more evidence that alcohol is not merely an innocent bystander in the gastrointestinal tract. Ethanol can be oxidized to acetaldehyde by many microbes representing normal gut flora and also by glands and mucosa of the digestive tract. However, as compared to the liver, the oxidation of acetaldehyde by either microbes or mucosal tissues is limited. This results in strikingly high salivary, intragastric, and intracolonic levels of reactive, toxic, and carcinogenic acetaldehyde. Therefore, intraluminal acetaldehyde generated locally in the digestive tract is a possible candidate in the pathogenesis of alcohol-related gastrointestinal symptoms and diseases such as diarrhea and alcoholic liver injury. More importantly, acetaldehyde has recently been shown to be a local carcinogen in the upper aerodigestive tract of humans.

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II. MICROBIAL ALCOHOL DEHYDROGENASES AND ENDOGENOUS ETHANOL

There is an array of microbial alcohol dehydrogenases. They display a variety of substrate specificities and they fulfill several vital but quite different physiological functions (1). Some are used in the production of alcoholic beverages, industrial solvents, or vinegar; others may participate in the degradation of naturally occurring and xenobiotic compounds (1). Under anaerobic conditions these microbes are capable of producing energy through fermentation (2). In alcoholic fermentation the end-product is ethanol, which is derived from acetaldehyde in a reductive reaction mediated by bacterial alcohol dehydrogenase (3). The reaction runs as follows:

Alcoholic fermentation

$$\text{Glucose} \longrightarrow \text{Acetaldehyde} \xrightarrow{\text{ADH}} \text{Ethanol}$$

Owing to the alcoholic fermentation, small amounts of endogenous ethanol have earlier been found in the contents of the alimentary tract and portal blood of normal rats (4). An enhanced production of endogenous ethanol as a result of bacterial overgrowth has been demonstrated in jejunal blind-loop contents of rats (5). In humans significant endogenous ethanol levels have been found in jejunal aspirates of patients with tropical sprue (6), and in venous blood of patients after jejunoileal bypass for morbid obesity (7). In gastric juice, ethanol levels up to 27 mM (>100 mg%) have been measured in patients using cimetidine or antacids (8). This was suggested to be caused by the raised gastric intraluminal pH associating with increased bacterial growth. Thirty-nine cases of intragastrointestinal alcohol fermentation syndrome, manifesting as alcohol intoxication after a carbohydrate-rich diet in patients with gastrointestinal abnormalities and overgrowth of *Candida albicans*, have been described in Japan (9). Endogenous blood alcohol levels up to 90 mg% could be demonstrated in these patients.

III. MICROBIAL ACETALDEHYDE PRODUCTION

A. Acetaldehyde in the Saliva and Upper Gastrointestinal Tract

In contrast to the conversion of acetaldehyde to ethanol during alcoholic fermentation, the reaction catalyzed by microbial alcohol dehydrogenase can also run into the opposite direction with acetaldehyde as an end-product. An example of this type of reversed reaction is acetaldehyde production in vitro by human mouth

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and bronchopulmonary washings (10). In vivo, microbial acetaldehyde production from ethanol has been reported to occur in the oropharynx of healthy subjects (11), and in the intestinal contents of rats with a jejunal blind loop (5).

Ethanol is present in saliva in concentrations comparable to blood ethanol levels after the consumption of alcoholic beverages (12). Marked amounts (up to 143 μM) of acetaldehyde can be detected in the saliva of healthy volunteers after ingestion of a moderate dose of ethanol (0.5 g/kg body weight) (Fig. 1) (13). This is 10–20 times more than systemic blood acetaldehyde levels after even considerably higher doses of alcohol (14). Salivary acetaldehyde levels show remarkable interindividual variation and are clearly reduced after a 3-day use of an antiseptic mouthwash (Fig. 1) (13). Smoking, heavy drinking, and poor dental status are well-known risk factors of the upper aerodigestive tract cancers, and they are also the strongest factors increasing salivary acetaldehyde production (15,16). Increased in vitro acetaldehyde production has been demonstrated by the mouthwashings of patients with oral cavity, laryngeal, or pharyngeal cancer (17). Especially, gram-positive aerobic bacteria and yeasts appear to be frequently associated with the high-acetaldehyde-producing salivas (15,18).

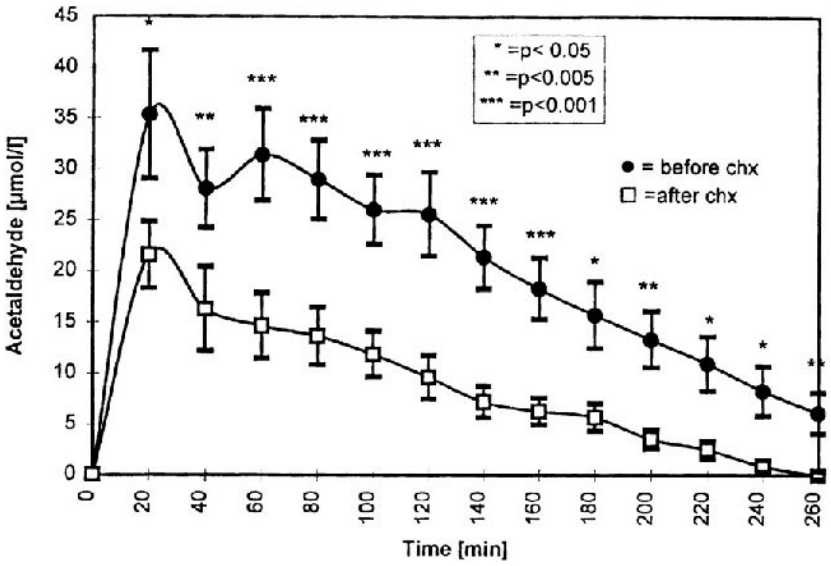

Figure 1 The effect of chlorhexidine (0.2%) mouth rinsing (twice daily for 60 sec) on acetaldehyde levels in the saliva after a moderate dose of ethanol (0.5 g/kg body weight). (Adapted from Ref. 13.)

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B. Acetaldehyde in the Stomach

1. Helicobacter pylori *Alcohol Dehydrogenase*

According to seroepidemiological studies, 20–50% of the population in developed countries is infected with *Helicobacter pylori* and in developing countries the prevalence is much higher (19). It is generally agreed that the presence of *H. pylori* in gastric mucosa almost invariably leads to chronic active gastritis. Furthermore, *H. pylori* is an important pathogenetic factor behind peptic ulcer disease and gastric cancer (20).

Initial studies in our laboratory revealed that two *H. pylori*–type strains possess significant alcohol dehydrogenase activity (21). The activity increased considerably with increasing ethanol concentrations, with the K_m for ethanol oxidation being in the range of 65–100 mM (22,23). The optimal pH for ethanol oxidation by *H. pylori* alcohol dehydrogenase is 9.6, but marked alcohol dehydrogenase activity can be observed already at neutral pH (22,23). 4-Methylpyrazole competitively inhibits the enzyme, but only at relatively high concentrations of the compound (23). A potent and competitive inhibitor of the *H. pylori* alcohol dehydrogenase is colloidal bismuth subcitrate (24,25). In line with their alcohol dehydrogenase activity *H. pylori*–type strains are capable of producing small amounts of ethanol when cultured in broth under microaerobic conditions (26). Compared to human alcohol dehydrogenase isoenzymes, *H. pylori* enzyme shows distinct kinetic characteristics (23) and a different pattern in isoelectric focusing (22).

2. *Acetaldehyde Production by* Helicobacter pylori

By virtue of possessing alcohol dehydrogenase activity cytosols prepared from two *H. pylori*–type strains produce considerable amounts of acetaldehyde from ethanol in a concentration-dependent manner (21,22,26). Among 30 *H. pylori* strains of human origin there was a positive association between the capacity of the bacterial cytosol to produce acetaldehyde and its alcohol dehydrogenase activity (27). Maximal acetaldehyde formation took place at pH 9.6, but it was marked also at physiological pH (22,23). 4-Methylpyrazole inhibited acetaldehyde production only at rather high concentrations (22). By contrast, colloidal bismuth subcitrate and omeprazole were fairly effective inhibitors of acetaldehyde formation by *H. pylori* (24,25). In addition to the bacterial cytosol, intact bacterial cells were capable of producing notable amounts of acetaldehyde from ethanol in a liquid culture medium (pH 7.4) (26). In contrast to somatic cells *H. pylori* appeared not to have any aldehyde dehydrogenase activity (22). Consequently, the microbe is not able to metabolize acetaldehyde efficiently produced from ethanol via its own ADH-pathway.

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3. Acetaldehyde Production in the Stomach In Vivo

Owing to its acidity normal human stomach is free of microbes. However, microbes may survive with a pH over 4.0 and bacterial proliferation can be expected when the pH rises over 5.0 (28,29). Therefore, gastric and duodenal microbial overgrowth is common during long-term use of gastric proton pump inhibitors (30,31) or H_2-receptor antagonists (32–34) as well as in certain similar conditions, e.g., atrophic gastritis with achlorhydria (35). Recently we were able to demonstrate in human volunteers that the use of gastric proton pump inhibitors for 1 week resulted in enhanced intragastric production of acetaldehyde from ingested ethanol (36). This was associated with a significant overgrowth of both aerobic and anaerobic bacteria in the stomach. Furthermore, there was a positive correlation ($r = 0.9$; $p < 0.001$) between gastric juice acetaldehyde level and aerobic bacterial count (36). High acetaldehyde production capacity has also been demonstrated in vitro from gastric juices of patients with atrophic gastritis (37). Seitz and Pöschl point to results with a similar trend in their 1997 review (38).

C. Acetaldehyde in the Large Intestine

1. Colonic Flora

The most richly colonized site of the digestive tract is the large intestine. More than 400 different bacterial species and 10^{14} individual bacteria inhabit a single human colon at a given time (39). Accordingly, the number of intestinal bacteria is 10-fold as compared to the number of cells in the human host (40). The metabolic capability of the colonic microflora has been estimated to be at least as great as that of the liver (41), or even exceed that of the whole human body (42). The volume of the large intestine is about 540 ml, and it receives approximately 1.5 kg of material from the small bowel each day. Most of this is water, which is rapidly absorbed. The adult large intestine contains around 220 g of contents (43), with an average daily fecal output of 120 g (44). Bacteria are the major component of feces, comprising approximately 55% of solids (45).

2. Alcohol and Aldehyde Dehydrogenases of the Bacteria Representing Human Colonic Flora

There are considerable differences in the alcohol dehydrogenase activity and acetaldehyde-producing capacity among the aerobic bacteria representing the normal human colonic flora (46). The mean cytosolic alcohol dehydrogenase activity of some bacteria can be up to 30 times higher than that of the rat liver when determined at similar conditions (Fig. 2) (46). The highly significant positive correlation between bacterial alcohol dehydrogenase activity and their acetalde-

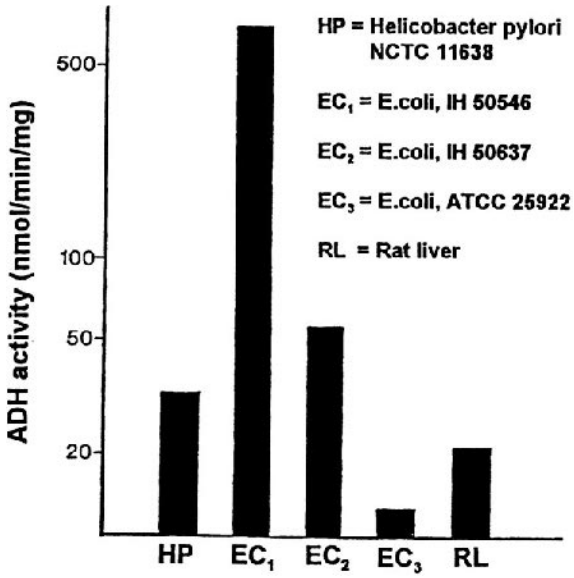

Figure 2 Alcohol dehydrogenase activity of some bacteria representing the human gut flora as compared to alcohol dehydrogenase activity of rat liver. (Adapted from Ref. 46.)

hyde-producing capacity from ethanol strongly suggests the catalytical role of microbial alcohol dehydrogenase in the production of acetaldehyde from ethanol (46).

Alcohol dehydrogenases of the bacteria tested thus far representing human colonic flora show a variety of K_m values for ethanol ranging from 0.06 to 29.9 mM (47). These values are comparable to the ethanol concentrations found in the large intestine during moderate drinking. Under these circumstances bacterial alcohol dehydrogenases are able to metabolize ethanol with a velocity close to maximal, and, accordingly, to produce marked amounts of acetaldehyde (47). On the other hand, the rather high K_m values for ethanol of some bacterial alcohol dehydrogenases (47) explain the close association between increasing intracolonic acetaldehyde and ethanol levels found in vivo in pigs (48).

Acetaldehyde administered intracolonically to the pigs is rapidly eliminated, and this is associated with an increase in intracolonic acetate and ethanol levels (48). Obviously intracolonic acetaldehyde is effectively metabolized partly by colonic mucosal cells and partly by intracolonic microbes. Indeed, many aerobic bacteria representing the normal flora of the human large intestine possess significant cytosolic $NADP^+$- and NAD^+-dependent aldehyde dehydrogenase

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(ALDH) activity (49). Furthermore, these bacteria metabolize acetaldehyde to acetate effectively in vitro (49). According to their apparent K_m values, aldehyde dehydrogenases of the aerobic colonic bacteria are able to metabolize endogenous acetaldehyde (50). However, the ability of bacterial ALDHs to oxidize the higher concentrations of intracolonic acetaldehyde associated with alcohol consumption is low. At ethanol and acetaldehyde concentrations found in the colon after moderate drinking, the ALDH activities of most of the bacteria are markedly lower than the ADH activities (50). This finding offers an additional explanation for the mechanism of the accumulation of ethanol-derived acetaldehyde in the colon after ethanol intake. Individual variation in the capability of colonic flora to produce or to remove acetaldehyde may thus be one factor regulating intracolonic acetaldehyde levels during ethanol oxidation. In addition, acetaldehyde is easily absorbed from the colon to the portal blood (51), and, accordingly, may be metabolized further also in the liver.

3. Acetaldehyde Production in the Large Intestine by Colonic Bacteria

As already mentioned, the last reaction catalyzed by microbial alcohol dehydrogenase in alcoholic fermentation can run also in the opposite direction with acetaldehyde as an end-product. Human intestinal strains of *Escherichia coli* possessing ADH activity are able to oxidize ethanol to acetaldehyde under both aerobic and microaerobic conditions (52). On the other hand, under anaerobic conditions these bacteria ferment glucose to ethanol (52).

The reversed microbial ADH reaction produces striking amounts of acetaldehyde also when human colonic contents are incubated in vitro at 37°C with increasing ethanol concentrations (53). It should be emphasized that this reaction is active already at comparatively low (10–100 mg%) ethanol concentrations known to exist in the colon during and after normal drinking (48,53). Moreover intracolonic acetaldehyde formation takes place at a pH normally found in the colon and is rapidly reduced with lowering of the pH (53). It remains to be established whether lactulose or a high-fiber diet, which can decrease fecal pH, might also be able to decrease colonic acetaldehyde production from ethanol in vivo.

After an acute dose of ethanol to rats, acetaldehyde levels higher than in the liver or in the blood have been demonstrated in colonic mucosa (54). Since in that study mucosal acetaldehyde levels were significantly lower in germ-free than in normal animals, mucosal acetaldehyde was suggested to be generated at least in part through bacterial ethanol oxidation (54). Our recent in vivo studies in pigs demonstrate that intracolonic levels increase in parallel with increasing blood and intracolonic ethanol concentrations (48).

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4. Bacteriocolonic Pathway for Ethanol Oxidation

Our results suggests the existence of a bacteriocolonic pathway for ethanol oxidation. In this pathway intracolonic ethanol is oxidized to acetaldehyde and further to acetate. The reaction runs as follows:

Bacteriocolonic pathway for ethanol oxidation

$$\text{Ethanol} \xrightarrow{\text{bacterial, mucosal ADH, bacterial catalase}} \text{Acetaldehyde}$$

$$\text{Acetaldehyde} \xrightarrow{\text{mucosal or bacterial ALDH}} \text{Acetate}$$

$$\text{Acetaldehyde} \xrightarrow{\text{Absorption to portal blood}} \text{LIVER}$$

In this pathway, intracolonic ethanol is at first oxidized to acetaldehyde in a reaction catalyzed by bacterial or colonic mucosal alcohol dehydrogenase. Moreover, a significant proportion of microbial acetaldehyde production from ethanol may be mediated by microbial catalase (55). In a subsequent reaction acetaldehyde is oxidized by colonic mucosal or bacterial aldehyde dehydrogenase to acetate. Furthermore, acetaldehyde may be metabolized by some other thus-far-undetermined microbial pathways existing even in other microbes to a variety of substrates. Acetate is further metabolized by either colonic mucosal cells or bacteria or it may be excreted via the fecal route. An additional possibility is that part of intracolonically produced acetaldehyde and/or acetate is absorbed to the portal blood and is metabolized further in the liver (51).

5. Inhibition of Bacteriocolonic Pathway by Ciprofloxacin

The quantitative role and rate-limiting steps of the bacteriocolonic pathway for ethanol oxidation have recently been clarified in studies in which antibiotics have been used to induce selective changes in normal gut flora. The reduction of aerobic gastrointestinal flora with ciprofloxacin decreases the total ethanol elimination rate by 9% in rats (56). This is associated with a significant decrease in the mean ADH activity of the fecal samples (56), in almost total abolishment of the formation of endogenous ethanol in the colon, and in a remarkable reduction of the intracolonic acetaldehyde production from ethanol (57). Interestingly, ciprofloxacin treatment also totally abolished the alcohol-induced enhancement in ethanol elimination rate in rats that had received ethanol chronically for 28 days (58). These findings have been confirmed also in humans. Ciprofloxacin 750 mg twice a day for 7 days inhibited the rate of ethanol elimination by 9.4% in human volunteers and concomitantly decreased fecal ADH activity and acetaldehyde production in vitro (59). Ciprofloxacin, however, inhibited neither hepatic alcohol dehydrogenase activity nor the microsomal ethanol oxidizing system in the liver (59).

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6. Enhancement of Intracolonic Acetaldehyde Production by Metronidazole

In contrast to ciprofloxacin, metronidazole is an effective antianaerobic agent leading to the overgrowth of aerobic flora in the large intestine. Long-term treatment with metronidazole induced a fivefold increase in the intracolonic acetaldehyde level of rats receiving an ethanol-containing liquid diet for 6 weeks (60). Metronidazole, however, did not affect either blood acetaldehyde levels or the hepatic or colonic mucosal ADH or ALDH activities (60). The increase in intracolonic acetaldehyde level was associated with increased growth of *Enterobacteriacae* in cecal cultures (60). The findings obtained in the studies with selective antibiotics suggest that the role of bacteriocolonic pathway in total ethanol elimination is quantitatively important and may explain most of the extrahepatic metabolism of ethanol known to be existing already for decades (61–63).

7. Some Other Characteristics of the Bacteriocolonic Pathway

The anaerobic conditions prevailing in the colon may not in general favor the oxidation of ethanol and acetaldehyde by bacterial enzymes. It is well known that in fecal flora anaerobic organisms outnumber facultative organisms by a factor of 1000. However, owing to the diffusion of oxygen from the colonic mucosa, the mucosa-associated flora contains as many or even more aerobes than anaerobes (64). Experiments with animals ensure that there is significant oxygen diffusion from the blood to the intestinal mucosa. Comparative studies with germfree and conventional rats suggest the presence of a flora that consumes O_2 and produces CO_2 in the animals of the latter group (65). One can therefore assume that the presence of microaerobic conditions for the mucosa-associated flora is enough for conversion of the microbial-ADH reaction to the direction of acetaldehyde (52).

As compared to the metabolism of ethanol in the liver, bacteriocolonic pathway for ethanol oxidation differs in some important respects. Mitochondrial and cytosolic aldehyde dehydrogenase activities of rat colonic mucosa are approximately six times lower than corresponding activities in the liver (66). Aldehyde dehydrogenase activity of the colonic mucosa may thus be sufficient for the removal of acetaldehyde produced by colonic mucosal alcohol dehydrogenase during ethanol oxidation, but it may be insufficient for the removal of acetaldehyde produced by intracolonic bacteria. This favors the accumulation of acetaldehyde in the colon during ethanol oxidation. Indeed, we have been able to demonstrate variable and sometimes strikingly high intracolonic acetaldehyde levels, up to 2.7 mM, after a moderate dose (1.5 g/kg/body weight) of ethanol to rats (57). The great variability in intracolonic acetaldehyde levels during ethanol oxidation probably can be explained by individual variations in alcohol- and acetaldehyde-metabolizing enzymes of the intestinal microbes.

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IV. IMPLICATIONS

A. Metabolic Implications

1. Contribution of Bacteriocolonic Pathway to Total Ethanol Elimination

Our studies with ciprofloxacin suggest that although liver is the main organ for ethanol oxidation at least 10% of the total ethanol elimination takes place in the digestive tract of both rats (56) and humans (59). The percentage may be even greater in chronic alcoholics, since at least in rats fed alcohol chronically ciprofloxacin treatment totally prevented the induction of ethanol elimination rate (58). It can be concluded that the digestive tract is probably the most important organ for extrahepatic elimination of ethanol, and that the induction of the bacteriocolonic pathway for ethanol oxidation during chronic alcohol administration may explain, at least in part, the enhanced rate of ethanol elimination observed even in the presence of relatively severe alcoholic liver injury in humans (67,68).

2. Microbial Origin for Metronidazole-Related Disulfiram Reaction

Several clinical studies, done in the 1960s, reported that metronidazole therapy in human alcoholics reduces the desire for alcohol and produces mild disulfiram-like reactions in some patients after ingestion of ethanol (69). Later controlled studies have, however, rather uniformly shown it to be of no benefit in the treatment of alcoholism (69). Nevertheless, patients taking metronidazole are still usually warned to avoid alcohol owing to the risk of disulfiram-like reaction. Metronidazole is an effective antianaerobic agent, which leads to the overgrowth of aerobic flora in the large intestine. This shift in the gut flora results in a fivefold increase in the intracolonic acetaldehyde level during both short- and long-term alcohol ingestion in rats (60,70). Unlike disulfiram, metronidazole neither inhibits liver ALDH nor increases blood acetaldehyde (60). Consequently, the mechanism behind metronidazole-related disulfiram reaction might be located to the gut flora instead of the liver (60).

B. Pathogenetic Implications

1. Acetaldehyde as a Local Carcinogen in the Digestive Tract

Chronic alcohol consumption is a strong determinant of enhanced cancer risk in the upper aerodigestive tract in humans (71). However, although epidemiological data for the increased cancer risk in alcoholics are convincing, the tumor-promoting effect of alcohol remains unclear since ethanol itself is not carcinogenic (71). In contrast, there is increasing evidence that the first metabolite of

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ethanol oxidation—acetaldehyde—is carcinogenic not only in animals but also in humans (see Homann, this volume).

Many recent epidemiological studies have shown that the risk of ethanol-associated digestive tract cancers is remarkably increased in Oriental subjects with partially inactive ALDH2 enzyme (72–74). The biochemical background of this association has so far been hypothesized to be due to the systemic effects of elevated blood acetaldehyde. Recently, however, we found that in addition to oral microflora parotid gland may also produce marked amounts of acetaldehyde from ethanol into the saliva in ALDH2-deficient subjects (75). After ingestion of a moderate dose of alcohol (0.5 g/kg body weight) Oriental flushers appeared to have two to three times higher acetaldehyde levels in their saliva than the nonflushers throughout the whole observation period of 240 min (75). Importantly, the flushers had nine times higher acetaldehyde levels in their saliva than in their blood (75), which indicates that the origin for salivary acetaldehyde is located in the parotid gland and not the blood. When this new information is combined with earlier epidemiological data, the Oriental human ''knockout model'' for deficient acetaldehyde oxidation proves that acetaldehyde produced from ethanol via microbes, mucosal cells, and/or glands is a local carcinogen in the digestive tract in humans.

2. Acetaldehyde as a Possible Local Toxic Agent in the Digestive Tract

As reviewed earlier, the bacteriocolonic pathway for ethanol oxidation results in strikingly high intracolonic acetaldehyde levels. High acetaldehyde concentration in the large intestine could act as a local irritant. Histological and ultrastructural specimens of rectal mucosa of heavy drinkers reveal marked pathological changes (76). On light microscopy there is a decrease in the number of goblet cells both in the surface epithelium and in the crypts. In addition, the lamina propria may be heavily infiltrated with mononuclear cells. Electron microscopy reveals swollen and distorted mitochondria and dilated vesicular endoplasmic reticulum. The abnormalities disappear after 2 weeks' abstinence (76). A possible candidate behind these pathological changes could be reactive and toxic acetaldehyde produced locally from ethanol by the aerobic gut flora.

The pathogenesis of endotoxemia induced by alcohol and/or liver injury is unknown (77). One possibility is that chronic alcoholism increases the intestinal permeability of the gut (78). In this process elevated intracolonic acetaldehyde level could be an important pathogenetic factor. This theory is supported by a recent finding showing that acetaldehyde increases the paracellular permeability of the Caco-2 cell monolayer (79). Accordingly, acetaldehyde generated in vivo in the gastrointestinal tract of alcoholics might cause a similar increase in the intestinal paracellular permeability, thus leading to enhanced endotoxin absorp-

tion (79). Interestingly, only the alcoholics with chronic liver disease do have increased gastrointestinal permeability, and it has been suggested that a ''leaky gut'' may be a necessary cofactor for the development of chronic liver injury in heavy drinkers (80). Alternatively, heavy drinking may also alter the composition of the intestinal (81) or fecal flora.

The variability in the characteristics of different microbial alcohol dehydrogenases may also have some other rare pathogenetic implications. For instance, the very low K_m of the ADH of *Hafnia alvei* for ethanol (0.06 mM) (47) allows it to metabolize intracolonic endogenous ethanol (0.4–0.6 mM) to acetaldehyde. In theory, the increased intracolonic endogenous acetaldehyde levels might explain, at least in part, the pathogenesis of *H. alvei*–associated diarrhea (82). It can also be hypothesized that the large variation in the levels of endogenous acetaldehyde (48) may reflect individual variability in the kinetics of microbial alcohol and aldehyde dehydrogenases.

3. Gut-Derived Acetaldehyde as a Possible Hepatotoxic Factor

Acetaldehyde is a highly reactive compound that has been linked to several organ-toxic effects of ethanol (83). Acetaldehyde binds covalently with macromolecules and proteins thus forming acetaldehyde adducts (84,85). Acetaldehyde binding with tissue proteins may initiate several biochemical and immunological reactions, which have been related especially to the pathogenesis of alcoholic liver injury (86,87) (see Jokelainen, this volume). Not only acetaldehyde formed in the liver, but also that generated by microbes in the gut could have hepatotoxic effects. Acetaldehyde is easily absorbed to the portal blood from the large intestine (51), and acetaldehyde-protein adducts are formed in the liver of animals following acetaldehyde administration in drinking water (88). Moreover, acetaldehyde delivered to the rats in drinking water may produce microvesicular fatty infiltration of the liver even in the absence of ethanol (51).

C. Clinical Implications

There is increasing evidence that the digestive tract with its flora forms an important organ for ethanol metabolism and acetaldehyde production. Under normal conditions about 10% of ethanol is metabolized by this extrahepatic pathway. In addition, a bacteriocolonic pathway for ethanol oxidation is induced by chronic alcohol consumption. In theory, an extrahepatic alcohol-oxidizing pathway may thus protect the liver by decreasing its alcohol load. On the other hand, the bacteriocolonic pathway may produce or release toxic factors via the portal circulation to the liver. Since intracolonic ethanol is partly oxidized in the colon by bacterial enzymes, all ethanol calories may not become available for the human body. Part of ethanol-derived calories may be excreted via the fecal route; therefore, the bacteriocolonic pathway offers a new explanation for the well-known disappearance of a proportion of ethanol calories (89).

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Diarrhea and flatulence are the most frequent gastrointestinal symptoms in chronic alcoholics (90), but their pathogenesis has so far, by and large, been unknown. Ethanol has been demonstrated to decrease both the frequency and amplitude of basal motility waves in the rectosigmoid in humans (91), and in alcoholics with diarrhea there is a rapid oral-cecal transit time (92). A possible candidate for this type of gastrointestinal motility changes could also be acetaldehyde derived from the bacterial metabolism of ethanol.

Many alcohol-associated hypersensitivity reactions, e.g., the Oriental flushing reaction, appear to be attributable to acetaldehyde rather than to ethanol itself. Alcohol sometimes causes urticaria and anaphylactoid reactions in Caucasian individuals as well (93). These hypersensitivity reactions can be blocked by antihistamines (94). In line with these observations, we found that acetaldehyde, at concentrations of 50–100 μM, significantly increases the release of histamine from rat peritoneal mast cells (95) and human blood basophils (96). Especially, basophils from allergic patients released histamine at acetaldehyde concentrations found under some conditions in human blood after alcohol consumption (96). It is conceivable that acetaldehyde-induced histamine release either from the cells of the digestive tract or from blood basophils may contribute to some adverse reactions caused by alcohol drinking, especially in humans with allergic diseases.

The most important clinical implication, however, is the local carcinogenic action of acetaldehyde in the digestive tract. Genetic factors, smoking habits, individual differences in the gut microflora, and blood and intestinal ethanol levels—either exogenous or endogenous—may all modify the carcinogenic action of acetaldehyde in the gastrointestinal tract. All these items provide a new genetic and microbiological approach for the screening and prevention of digestive tract cancers.

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5

Distribution of Alcohol Dehydrogenase in Human Organs

Relevance for Alcohol Metabolism and Pathology

Xavier Parés, Susana E. Martínez, Abdellah Allali-Hassani, Emma Borràs, Jaume Farrés, Sílvia Martras, Albert Rosell, and Julia Vaglenova

Universitat Autònoma de Barcelona, Bellaterra, Barcelona, Spain

I. INTRODUCTION

Alcohol dehydrogenase (ADH) exhibits multiple forms in humans. At protein level, four ADH classes (I–IV) have been identified in human tissues, while an additional class (V) has been detected at the mRNA level (1). Human ADH classes I, II, and IV exhibit appropriate kinetic constants for an effective ethanol metabolism at the blood ethanol concentrations attained after social drinking (2). Class III activity with ethanol is detected only at very high concentrations ($>$100 mM) and, therefore, its contribution to ethanol metabolism should normally be minimal. The presence of ADH classes I, II, or IV in a tissue indicates an active ethanol metabolism after consumption of alcoholic beverages. Most ADH activity in the human body is concentrated in liver, due to high content of classes I and II (about 3% of the hepatic soluble protein). However, many organs other than liver exhibit a detectable ADH activity, because of the presence of classes I and/or IV—at the protein level, class II has been detected only in liver and skin (3). The contribution of extrahepatic organs to ethanol metabolism is small but significant. The study of the activity and ADH forms of each organ should serve to estimate the amount of local ethanol metabolism, and the consequent production of acetaldehyde and increase in NADH concentration. This may have pathological consequences and may explain some of the effects of ethanol in the organ.

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Moreover, distribution of ADH in nonliver organs should reflect a role of the enzyme other than ethanol elimination. The wide substrate specificity of ADH makes the enzyme suitable for the transformation of many alcohols and aldehydes of physiological interest: ω-hydroxyfatty acids, 4-hydroxynonenal and other aldehydes of lipid peroxidation (4,5), steroids (6), and intermediate compounds in the metabolism of dopamine (7), norepinephrine (8), and serotonine (9). ADH oxidizes retinol and reduces retinal (4,5,10) and probably performs an important role in the pathway of retinoic acid formation (11,12). Retinoic acid is the active metabolite of vitamin A and, through the binding to specific nuclear receptors, plays an indispensable function in the regulation of development, differentiation, growth, and epithelial maintenance (13). The presence of ADH in many organs may be essential to regulate the production of retinoic acid from retinol. The metabolism of any of these mentioned physiological compounds by ADH could be disturbed by the competitive presence of ethanol, with possible effects on the organ physiology. The present review, however, will focus on the relationship between ADH localization and ethanol and retinol metabolism.

The distribution of ADH classes and activities in organs is best known for the rat (14) and mouse (15,16), and can be used as a model of what may occur in humans. However, study in humans reveals that the enzyme distribution exhibits remarkable differences from that of rodents, and that activity of some ADH forms notably differs between species. Thus K_m of class IV for ethanol is 40 mM in humans and 2.4 M in rats (17). This makes the information on rodent ADH only partially useful, and it is necessary to study human tissues.

In the present review we will emphasize the enzymatic data obtained in tissue homogenates of adult individuals, either by activity measurement in the spectrophotometer or by activity staining of electrophoretic separations. This gives an estimate of the contribution of the enzyme to ethanol metabolism in each organ considered. Analysis of ADH expression by immunohistochemistry and Northern blot will be also summarized.

II. ADH CLASSES, ISOZYMES, POLYMORPHISM, AND SUBSTRATE SPECIFICITY

As mentioned earlier, only classes I, II, and IV have appropriate kinetic constants for an effective contribution to ethanol metabolism (Table 1). Class I constitutes a group of multiple isozymes, homodimers or heterodimers of the α, β, and γ subunits, coded by *ADH1*, *ADH2*, and *ADH3* genes, respectively. *ADH2* and *ADH3* exhibit polymorphism, encoding the monomers β_1, β_2, and β_3, and γ_1 and γ_2, respectively. Since all monomers of class I can cross-hybridize to form dimers, this results in a complex isoenzymatic pattern in organs like liver, where all class I genes are expressed (Fig. 1). Class II comprises homodimers of the π-ADH

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Table 1 Kinetic Constants with Ethanol and All-*trans*-Retinol of Human Alcohol Dehydrogenase Forms

		Ethanol			All-*trans*-retinol		
Gene (allele)	Class, isozyme	K_m (mM)	k_{cat} (min^{-1})	k_{cat}/K_m ($mM^{-1}\cdot min^{-1}$)	K_m (mM)	k_{cat} (min^{-1})	k_{cat}/K_m ($mM^{-1}\cdot min^{-1}$)
ADH1	I, αα	4.2	24	5.7	0.056	5.1	92
*ADH2*1*	I, $\beta_1\beta_1$	0.05	9	180	0.045	0.9	20
*ADH2*2*	I, $\beta_2\beta_2$	0.94	340	361.7	0.061	0.48	7.8
*ADH2*3*	I, $\beta_3\beta_3$	36	320	8.9	0.06	6.6	110
*ADH3*1*	I, $\gamma_1\gamma_1$	1	88	88	0.29	5.5	19
*ADH3*2*	I, $\gamma_2\gamma_2$	0.6	35	58.3	0.043	18.1	420
ADH4	II, ππ	34	20	0.6	0.014	9.1	650
ADH7	IV, σσ	37	1510	40.8	0.015	67	4500

Kinetic constants were determined at pH 7.5, 25°C, except for class I $\gamma_2\gamma_2$ with retinol (pH 7.3, 37°C). *Source*: Constants for ethanol were from Ref. 2 (classes I and II) and from Ref. 17 (class IV). Results for all-*trans*-retinol were from Refs. 10 and 18 (classes I and II) and Ref. 5 (class IV).

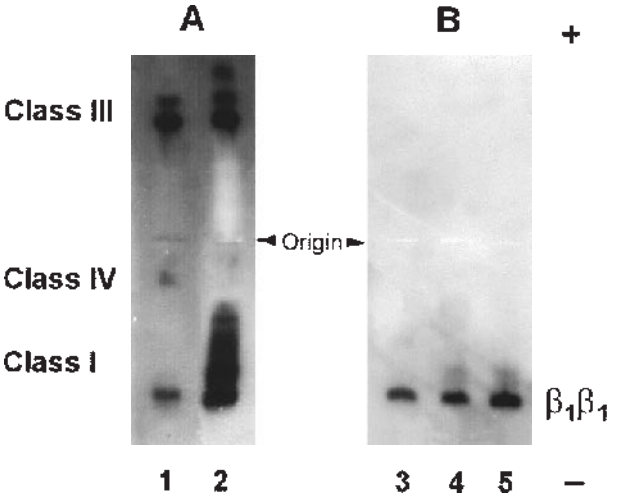

Figure 1 Starch gel electrophoresis analysis and activity staining with 0.1 M 2-buten-1-ol (A), and 0.2 M ethanol (B) of different human sample homogenates. 1, aorta; 2, liver; 3, 4, and 5, superior vena cava, portal vein and aorta, respectively, from the same individual. (From Ref. 19.)

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subunit, while class IV is a homodimer of σ-ADH (also called μ-ADH) (20). Interclass pairwise comparisons of amino acid sequences show about 60–70% identity. The three class I ADHs share about 94% sequence identity, while the allelozymes from the same gene differ in only one or two positions. In spite of the close structural relationships between ADH forms, kinetic constants show significant differences (Table 1). Considering the polymorphic character of *ADH2* and *ADH3*, the differences in kinetic constants with ethanol (mostly k_{cat}) between the corresponding allelozymes may result in variations in the capacity of oxidizing ethanol. The most relevant difference occurs between $\beta_1\beta_1$ and $\beta_2\beta_2$, both having a low K_m for ethanol but $\beta_2\beta_2$ showing about 40 times higher k_{cat}. This polymorphism is frequent in the Asian population with frequencies of the *ADH2*2* allele, coding for β_2, of 60–80% (21). In nonalcoholic Europeans the *ADH2*2* frequency is only about 4% (22).

Except for class III, all other ADH forms can reversibly oxidize retinol to retinal (Table 1), which is the basis for the proposed role of ADH in retinoid metabolism. At the physiological concentrations of retinol in the lower micromolar range, ADH forms are far from being saturated and then their activity depends on the k_{cat}/K_m ratio. By far the most efficient ADH is class IV, which will be the most active human ADH under cellular conditions. The impaired retinol utilization in class IV ADH mutant mice also supports a relevant function of this enzyme in the generation of retinoic acid (12). It has been suggested that the true substrate for retinol oxidation is retinol bound to cytosolic retinol-binding protein (CRBP), which is the substrate for microsomal retinol dehydrogenase (13) but not for ADH (18). However, knockout experiments in mice demonstrate that CRBP is not indispensable for normal retinol oxidation (23). Moreover, 9-*cis*-retinol, a physiologically important retinoid in addition to the all-*trans* isomer (13), is a good substrate for human ADH (5) and does not bind to CRBP. These data support the involvement of ADH in the generation of retinoic acid.

Retinol oxidation activity of ADH is inhibited by low or moderate alcohol concentrations, with inhibition constants ranging from 0.04 to 10 mM ethanol (5,24). It has been estimated that the oxidation of retinol can be blocked 50–100% through the ADH-linked pathway by concentrations of ethanol (5–50 mM) physiologically attainable in moderate and heavy drinkers (24). Moreover, inhibition with ethanol may be enhanced *in vivo* because of the existence of CRBP, which can reduce the concentration of the free form of retinol in tissues (13,24). All these data strongly suggest that chronic ethanol consumption can interfere with the normal production of retinoic acid by the ADH pathway. Studies using homogenates of human fetuses support this notion (25). This finally could result in abnormalities produced by chronic ethanol consumption, in organs and tissues that need retinoic acid for maintenance and function. Interference with fetal and maternal metabolism of retinoids by ethanol, through the ADH pathway, has been also proposed to contribute to the development of the fetal alcohol syndrome (26).

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III. ADH IN LIVER

Most of the ethanol oxidation occurs in liver—probably more than 90% of the total oxidation (27). A hepatic activity of about 50 nmol/min/mg protein can be estimated (pH 10.0, 33 mM ethanol, 25°C, for *ADH2*1* individuals), which obviously will change between different subjects depending on their isozyme composition (Table 1). Liver expresses all class I and class II genes in large amounts. Except for $\beta_3\beta_3$, which has been detected only in some Afro-Americans (2), all other class I isozymes will be readily saturated after a moderate dose of ethanol, and they will work at maximum velocity. Under these conditions, class II will be the only ADH partially saturated. The contribution of class II will increase at elevated, highly toxic ethanol concentrations. At moderate ethanol concentrations, with class I saturated and class II partially saturated, an additional ethanol overload from the gastrointestinal tract through the portal vein could increase the liver oxidation velocity by raising the saturation level and, therefore, the activity of class II. This has been proposed as the origin of the hepatic contribution to the first-pass metabolism of ethanol (24).

The fact that acetaldehyde is in the low micromolar range during ethanol consumption (in *ALDH2*-active individuals) suggests that ALDH capacity in eliminating acetaldehyde exceeds that of ADH in generating this toxic compound. It is now believed that the major rate-limiting step in ethanol elimination is the liver ADH activity, while the NADH concentration responds to, rather than regulates, the rate of alcohol oxidation by ADH (28). This may change in *ALDH2*-deficient individuals, where an increase of hepatic and circulating acetaldehyde occurs. In this case the ALDH activity may be as important as the ADH activity for the regulation of ethanol metabolism (29). In alcohol liver disease, the total ADH activity decreases in proportion to the severity of the liver damage (30).

Liver ADH accounts also for the elimination of xenobiotic alcohols such as the highly toxic methanol and ethylene glycol. It is well proven that hepatic class I ADH and extrahepatic class IV are the major enzymatic systems for the elimination of retinol after an overdosis of this compound (12). Inhibition of ADH by 4-methylpyrazole alleviates the vitamin A toxicity by blocking the generation of retinoic acid, the most toxic retinol metabolite when present in excess (31).

IV. GASTROINTESTINAL ADH

ADH activity resulting from one or more ADH forms is present throughout the gastrointestinal tract, from mouth to rectum. Enzymatic analysis (Table 2) and immunohistochemical techniques (34) show that the mucosa is the layer with higher ADH content. The highest activity corresponds to the upper part of the tract, mouth, and mainly esophagus, owing to the high amount of $\sigma\sigma$ (class IV)

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Table 2 Alcohol Dehydrogenase in Gastrointestinal Tract

Organ	*N*	ADH activity (nmol/min/mg protein)	Major ADH forms
Mouth	3	14 ± 10	σσ
Esophagus	4	69 ± 14	σσ
Stomach	19	6.7 ± 1.7	σσ, γγ
Duodenum	3	5.5 ± 3.6	γγ
Colon	19	3.4 ± 1.1	γγ
Rectum	55	6.1 ± 0.8	γγ

Activities were measured with 100 mM ethanol, pH 10.0, 25°C (32), except for colon and rectum (580 mM ethanol, pH 9.6, 22°C) (33), using biopsy homogenates, except for oral mucosa samples, which were obtained at autopsy. Stomach samples were from men. Isozyme composition was determined by starch gel electrophoresis.

present in these areas. The activity results of Table 2, obtained from Refs. 32 and 33, are consistent with those from other reports, taking into account the methodological differences (ethanol concentration, pH, and temperature of the assay) (20,35–39). The high activity (per mg protein) in esophagus is impressive, of the same order as that in the liver. The activity decreases sharply in the stomach, with about 10 times less enzyme than in the esophagus. In the different parts of the intestine, the values of ADH activity are similar to those in stomach.

Class IV ADH (σσ) is the typical enzyme of the upper gastrointestinal tract, from mouth to stomach (Fig. 2). Stomach also exhibits the γγ form of class I, which is the characteristic isozyme of the intestine. While σσ and γγ are localized in the mucosa, ββ has been localized in muscular layers (36). The fact that βγ heterodimers have never been found in the digestive tract homogenates supports the suggestion that ββ and γγ are expressed in different cell types (36).

A gastric ADH contribution to the first-pass metabolism of ethanol (40) is possible since both γγ-ADH and σσ-ADH have appropriate kinetic constants for ethanol oxidation. But it is also clear that the gastric total activity is very small, not higher than 1% of the total hepatic activity (41). From this basis and also from metabolic estimates (24,28) it can be concluded that the contribution of gastric ADH to ethanol metabolism is negligible.

Different parameters appear to influence gastric ADH activity (29,41,42). One of the most obvious is the polymorphism at the *ADH3* locus. Individuals with the $\gamma_1\gamma_1$ phenotype (see Table 1) exhibit higher activity than individuals with any of the other phenotypes (32,39). A polymorphism affecting the expression of σσ has been suggested (43) but not confirmed. A lower expression of σσ in stomach, but not in other gastrointestinal organs, has been suggested in Chinese, as compared to Europeans. The activity values were consistently related to the

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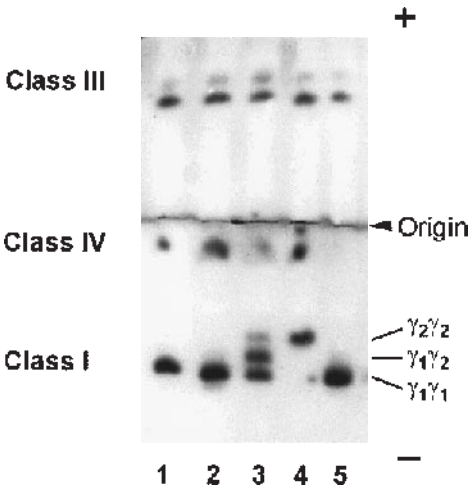

Figure 2 Starch gel electrophoresis analysis and activity staining with 0.1 M 2-buten-1-ol of homogenates from human gastric body biopsies. 1, 2, and 5, individuals homozygous for *ADH3*1*; 3, heterozygous at the *ADH3* locus; 4, homozygous for *ADH3*2*.

σσ expression levels (39). Age, gender, gastric region, medication, and gastric illness may also influence gastric ADH activity (see Refs. 29, 41, and 42 for revisions). A more recent report on a large sample of Asian individuals has not found any effect of age or gender on gastric ADH activity (39). *Helicobacter pylori* infection (44,45) and atrophic gastritis (45,46) decrease activity.

V. ADH IN BLOOD VESSELS

ADH activity has been detected in all arteries and veins examined from human biopsy and autopsy samples. The aorta is the most active vessel, exhibiting more activity than the stomach mucosa (9.9 nmol/min/mg protein) (19). The ADH enzyme characteristic of human blood vessels is ββ, present in all samples and vessel types analyzed (19,47) (Fig. 1). In about 10% of the samples, the σσ form was also observed. Retinoid metabolism and elimination of lipid peroxidation products have been suggested as the roles of ADH in this tissue (19). The significant activity and the extension of the vascular system indicate a contribution to ethanol metabolism, when ethanol is present in blood. This provides a new mechanism to explain the effects of ethanol on the cardiovascular system. Moreover, the presence of the highly active $\beta_2\beta_2$ in blood vessels of individuals with the *ADH2*2* allele may result in a locally very active ethanol oxidation, and in

unpleasant cardiovascular symptoms that may contribute to the protective effect of this allele against excessive ethanol consumption.

VI. ADH IN LUNG

Human lung expresses the class I ββ-isozymes (48). The activity levels in lung homogenates greatly depend on the individual phenotype. The ADH activities for the homozygous phenotypes, $\beta_2\beta_2$ and $\beta_1\beta_1$, were 14 and 0.7 nmol/min/mg protein, respectively, at pH 7.5, while the heterozygous phenotype, $\beta_2\beta_2$, $\beta_2\beta_1$, and $\beta_1\beta_1$, showed an intermediate value, 7 nmol/min/mg.

VII. ADH IN SKIN

Western blot and immunohistochemical analyses reveal class I and class II ADH in skin, mainly detected in the epidermis (3). The local ethanol metabolism in skin most probably originates some of the many effects of chronic ethanol consumption on this tissue (49). Ninety-four percent of patients who flush after oral ethanol have the cutaneous vascular response of fast flushing to topically applied ethanol and acetaldehyde (50). Pretreatment of the skin with the ADH inhibitor 4-methylpyrazole has been shown to decrease the cutaneous response to topical ethanol (51), suggesting that the local oxidation of ethanol to acetaldehyde is the origin of this erythema response. Moreover, since retinoic acid is necessary for normal epithelial differentiation and maintenance, some of the observed effects may be due to the decrease of retinoic acid production by inhibition of retinol oxidation by ADH in the presence of chronic ethanol.

VIII. ADH IN OTHER TISSUES

The presence of distinct ADH forms in many human tissues is known by Northern-blot (52,53) and immunohistochemical analyses (54). Class I transcripts were found by Northern-blot analysis in many tissues. Among class I genes, *ADH2* exhibited the broadest expression, being detected in heart, skeletal muscle, pancreas, spleen, prostate, ovary, adrenal gland, thyroid, lymph node, and lung, while class II was detected in liver, pancreas, stomach, and small intestine (52). The presence of class I protein has been reported in pancreas, kidney, and testis by immunohistochemistry (54). Interestingly, this technique also showed class I in specific regions of adult human brain such as cerebellum (54), although neither Northern-blot (52) nor biochemical methods (55) were able to detect ADH, other

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than class III, in whole brain homogenates. Starch gel electrophoresis demonstrated the presence of class I in human testis (56) and of class IV in human cornea but not in the lens (57).

IX. ADH POLYMORPHISM AND DISEASE

Many reports demonstrated that Asian individuals with the *ADH2*2* allele have lower risk of becoming alcoholic (58). Recently, this has been also demonstrated for European (22,59) and Jewish persons (60). Several studies in Asians indicated that *ADH3*1* was also more prevalent in nonalcoholic than in alcoholic individuals (see Refs. 22 and 58 for references). However, it has been recently shown that linkage disequilibrium exists between the *ADH2*2* and *ADH3*1* alleles (22,61), which is explained by the close localization of these genes in chromosome 4q21–23. When this linkage was considered, no relationship was found between *ADH3* and alcoholism (58). A simple explanation for the protective effect of *ADH2*2* could be based on a faster ethanol elimination by the isozymes containing β_2. However, no differences in the rate of alcohol elimination have been reported between individuals with the *ADH2*1* and *ADH2*2* alleles (62,63). In short, the basis for a protective effect of *ADH2*2* against alcohol misuse (''the ADH effect'') (64) is not clear yet (58). An appealing hypothesis is a role of the extrahepatic ethanol metabolism since the *ADH2* gene is expressed in many organs other than liver, such as blood vessels and skin (19,52,65). Thus, although the total extrahepatic metabolism is small when compared to the liver contribution, the local ethanol oxidation may significantly influence the normal function of these tissues. This could be a basis for unpleasant symptoms after ethanol intake in individuals with the most active *ADH2*2* allele. This would also explain the ethanol-induced cutaneous erythema in *ADH2*2* subjects and the increased facial flushing in *ADH2*2* individuals who simultaneously exhibited the *ALDH2*1*2* genotype (66). The fact that in several cases the presence of the *ADH2*2* genotype reinforces the effect of *ALDH2* deficiency (66–68), supports a metabolic basis for the *ADH2*2* effect.

An important effort has been made to find a relationship between ADH polymorphism and alcohol liver disease, mostly cirrhosis. Although some correlation was found in Asians (69), most studies with both Asians and Europeans could not find any relationship (22 and references cited therein).

The *ADH2*2* frequency has been found higher in patients with alcoholic pancreatitis than in other alcoholic groups in Asian individuals (69–72). Human pancreas exhibits detectable amounts of class I ADH, concentrated in the endocrine cells (54). The $\beta\beta$ isozymes seem to be the major pancreatic ADH form (52), which may explain the sensitivity of this organ to alcohol damage in individuals expressing the highly active β_2 subunit.

Interestingly, recent studies on ADH allele frequencies in alcoholics with or without different types of cancer—laryngeal (73), pharyngeal (74), breast (75), esophageal (76)—show a correlation between the cancer incidence and the prevalence of the most active allele (*ADH3*1* or *ADH2*2*). In contrast, another report found a high risk of esophageal squamous cell carcinoma in *ADH2*1* individuals (77). In alcoholic patients, the oxidation of ethanol in extrahepatic tissues would result in local acetaldehyde accumulation. The cytotoxic acetaldehyde might be an important factor inducing cell proliferation, and increasing the risk of neoplastic transformation, particularly within epithelial cells in direct contact with the ingested ethanol (42,73). A detailed knowledge of the localization of the ADH forms in each cell type of normal and tumoral tissue seems necessary to understand the relationship between ethanol oxidation and cancer development.

X. CONCLUSIONS

About 90% of the alcohol dehydrogenase activity is localized in liver (classes I and II), where it accounts for most of the elimination of ingested ethanol, and for the metabolism of xenobiotic as well as endogenous alcohols and aldehydes of a variety of structures.

The spread of about 10% of the total ADH activity (classes I and IV) over many extrahepatic organs, although it is of little relative importance for the global ethanol elimination, demonstrates a level of active ethanol oxidation in distinct cells and tissues throughout the body. As in liver, where ethanol oxidation is a major cause of alcoholic liver disease (78), extrahepatic ADH activity may also constitute the origin of many pathologies (cancer, alcoholic pancreatitis, skin diseases) in nonhepatic organs from alcoholics. The metabolic basis may not be only the local accumulation of acetaldehyde and NADH, but also the inhibition by ethanol of the ADH activity with important endogenous substrates, such as retinol, thus impairing the normal regulation of relevant functions in organs and tissues.

Polymorphism at the *ADH2* gene has important consequences for drinking habits and for the pathological effects of ethanol. The *ADH2*2* allele represents a protection against excessive drinking, in both Asians and Europeans. The basis, not yet understood, of this effect might be the fast ethanol oxidation in extrahepatic tissues, where *ADH2* is widely expressed.

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6

Genetics and Alcoholism: The COGA Project

Victor M. Hesselbrock
University of Connecticut School of Medicine, Farmington, Connecticut

Tatiana Foroud, Howard Edenberg, and John I. Nurnberger, Jr.
Indiana University School of Medicine, Indianapolis, Indiana

Theodore Reich and John P. Rice
Washington University School of Medicine, St Louis, Missouri

I. INTRODUCTION

The observation that alcoholism runs in families has been frequently noted, even from ancient times. A variety of studies from the United States and other countries have consistently found a higher prevalence of alcoholism among the family members of alcoholic patients than in the general population. Studies of family pedigrees have found that about 25% of the biological fathers and brothers of an alcoholic patient also have significant alcohol-related problems (1). One study has reported that 80% of male and female alcoholics in treatment have at least one biological first- or second-degree relative also affected with alcoholism (2). It is important to note that the familial nature of alcoholism seems to hold regardless of the nationality of the samples studied, or whether the proband has an additional comorbid psychiatric disorder. More recent epidemiological studies have documented the familial nature of alcoholism even among untreated persons with alcohol dependence drawn from the community (3). Adoption and twin studies also point to the importance of genetic factors in the transmission of alcohol dependence (3). From adoption studies, we know that the risk for alcoholism among sons of alcoholic fathers is three to five times higher than in the general population. Further, an examination of published twin studies of alcohol depen-

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dence indicates that genetic influences seem to explain about one-half of the total alcoholism liability variance in men and as much as 25% of the variance in women (4). However, the heritability of the disorder may vary according to the definition of alcoholism used (5), with higher heritabilities being associated with more severe forms of the disorder, at least for women (6). Recent twin studies and parent-twin studies (7) have documented the importance of genetic influences, not only for the risk for developing the disorder, but also in relation to the onset and individual differences found in alcohol use. Although the evidence from these studies clearly points to the importance of genetic factors in the development of alcohol dependence, to date, specific genes that predispose toward alcoholism have not been identified.

Based upon the substantial evidence that genes influence the vulnerability to alcoholism, the Collaborative Study on the Genetics of Alcoholism (COGA) was initiated. Funded by the National Institute on Alcohol Abuse and Alcoholism (NIAAA) of the National Institutes of Health, the primary goal of the COGA project is identification of genes that influence susceptibility to alcohol dependence and its related phenotypes. Additional goals of the COGA project include the use of multivariate statistical methods to refine alcohol dependence phenotypes based upon clinical diagnostic systems and the use of electrophysiological and alcohol challenge strategies to identify genetic factors that may contribute to the risk for developing alcohol dependence.

II. METHODS

A. Sample and Assessment

COGA is a large-scale family study being conducted at university medical centers located in six sites spanning the continental United States. The participating sites are located in Farmington, Connecticut, Brooklyn, New York, Indianapolis, Indiana, St. Louis, Missouri, Iowa City, Iowa, and San Diego, California. The study was designed to include both alcoholic and community control probands and their biological relatives. All potential alcoholic probands were recruited from consecutive admissions to both inpatient and outpatient alcohol treatment facilities. To be identified as a potential proband, the individual was required to meet both DSM-III-R criteria (8) for alcohol dependence and the Feighner (9) criteria at the definite level for alcoholism. This combination of alcoholism diagnoses defines a phenotype known as ''COGA alcohol dependence'' and was used to minimize diagnostic errors. Potential probands were excluded if they were habitual intravenous-drug users, had any life-threatening illness not related to alcoholism, were non-English speaking, were known to be HIV+, or if their biological relatives did not live close to one of the six COGA testing centers (so that they

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could be personally interviewed). Based upon a multi-staging-sampling scheme, probands and their families were classified into one of two types.

1. Stage I

Stage I probands included those who met the inclusion criteria described above and who had at least two first-degree relatives living within a 150-mile radius of one of the six COGA catchment areas. Stage I probands and their biological relatives (aged 6 years and older) who were willing to participate completed a formal, structured psychiatric diagnostic interview, provided information on the psychiatric status of other family members via family history/family story methods, and completed several standardized measures of personality traits. Age-appropriate assessments were used for younger family members.

2. Stage II

Stage I families in which the proband and at least two other first-degree relatives who met inclusion criteria and who also were affected with COGA-defined alcohol dependence were designated as Stage II families. Probands and all first-, second-, and third-degree biological relatives over the age of 6 years completed the Stage I assessments mentioned earlier. In addition, each subject completed a battery of neuropsychological tests and an electrophysiological/event-related potential (ERP) assessment that employed visual and auditory paradigms. Blood samples were obtained for biochemical analysis, for extraction of DNA, and for the establishment of lymphoblastoid cell lines.

3. Control Families

Probands of control families were identified through consecutive admissions to dental clinics, from driver's license bureaus, health maintenance organizations, church congregations, and large corporations. Selected families consisted of two parents and at least three biological children over the age of 14 years. All other children 6 years of age or older who consented to participate were also evaluated. Potential subjects were excluded if they had any life-threatening illness, had a history of serious head injury or neurological disease, were non-English speaking, or were known to be HIV+. Control probands and their family members were not excluded if they had a history of DSM-III-R alcohol dependence or another Axis I disorder. Control families were evaluated using the Stage II family protocol, excluding neuropsychological testing and blood samples for establishment of cell lines.

Informed consent was obtained from all adult subjects prior to their participation; minor children provided informed assent and parental consent was ob-

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tained for each child. All subjects were paid for their participation. A Certificate of Confidentiality was obtained from NIAAA to cover the information provided by subjects.

Subject ascertainment and assessment procedures were standardized across all sites and an extensive quality assurance program was instituted to ensure the reliability of the diagnostic information over time (10) and the reliability of the evoked EEG/ERP protocol (11).

B. Instruments

In both the initial assessment (1989–1999) and the 5-year follow-up assessment currently underway (1997–2004), subjects have been examined across a variety of possible phenotypic dimensions thought to be related to alcohol dependence or to the susceptibility to develop alcohol dependence. This information, e.g., personality traits, psychological problems, psychiatric symptoms, deviant behaviors, has been employed in the construction of different alcoholism phenotypes, including those based upon different standardized diagnostic systems, for use in the search for potential genetic vulnerabilities for developing alcohol problems.

The present 5-year follow-up assessment will continue the repeat evaluation of these factors to identify incident cases of alcohol dependence, to determine phenotypic stability over time, and to assist in the examination of the course of illness. The follow-up battery will also include certain environmental, individual, and family factors that may also influence the onset and expression of drinking behavior among the children, adolescents, and young adults sampled. Since the follow-up is currently underway, those data will not be reported here.

1. Mental Health Interview

A complete and detailed lifetime psychiatric history was obtained from each adult subject using the Semi-Structured Assessment for the Genetics of Alcoholism (SSAGA) (10) at the initial evaluations conducted to date. The SSAGA interview schedule covers the major DSM-III-R-defined Axis I psychiatric disorders, as well as antisocial personality disorder (ASPD). The SSAGA has been shown to have good within- and between-site reliability (10), to have good item reliability (12), and to be valid (13). All interviews were conducted by trained technicians and audio-recorded to monitor the reliability of the assessment procedure. Psychiatric diagnoses were made based upon computer algorithms developed to make Feighner, DSM-III-R, DSM-IV and approximate ICD-10 diagnoses. Age-appropriate companion versions of the SSAGA were used for assessing the psychiatric status of children 7–12 years of age (C-SSAGA-C), and adolescents 13–17 years old (C-SSAGA-A).

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2. Psychiatric Family History by Interview

The psychiatric status (including alcohol abuse/dependence, affective disorder, ASPD, etc.) of all first- and second-degree biological relatives was also obtained from each subject, including the proband, using the family history method (FHAM-Family History Assessment Module) (14). DSM-III-R criteria were used to make diagnoses for all family members not directly interviewed. Rice et al. (14) have found that the sensitivity/specificity of the FHAM for the diagnosis of alcohol dependence and the other disorders covered to be quite good.

Families with evidence of obvious bilineal alcohol dependence (i.e., two affected parents) were not included in the extended pedigree assessment protocol. Importantly, alcohol dependence, drug dependence, or other psychiatric disorders were not used as exclusionary criteria in the selection of control subject probands.

3. Sample

The current sample (masterfile 90, November 1999) includes 9032 adults (18+ years old), 627 adolescents 13–17 years old, and 668 children (7–12 years old) representing 1439 families. (See Table 1.) Of this number, 4446 adults, 157 adolescents, and 221 children are from 887 Stage I families. Biological material is available on 3541 adults, 291 adolescents, and 398 children from 318 Stage II families. In the total sample, 53.5% of all subjects are female. The ethnic composition of the sample includes 74.0% Caucasians, 17.2% African-Americans, and 6.0% Hispanics. Native Americans represent less than 1% of the total sample.

4. Genotyping

To provide a replication sample similarly ascertained and assessed the Stage II family sample was divided. The initial sample, which has been reported previously by Reich et al. (15), contained 105 families, including 987 individuals with genotype data. In each family, on average, 9.5 individuals were genotyped. The

Table 1 Current COGA Sample Description

	Interviewed subjects			
Family type	Adults	Adolescents	Children	# Families
Stage I	4446	157	221	887
Stage II	3541	291	398	318
Controls	1045	179	49	234
Totals	9032	627	668	1439

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replication sample that was collected using the same ascertainment, assessment, and genotyping criteria consisted of 157 families with genotypic data available on 1295 individuals. The average number of individuals genotyped in the replication families was 8.3 persons. In the initial sample, 291 markers with an average intermarker interval of 13.8 cM were genotyped. The average heterozygosity was 0.72. Additional markers were added in chromosomal regions linked to phenotypes of interest to decrease intermarker distance. Consequently, 336 genetic markers were genotyped on both the initial and the replicate samples, with an average intermarker distance of 10.5 cM.

The genotyping was conducted in two separate laboratories with each responsible for approximately half of the chromosomes. Most markers were tri- or tetranucleotide repeat polymorphisms developed by the Cooperative Human Linkage Center. Additional markers were obtained from Genethon, the Marshfield Clinic, the Massachusetts Institute of Technology, and the University of Utah. Polymerase chain reaction (PCR) was used to amplify the markers, and allele sizes were determined using either radioactively labeled markers separated on DNA sequencing gels or fluorescently labeled markers on an ABI-373A automated DNA sequencer (Applied Biosystems, Inc., Foster City, CA). See Reich et al. (15) for additional details.

The genotypic data for both the initial and the replicate samples were stored separately using the GeneMaster Database Management System (J. Rice, personal communication) and checked for Mendelian inheritance of marker alleles with the CRIMAP (16) and USERM13 (17) option of the MENDEL linkage computer programs. Marker allele frequencies were estimated using maximum-likelihood estimates from the USERM13 program. Marker recombination and distance were estimated using CRIMAP and compared to other published maps.

Families with an identified noninheritance were reviewed. If the apparent discrepancy was not resolved by review, the genotypic data from individuals incompatible with the remainder of the family were removed. Determination of allele sizes and the review of potential discrepancies were made blind to diagnostic phenotype.

III. RESULTS

A. Genomic Scan

Recent psychiatric genetic studies have utilized a genome-wide screen approach to identify genetic loci contributing to susceptibility for a variety of psychiatric disorders. The genomic scan approach allows for the detection of novel genetic loci that might otherwise be missed with a candidate gene approach. To date, two separate genome-wide scans for genes affecting the risk for alcohol depen-

dence have been conducted. Both used an ''alcohol dependence'' phenotype based upon formal diagnostic criteria.

The initial genomic scan of the COGA data set by Reich et al. (15) examined 291 markers in 987 individuals from 105 Stage II families. Two-point and multipoint nonparametric linkage analyses were performed to detect susceptibility loci for the COGA phenotype (i.e., DSM-III-R alcohol dependence and Feighner definite alcoholism). The multipoint methods provided the strongest suggestions of linkage with susceptibility loci located on chromosomes 1 and 7, and more modest evidence for a locus on chromosome 2. A lod score of 2.93 was found on chromosome 1, near D1S1588, using multipoint analyses. Two-point affected sib-pair analyses in this region also provided evidence of significant (55%+) allele sharing. On chromosome 7, a maximum multipoint lod score of 3.49 was found near marker D7S1793; allele sharing was approximately 64%. This finding was supported by two-point analyses of affected sib pairs. Also, a region on chromosome 2 near D2S1790 produced a multipoint lod score of 1.81, although there was no evidence of increased allele sharing at this locus using two-point analyses.

Interestingly, evidence of a modifying or *protective* factor was found on chromosome 4, near the alcohol dehydrogenase (ADH) gene cluster region. Subjects who were regular consumers of alcohol but who reported no lifetime symptoms of alcohol abuse or dependence by any diagnostic system covered were considered ''unaffected.'' Two-point analysis found increased allele sharing (69%) among unaffected sibpairs at D4S2393, and reduced sharing among affected-unaffected (i.e., discordant) sib pairs, as would be expected if there was a protective locus linked to this marker. To increase the statistical power for detection of linkage, a modified ''unaffected'' phenotype was constructed to increase the number of sibling pairs. The modified ''unaffected'' phenotype included subjects who regularly drank alcohol and may have reported as many as eight lifetime symptoms of alcohol dependence, but they never met any formalized criteria for alcohol dependence. Using this definition, 126 unaffected sib pairs were identified. Multipoint analyses identified a lod score of 2.50, with a peak near D4S2361. This finding is particularly interesting as it lies in a region on chromosome 4 that includes the alcohol metabolizing genes *ADH2* and *ADH3*. Certain *ADH2* alleles have been hypothesized to be protective against heavy drinking in several Asian populations (18), but these alleles are rare among Caucasians and persons of African descent (18). This finding is particularly exciting as the COGA family study sample is composed primarily of Caucasians (~74%), African-Americans (~17%), and Hispanics (~6%), rather than persons of Asian ancestry (<1%). The region also overlaps with a finding of linkage for alcohol dependence based upon a Southwest American Indian population (19).

More recently, Foroud et al. (20) conducted a confirmation study of the COGA findings reported in Reich et al. (15). The data used by Foroud et al.

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included the initial data set of Reich et al. (15), plus the additional COGA families that were collected to provide a replication sample. Additional genotypic markers were added in regions of interest on several chromosomes. In this new analysis two phenotypes of alcohol dependence were examined: individuals meeting the COGA definition [similar to Reich et al. (15)], and individuals meeting ICD-10 criteria for alcohol dependence. These two phenotypes have considerable overlap (~93% of probands and ~46% of affected relatives in the COGA sample). The COGA definition provides a broader definition of the disorder, while the ICD-10 criteria are thought to identify individuals with a more severe form of alcohol dependence. Using an affected sibling pair design, the genetic analyses again found support for linkage to chromosome 1 with a lod score of 1.6 in the replication sample based upon the ICD-10 phenotype, and a lod score of 2.6 in the combined sample based upon the COGA phenotype. In relation to chromosome 7 and the COGA phenotype, linkage in the combined set had a lod score of 2.9. The lod score on chromosome 2 for the COGA phenotype in the initial dataset increased to a lod score of 3.0 with 64.5% allele sharing following the genotyping of additional markers. However, when the two data sets were combined, a lower overall lod score of 1.8 was found. A new finding of linkage on chromosome 3 was identified in the replication sample with a lod score of 3.4. Allele sharing among the COGA-defined affected sib pairs reached 72.5% and was nearly 72% using the ICD-10 phenotype of alcohol dependence. In general, the analyses of the second larger sample of replication families and the analyses of the combined sample have provided evidence of genetic susceptibility loci on both chromosomes 1 and 7. Susceptibility loci located on chromosomes 2 and 3 were restricted to a single data set; thus genes at these loci might be acting in only one of the two data sets.

Unfortunately, the report of linkage on chromosome 4 by Reich et al. (15), suggestive of a protective factor for alcohol dependence, could not be adequately tested in the replication sample owing to the insufficient number of sib pairs representing the 'unaffected' phenotype.

The above analyses are summarized in Table 2.

B. Features of Alcohol Dependence

To date, our efforts to detect linkage using a phenotype based upon formal diagnostic criteria, such as the COGA definition, DSM-III-R, and ICD-10, although promising, have not been definitive. However, alcohol dependence is a complex trait and is likely influenced by a variety of genes. In an attempt to improve the power to detect linkage, we have undertaken several studies using different alcohol-related phenotypes to define affectation status. Three different features (phenotypes) of alcohol dependence have been examined to date including phenotypes based upon alcohol dependence severity, the presence of alcohol with-

Table 2 Summary of Linkage Analysis Using Different Alcoholism Phenotypes

		Initial sample			Replication sample			Combined sample		
Chromosome	Phenotype	Lod score	% IBD	cM	Lod score	% IBD	cM	Lod score	% IBD	cM
1	COGA	2.5	63.5	145.7	0.7	55.2	127.4	2.6	59.2	143.8
1	ICD-10	1.0	63.2	145.7	1.6	61.2	127.4	1.9	60.1	139.0
1	COGA or DEP	5.1	61.5	120.0	1.5	56.0	150.0	4.7	57.0	122.0
2	COGA	3.0	64.5	98.8	0.0	50.0	98.8	1.8	56.6	120.6
3	COGA	0.11	54.0	97.7	3.4	72.5	97.7	2.4	64.0	100.5
3	ICD-10	0.0	50.0	97.7	1.6	71.6	97.7	0.8	62.1	100.5
4	HIGH SXS[a]	2.5	57.0	86.6						
4	MAX DRINK[b]	2.2		124.0	1.5		119.0	3.5		121.0
7	COGA	2.0	58.4	91.4	0.2	53.0	91.4	2.1	58.1	105.2
7	COGA	1.6	58.3	128.0	1.3	57.5	129.1	2.9	57.9	128.5
8	ICD-10	1.6	61.4	88.2	0.4	55.7	88.2	1.8	58.5	88.2
8	COGA+WITH	2.2	54.0	20.0						
16	LCA	3.2	>65.0	5.2						
16	COGA+WITH	2.9	>60.0	6.7						
18	COGA+WITH	2.0	56.1	90.6						
22	ICD-10	0.0	50.0	58.3	1.9	64.4	58.3	1.1	58.1	58.3

[a] <8 alcohol symptoms but no diagnosis.
[b] Max drink = maximum number of drinks consumed in a 24-hr period.

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drawal symptoms, and the maximum number of drinks consumed in a 24-hr period.

In an attempt to refine the alcohol dependence phenotype based upon a formal diagnosis that was used in the two genomic scans mentioned above (15,20), a latent class analysis (LCA) (21) was applied to 37 symptoms commonly associated with alcohol abuse and dependence queried by the SSAGA. This study, by Bucholz et al. (22), of the alcoholism symptoms reported by the adult subjects in the original COGA sample produced four different latent classes of alcoholism that appeared to classify subjects based upon the severity of their disorder. The initial LCA analysis was further refined by Foroud et al. (23). Items related to adverse social consequences of drinking were excluded, leaving only 11 symptoms that more closely reflected the DSM-IV definition of alcohol dependence. Four ''classes'' resulted from the LCA applied to 830 Stage II adults in the initial COGA sample: an unaffected class, a mildly problematic group, a moderately affected group, and a severely affected group. Classes 3 and 4 were combined (241 subjects and 101 affected sib pairs) to form a group considered to have more severe alcohol dependence. Of this more severely affected group, 88% met the COGA definition of alcohol dependence while 99% met ICD-10 criteria for alcohol dependence. A genome-wide survey produced evidence of linkage on chromosome 16 with a multipoint lod score of 4.0 near D16S675. When additional markers near this locus on chromosome 16 were examined, the critical region was narrowed between D16S475 and D16S675, producing a lod score of 3.2.

A genomic scan has also been conducted by Goate et al. (24) using lifetime endorsement of a variety of alcohol withdrawal symptoms as the basic phenotype. Two-point linkage analyses were conducted on the initial COGA sample using 216 affected-affected, 113 affected-unaffected, and 20 unaffected-unaffected sibling pairs. ''Affected'' individuals were defined as those who met COGA criteria for alcohol dependence and also endorsed symptoms of withdrawal upon cessation of drinking. ''Withdrawal'' was defined as reporting two or more withdrawal features including anxiety, autonomic hyperactivity, tremor, insomnia, nausea, psychomotor agitation, hallucinations, or seizures. Individuals identified as ''unaffected'' in these analyses drank four drinks a day or more for at least 4 weeks, but did not report any symptoms of withdrawal. Sibling-pair analyses found suggestive linkage on chromosomes 8, 16, and 18. Increased sharing was also observed on chromosome 8, with three of four adjacent markers providing sharing estimates of 54–55%. A lod score of 2.8 was found between D16S475 and D16S675, with an estimated allele sharing greater than 60%. On chromosome 18, a single marker, D18S541, was identified using a two-point analysis, with 56% allele sharing among affected siblings.

The finding on chromosome 16 was further examined using six additional markers. Five of the markers were between D16S2622 and D16S298. Multipoint

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analyses of the expanded set of markers on chromosome 16 resulted in a maximum lod score of 2.5 at D16S475. The finding of linkage using a ''withdrawal symptoms'' phenotype in this region of chromosome 16 is consistent with the finding of linkage on chromosome 16 reported above by Foroud et al. (23) using an alcohol dependence ''severity'' phenotype.

A third trait highly related to the phenotype of alcohol dependence has been examined by Saccone et al. (25). The maximum number of drinks ever consumed in a 24-hr period (max drinks) is closely related to a diagnosis of alcohol dependence, yet provides a quantitative measure to assess individuals who do not meet formal diagnostic criteria. Heritability of this phenotype has been estimated between 30 and 50% (26). Multipoint linkage analyses were conducted in the initial sample, the replication sample, and the combined sample. In each analysis, the maximal lod score was obtained on chromosome 4 in the region of D4S2407. The largest lod score was obtained in the combined sample with a value of 3.5. Even though this finding is proximal to the *ADH3* genotype in our current map, the ''maximum drinks'' signal does not appear to vary by *ADH3* genotype. The ''maximum drinks'' finding is consistent with linkage to chromosome 4 reported in this same region by Reich et al. (15), using a sample of ''unaffected'' for alcohol-dependent sibs, and by Long et al. (19) from a sample of alcohol-dependent Native Americans from the southwest United States. These analyses are summarized in Table 2.

C. Candidate Gene Analyses

Because of their hypothesized role in relation to personality traits and a variety of psychiatric disorders, the neurotransmitter systems have been targets for genetic scrutiny. Indeed, both the dopamine and the serotonin systems have been implicated as potentially contributing to the risk for the development of alcohol dependence. Over the past 10 years, the dopamine system, and particularly the dopamine D_2 receptor (DRD_2) has received considerable attention in relation to alcohol dependence. The initial study by Blum et al. (27) reported an association between the *Taq I*-A1 polymorphism in the *DRD2* gene and alcoholism. However, a number of reports attempting to replicate this initial finding have been negative (cf. 28,29). Variations in findings across studies have been attributed to failure to control for differences in allele frequencies in the different populations tested as well as to variations in the phenotype of alcoholism examined (29).

The initial 105 pedigrees of the COGA data set were examined for a possible association or linkage between the *DRD2* locus and alcohol dependence by Edenberg et al. (30). ''Alcoholism'' was defined according to three separate phenotypes: the COGA definition of alcohol dependence, alcohol dependence as defined by DSM-IV, and alcohol dependence in relation to ICD-10 diagnostic criteria. Separate analyses were conducted for the *Taq I*-A locus and a highly

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polymorphic single tandem repeat polymorphism in Intron II. Possible problems associated with population differences in allele frequencies are avoided, to some extent, by using the large family-based COGA sample. As expected, frequencies of the *TAQI*-A allele did vary across ethnic groups in the COGA sample. However, neither the extended Transmission/Disequilibrium Test (TDT) nor the Affected Family-Based Controls (AFBAC) Test was able to detect either an association or linkage between the *DRD2* locus and any of the three phenotypes of alcohol dependence considered.

Edenberg et al. (31) have also examined a second candidate gene, the serotonin transporter gene *HTT*, using the COGA sample. Two variants of the *HTT* promoter have been reported: a shorter, 484-bp allele; and a longer variant, a 528-bp allele. The shorter allele was initially reported to be associated with an increase in anxiety-related traits, including harm avoidance (32). A recent population-based association study found the phenotype of severe alcoholism, marked by withdrawal seizures or delirium, to be associated with the *HTT* promoter polymorphism with alcoholics having an excess of the shorter allele as compared to population controls (33). Three separate phenotypes of alcohol dependence were examined by Edenberg et al. (31). These included all individuals who met the ICD-10 criteria for alcohol dependence, subjects who met ICD-10 alcohol dependence with at least one alcohol-related withdrawal symptom, and ICD-10 alcohol-dependent individuals reporting at least two withdrawal symptoms. For each of the three separate phenotypes examined, no evidence for linkage was found between the *HTT* promoter polymorphism and alcohol dependence. Further, there was no significant elevation of allele sharing among sibling pairs defined as ''affected'' by any of the three separate models. These findings were consistent with an earlier report by Gelertner et al. (34).

D. Alcohol Dependence and Comorbid Conditions

A variety of studies of clinical samples, as well as general population epidemiological studies, have indicated that persons with alcohol dependence often have additional psychiatric conditions. Major depressive disorder, anxiety disorder, other types of substance dependence, and antisocial personality disorder are frequently reported as co-occurring in individuals also affected with alcohol problems (35,36). Nurnberger et al. (37) conducted a genomic survey of the COGA sample using three phenotypes: alcoholism and depression, alcoholism or depression, and depression alone to identify potential chromosomal regions that link to these phenotypes. A two-stage genomic scan was conducted on the initial sample of 987 genotyped individuals from 105 families and then again in the replication sample of 1295 individuals from 157 families. The phenotype of alcoholism was based upon the COGA definition of alcohol dependence, while the depression phenotype was defined as the presence of a lifetime diagnosis of either DSM-III-

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R major depressive disorder or depressive syndrome. Subjects defined as having depressive syndrome met all criteria for a major depressive disorder, except for the organic exclusion. The phenotype ''alcoholism *or* depression'' produced a multipoint lod score of 5.1 on chromosome 1 in the initial sample. Allele sharing in this area was 62%. The replication sample produced a lod score of 1.5 in the same chromosomal region; the combined sample yielded a lod score of 4.7, with 57% allele sharing among affected sibling pairs. This is substantially larger than the lod score for alcohol dependence alone in the same location (lod = 2.9), but the allele sharing is similar (58%). This indicates that the sample size is increased by including siblings with depression, and may suggest a common genetic locus for the two conditions, at least in the COGA sample.

The findings for the ''alcoholism *and* depression'' phenotype were less consistent. A lod score of 4.1 was found on chromosome 2 near the marker D2S1371, but only in the replication sample. The combined sample produced a lod score of 2.2. Evidence of linkage for the ''alcoholism and depression'' phenotype was not found on other chromosomes. The ''depression only'' phenotype produced a lod score of 2.4 in the initial sample on chromosome 7, but this value decreased to 1.7 in the combined sample. No evidence of linkage for the ''depression only'' phenotype was found on chromosome 1.

Phenotypes related to habitual smoking and alcohol dependence have also been examined in the COGA sample by Bierut et al. (38) using a genome-wide survey. The phenotype of habitual smoking was defined as having ever smoked at least one pack daily for 6 months or more and was found to be widely prevalent in the adult COGA sample. The alcohol dependence phenotype was defined according to the COGA criteria. The analyses of the habitual smoking and alcohol dependence phenotypes were based upon 67 multigenerational families in the initial sample with 154 independent sibling pairs affected with habitual smoking. Although several chromosomal regions had evidence of increased allele sharing, the highest lod score for habitual smoking was found on chromosome 9 with a lod score of 2.02 and allele sharing of 58.9%. Other chromosomal regions provided modest evidence for linkage to the comorbid phenotype of habitual smoking and alcohol dependence. Strongest evidence for the comorbid phenotype was found on chromosome 2 with a lod score of 2.79 and allele sharing at 69.1%. Previously identified regions on chromosomes 1 and 7 that had been implicated for alcohol dependence in the same sample by Reich et al. (15) described earlier did not provide evidence of linkage with habitual smoking.

E. Putative Risk Factors for Alcoholism

Over the years, a number of biological traits have been suggested as potential ''markers'' for the risk for developing alcoholism. Typically, these ''markers'' are highly prevalent among persons diagnosed with the disorder, do not disappear

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following recovery from the disease state, and appear more frequently among the biological relatives than among subjects from families where the disease is known to be absent. Two of the more widely studied markers for the risk of alcoholism have been examined in the COGA project. These include diminished platelet monoamine oxidase (MAO) enzyme activity [cf. Major and Murphy (39)] and the amplitude of the P300 waveform of the electroencephalographic evoked response [cf. Begleiter et al. (40); O'Connor et al (41)].

Two studies of platelet monoamine oxidase B activity have been conducted using the COGA sample. To address the question of whether reduced platelet monoamine oxidase (MAO) activity was a marker of alcoholism, Anthenelli et al. (42) compared platelet MAO-B activity in subgroups of DSM-III-R alcoholics and controls. Subjects meeting DSM-III-R criteria for alcohol dependence were further subtyped in relation to type 1/type 2, type A/type B, and the primary/secondary distinction. No between group differences in platelet MAO-B activity were found for any of these additional phenotypes, suggesting that MAO-B activity is not a trait marker of alcohol dependence. Gender (being male) and current smoking status were found to be the most important factors contributing to MAO activity.

Saccone et al. (43), also using the COGA sample, conducted a genomic survey to identify loci involved in the control of MAO-B activity. The analysis focused on 148 nuclear families (from 95 extended pedigrees in the initial COGA sample) containing 1008 nonindependent sib pairs and controlled for gender and for smoking status. MAO activity was determined by the method described by Anthenelli et al. (42). Modest evidence for linkage was found on chromosomes 2 and 6 based upon two separate sets of analyses. A two-point linkage analysis identified three markers (D6S1018, D2S1328, and D2S408) with $p < .01$. Additional analyses were done using all sib pairs. However, the multipoint analysis of the independent pairs produced a maximal lod score of only 2.0 on chromosome 6; lod scores for the earlier chromosome 2 findings were 1.0 and 1.3.

As indicated above, a large number of studies from a variety of different populations have identified the amplitude of the human P300 electroencephalographic event-related potential as a marker of susceptibility for developing alcoholism (44). However, studies of several other psychiatric conditions suggest that dampened amplitude of the P300 ERP component is not a specific marker for alcoholism. More likely, the P300 waveform is a marker for a variety of poor adult outcomes (cf., schizophrenia, antisocial behavior), as well as alcoholism (45). Importantly, the amplitude of the P300 has been demonstrated to be highly heritable [cf. OConnor et al. (46); Van Beijsterveldt (47)].

A quantitative trait loci (QTL) analysis of the amplitude of the P300 event-related potential (ERP) waveform has been conducted by Begleiter et al. (48) using the COGA sample. ERP and genetic data were available on 607 individuals from 103 families in the initial sample, resulting in 758 sib pairs. The ERP data

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were collected from 21 scalp leads of the 10/20 international system using a set of visual stimulus 'oddball' tasks. These have been described in detail elsewhere (11). A genome-wide QTL analysis was conducted using SOLAR (49), a set of variance component-based linkage programs, well suited for examining quantitative traits. Lod scores greater than 2.0 were found for several different electrode sites on four different chromosomes. These included: chromosome 2 (T8 electrode site, lod = 2.38; O2, lod = 3.28), chromosome 6 (Cz, lod = 3.41), chromosome 5 (T8, lod = 2.10), and chromosome 13 (T8, lod = 2.07). Peak multipoint lod scores of 3.28 were found on chromosome 2 between D2S425 and D2S434 for P300 amplitude measured at the O2 electrode, and a score of 3.41 on chromosome 6 close to marker D6S495 for P300 amplitude measured at the Cz electrode.

IV. DISCUSSION

This chapter has reviewed the genetic findings to date from COGA presented by its investigative team. COGA has systematically collected and assessed a large sample of families suitable for the detection of susceptibility genes for ''alcohol dependence'' and its related phenotypes. The clinical and electrophysiological data have been collected using reliable, valid, and standardized assessment methods. Currently, the COGA data set contains 10,324 directly interviewed probands and relatives representing 1439 families. Participants, including probands and other related individuals, represent a range of both drinking behavior and alcohol-related problems. Women comprise 53% of the sample. The sample is also ethnically diverse. Thus, the COGA sample provides a unique opportunity and a solid basis for examining alcohol problems and alcohol dependence across different age groups and in relation to ethnicity. Linkage findings from the initial sample, the replication sample, and the combined samples have been described. Although specific genes for alcohol dependence susceptibility have not yet been identified, regions on chromosomes 1 and 7 show particular promise. Additional markers are being added to these regions and fine mapping is continuing.

Previous psychiatric genetic studies have often been criticized for their lack of replicability, with variations in specifying the phenotype often cited as a primary culprit for the lack of consistency of findings. Lack of replication may arise due to differences in phenotypic definition and assessment, as well as due to differences in ascertaining subjects. The COGA project has used robust family study sampling methods and formal, standardized diagnostic criteria for identifying probands. Phenotypic information was collected using a standardized diagnostic instrument, the SSAGA, with proven reliability and validity. Even with these procedures in place, the initial Reich et al. (15) linkage findings were not consistently found in the replicate sample. Two possible explanations can be offered for differences in findings in the two samples; these include the likely

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genetic heterogeneity of the alcohol dependence phenotypes examined in the studies reported as well as possible between-sample differences, e.g., family size, number of affected sib pairs, etc. Further, separate features of the diagnostic phenotype [e.g., severity of the disorder (23), withdrawal behavior (24), maximum drinks ever consumed per 24 hr (25)] have provided significant linkage findings in locations different from those found based solely on a diagnosis. This suggests that a phenotype based upon a clinical diagnosis of alcohol dependence is quite complex and may be inadequate for identifying all relevant susceptibility genes.

Comorbid psychiatric conditions can also complicate the search for genes that may specifically contribute to the development of alcohol dependence. A variety of other co-occurring psychiatric conditions have been documented in both clinical (35) and community samples (36) of persons with alcohol dependence. In one report, only about 20% of persons receiving inpatient treatment for alcoholism did not report another psychiatric disorder sometime in their life (35). Similarly, in the COGA sample, anxiety disorders (50), affective disorders (51), antisocial personality disorder (52), and other substance abuse/dependence (53) [including tobacco (38,53)] have been noted in persons who also have met criteria for a diagnosis of alcohol dependence. Interestingly, when multiple clinical phenotypes were examined, a region on chromosome 1 was identified that may influence vulnerability for both alcoholism and depression (37). Habitual smoking, however, seems to be influenced by genes separate from those that influence habitual drinking (38). In addition, Bierut et al. (53) found evidence for independent causative factors in the familial transmission of alcohol, cocaine, and marijuana dependence as well as habitual smoking. These findings highlight the likelihood that there may be genes that contribute to the general vulnerability for poor adult psychiatric outcomes, while other genes may contribute to the vulnerability for a specific disorder (54,55).

In addition, COGA has examined the genetic bases of two possible biological markers of a vulnerability for alcohol dependence, platelet MAO activity (42,43) and the amplitude of the P300 waveform of the event-related potential (48). Modest evidence for linkage was found on chromosomes 6 and 2 for MAO activity (43), while a QTL analysis of the amplitude of the P300 ERP waveform identified genetic loci on chromosomes 2, 5, 6, and 13 (48). The regions on chromosomes 2 associated with the P300 amplitude are homologous with regions on mouse chromosome 1, while the chromosome 6 region (6q21–23.2) is homologous with mouse chromosomes 10 and 17. Interestingly, E. M. Simpson (personal communication, 1999) has developed a mouse model deleted for a gene called *tailless*. This mouse model has a number of features that make it a potential model for studying risk for addiction. The mouse is quite aggressive and has abnormal social behavior—characteristics often found in persons with antisocial personality disorder, a known risk factor for alcoholism (56,57). Forebrain hypo-

plasia is also seen in this mouse. The human homolog of *tailless* maps to 6q21.1 and corresponds with the QTL linkage reported by Begleiter et al. (48) in relation to P300 ERP waveforms in the COGA sample. Another potential candidate gene, the ionotropic glutamate receptor gene (*GRIK2*), resides in this same general area. The serotonin 5HT1B (*HTR1B*) receptor gene is located within 15cM at 6q13–15 and has been linked to antisocial alcoholism (58).

The COGA findings presented here, along with findings from other investigations of alcohol dependence [cf. Johnson et al. (4); Hill et al. (59); Tsuang et al. (55); Merikangas et al. (60)], are beginning to address the question of how a family history of alcoholism influences the susceptibility for developing alcohol-related problems, including alcohol dependence. Results from the COGA project and from other published studies indicate that there is considerable variability in the vulnerability for developing alcoholism. The variability in susceptibility for alcoholism appears to result from two sources. The first source is the likelihood of genetic heterogeneity, suggesting that the genetic mechanisms contributing to the predisposition for alcohol dependence may not be the same in all pedigrees. Given the variable clinical expression of patients with alcohol related problems (22,61,62) it is likely that some families might demonstrate a polygenic form of inheritance. The evidence to date indicates that alcohol dependence susceptibility is the result of multiple genes, each contributing a small effect. In recognition of this variability in clinical expression, additional efforts are being made by COGA to refine the alcohol dependence phenotype. Biologically based features of the syndrome are currently being assessed as quantitative traits during the follow-up portion of the study to take advantage of more new, powerful quantitative genetic methods.

A second source of variability in the susceptibility to alcohol dependence may come from the clinical course of the disorder. Additional analyses of the COGA data set have also revealed that the alcohol-dependent probands and their biological relatives are quite heterogenous in terms of the etiology of their disorder, their demographic characteristics, alcohol use patterns and history, the presence of comorbid psychiatric disorders, and patterns of other substance abuse. Different family backgrounds, different rearing environments, and a variety of biological, social, and psychiatric problems (including comorbidity) have been associated with chronic alcohol use. These factors may influence treatment-seeking behavior, treatment outcomes, and the life course of alcoholism including its genetic expression. Multivariate statistical methods, such as cluster analysis (61,62) and latent class analysis (22,52), are being used to identify subtypes (phenotypes) of alcohol dependence that are not based solely upon formal diagnostic criteria. Importantly, information from the follow-up portion of the COGA study will be invaluable for determining the stability of different alcohol dependence phenotypes, whether based upon clinical features or the course of the disorder over time.

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The initial data set has been subjected to a large number of additional genetic analyses as part of the Genetic Analysis Workshop 11 (GAW11) by non-COGA investigators. The results of these analyses can be found in *Genetic Epidemiology* (63).

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7

The Genetic Epidemiology of Alcoholism

Sex Differences and Future Directions

Carol A. Prescott
Virginia Institute for Psychiatric and Behavioral Genetics, Medical College of Virginia of Virginia Commonwealth University, Richmond, Virginia

I. INTRODUCTION

It has long been recognized that alcoholism is familial. First-degree relatives of alcoholics have significantly increased risk for alcoholism compared with control samples (1). The basis for this familiality has been studied in over 20 twin and adoption studies, and the evidence supports the role of genetic influences on alcoholism in men (2,3). The contributions of the more recent studies are in addressing the etiology of alcoholism in women and for understanding sex differences. The study of sex differences in alcoholism is useful because sex is associated with variation in both biological and cultural risk factors. An understanding of the mechanisms influencing sex differences in liability can also help to illuminate the basis for variability within the sexes.

The goals of this chapter are: (1) to overview the methods of genetic epidemiology; (2) to present types of sex differences; (3) to summarize the results from adoption and twin studies of alcoholism; (4) to evaluate the evidence and explanations for apparent sex differences in genetic contributions to alcoholism; and (5) to discuss future directions in genetic epidemiological research on the etiology of alcoholism.

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II. METHODS USED IN GENETIC EPIDEMIOLOGY

The discussion of the literature presented here assumes that alcoholism is a multifactorial disorder, with liability to develop alcoholism arising from a combination of multiple genetic and environmental factors. Liability is assumed to be continuous and normally distributed in the population, with individuals who exceed a theoretical threshold expressing the disorder. Individual differences in liability are assumed to arise from three components of variance: additive genetic variation (also known as heritability, h^2) comes from genes whose allelic effects combine additively; family or common environment (c^2)includes all sources shared by family members, including family environment, social class, familial attitudes about alcohol use, and, for twins, the intrauterine environment; and specific environment (e^2) includes all remaining factors not shared by family members.

In studies of intact families, the effects of genetic and common environment are not separable. Adoption studies separate these effects because adoptees receive their genetic heritage from one set of parents and their rearing environment from another set. Assuming an absence of intrauterine effects, and no correlation between the adoptive and biological family environments, the degree to which adoptees resemble their biological relatives is a direct measure of genetic influence, while the degree to which they resemble their adoptive relatives is a measure of the influence of family environment.

Estimates of genetic and environmental contributions to liability are obtained from twin studies by comparing the resemblance of identical (monozygotic, MZ) and fraternal (dizygotic, DZ) twin pairs. MZ twins within a pair resemble one another because they share all of their genetic and common environmental factors, while DZ pairs share (on average) half of their segregating genes and all of their common environment. Estimates from twin studies assume that MZ and DZ pairs are equally similar in their environments relevant to the development of alcoholism.

The studies chosen for review included assessment of alcoholism based on clinical interviews or reliable archival information (such as hospital diagnosis or temperance board registrations). Not included are studies of alcohol consumption [reviewed elsewhere (4)], animal studies, or molecular genetic studies (see chapter by Hesselbrock, this volume).

III. SEX DIFFERENCES IN ALCOHOLISM

There are several ways in which sex differences in alcoholism etiology may be manifest, including differences in prevalences, in the magnitude of genetic influences, and in the sources of genetic influences, i.e., sex-specific transmission.

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In most cultures, men have higher prevalences of alcoholism and heavy drinking than women (5). Both cultural and biological explanations have been invoked to explain this difference, but the mechanisms remain unclear. It is important to note that it is not necessary to invoke different underlying mechanisms in the sexes to explain differences in prevalences. An alternative explanation is that the same genetic factors operate in men and women but that other sex-specific genetic and/or environmental factors influence whether alcoholism is manifest. Cloninger et al. (6) described this in terms of sex-specific thresholds for the development of alcoholism, with women needing a higher level of liability before they manifest alcoholism.

Another type of sex difference is in magnitude of genetic influence. The evidence from twin and adoption studies in men consistently indicates genetic influences account for about half the population variation in liability to develop alcoholism. However, as described later, the evidence regarding alcoholism in women has varied across studies.

A final type of sex difference is sex-specific etiological factors. Such a process could be due to genetic factors that had different levels of expression in the two sexes or were modified by other sex-specific genetic or environmental factors to make male and female relatives less similar. This effect could also arise from environmental modes of transmission, such as if girls were more likely to model their drinking behavior after their mothers and boys after their fathers. The existence of sex-specific etiology is indicated when (after adjusting for sex differences in prevalences) the risk to a relative is greater when the relative and affected individual (proband) are the same sex than when they are of different sexes.

IV. ADOPTION STUDIES OF ALCOHOLISM

The first published adoption study of alcoholism was by Roe and Burks (7), who used adoption agency records in New York City to identify 32 adoptees with alcoholic biological parents and 25 adoptees with nonalcoholic parents. When interviewed in early adulthood, none of the adoptees had alcoholism. The small sample sizes in this study limit its power to make strong conclusions about a lack of genetic influence on alcoholism. The remaining adoption studies are summarized in Table 1 and described below.

A. Danish Adoption Studies

The first large-scale adoption studies of alcoholism were reported by Goodwin and colleagues (8,9). These studies identified subjects by crossing Danish national

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Table 1 Prevalences of Alcoholism Among Adopted-away Offspring of Alcoholics Compared to Control Adoptees by Sex of Adoptee and Sex of Affected Parent

Study	Ascertainment and assessment	Diagnosis	Birth cohort	Male offspring			Female offspring		
				Alcoholic father	Alcoholic mother	Controls	Alcoholic father	Alcoholic mother	Controls
Bohman et al., 1981; Cloninger et al., 1981[a]	Sweden, TBR (Stockholm)	TBR, medical and government records	1930–1949	.22 (259)	.26 (23)	.15 (571)	.04 (285)	.10 (29)	.03 (577)
Sigvardsson et al., 1996[a]	Sweden, TBR (Gothenburg)	TBR	1930–1949	.23 (78)	.32 (22)	.13 (469)	.01 (106)	.00 (7)	.01 (546)
Goodwin et al., 1973, 1977[b]	Denmark, Psychiatric registry + IV	Alcoholic Alc + PD	1924–1947	.18 (55) .27		.05 (78) .19	.04 (49) .09		.09 (47) .09
Cadoret et al., 1985[b,c]	Iowa, Adoption records + IV	DSM-III-R AAD	1938–1962	.61 (18)		.24 (109)	.33 (12)		.05 (75)
Cadoret et al., 1987[b,c]	Iowa, Adoption records + IV	DSM-III-R AAD	1938–1964	.63 (8)		.20 (152)	—		—

Entries are prevalences and (*N*); TBR = temperance board registration; IV = interview; AAD = alcohol abuse and/or dependence; PD = problem drinking

[a] Adoptees with both alcoholic biological parents excluded in prevalences.

[b] Results not reported separately by sex of affected relative.

[c] Samples are independent; affected relatives were primarily parents but also include other first-degree biological relatives.

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registries of psychiatric diagnoses with records of nonfamily adoptions in Copenhagen between 1924 and 1947. Based on clinical interviews, sons of alcoholics were significantly more likely to have alcoholism than controls (whose parents had no disorder or were hospitalized for disorders other than alcoholism), although less difference was found using a broader definition of problem drinking (8). Results were not separated by gender of affected parent, but 85% of the alcoholic parents were fathers. The 1977 study (9) used a similar design with women and found no significant differences between the probands and controls in the prevalence of alcoholism or problem drinking.

Goodwin et al. (10,11) also reported on the prevalences of alcoholism among the siblings and half-siblings of the probands. These individuals were not adopted but were raised by their alcoholic biological parents. Compared to prevalences in the adoptees, alcoholism was not more prevalent among either male (17% vs. 25%) or female (2% vs. 3%) siblings, providing further evidence that any increased risk for alcoholism among offspring of alcoholics was due to genetic rather than social factors.

B. Swedish Adoption Studies

In 1981, Bohman, Cloninger, and colleagues reported the results of studies based on merging adoption records from Stockholm with national Temperance Board registrations (TBR) and other medical and public records (12,13). TBR occurred for a variety of offenses including public drunkenness, disorderly conduct, and illegal manufacture of alcohol. In addition, the board maintained records on treatment and supervision of alcoholics. These studies are unique among adoption studies for reporting separate prevalences by sex of parent and adoptee. These studies began with adoptees with alcohol abuse and examined the prevalences of alcoholism in their biological parents. In the Bohman et al. study (12), 31 female adoptees with a history of alcohol abuse (based on one or more TBR) were more likely to have alcoholic biological mothers than 882 female adoptees without alcohol abuse. There was an association with fathers' alcoholism only if the fathers did not also have criminal histories.

In a parallel study of male adoptees, Cloninger et al. reported on the family history of male adoptees with varying degrees of alcohol abuse compared to that of adoptees with no TBR (13). Alcoholic men were more likely to have alcohol abuse in their biological fathers and biological mothers. Men with moderate alcohol abuse (defined as repeated TBR but no treatment) had greater paternal alcoholism and criminality and lower maternal alcohol abuse than male adoptees with mild or severe alcohol abuse. These results contributed to the development of Cloninger's typology of alcoholism, in which one form (type II) is hypothesized to have moderate severity, greater heritability, early onset, greater association with antisocial traits, and be infrequent in women. In contrast, type I is associated

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with mild or severe disorder, less heritability and criminality, and is the predominant form among female alcoholics (14).

Sigvardsson et al. (15) reported results from a replication study using the same methods applied to adoptees born in Gothenburg, Sweden. The results among male adoptees were similar to those in the original study, with type II alcoholic men more likely to have affected biological parents. Adopted men were at risk for type I alcoholism only if they had an affected biological parent plus a high-risk adoptive family environment. The findings were not replicated for female adoptees, but the study had limited power as only 7 of 660 women studied had any TBR.

C. Iowa Adoption Studies

Cadoret and colleagues conducted studies of alcoholism, substance abuse, and antisocial behavior among adoptees ascertained from adoption agencies in the state of Iowa in the United States. In the first sample (16), alcohol abuse in both male and female adoptees was associated with problem drinking in biological first-degree relatives (usually parents). There was significantly increased alcohol abuse in male adoptees who had adoptive family members with alcohol problems. The effect of adoptive family alcohol problems was of similar magnitude among female adoptees, but was not significant in this smaller sample. The results from a second sample of male adoptees (17), were similar.

V. TWIN STUDIES OF ALCOHOLISM

The results of twin studies of alcoholism are summarized in Tables 2–4, grouped by method used to identify affected individuals.

A. Studies Based on Ascertainment of Affected Twins with Cotwin Follow-up

Table 2 summarizes the results from four twin studies that identified affected twins from treatment settings or archival records and then assessed their cotwins. Based on TBR records in Sweden, Kaij (18) reported twin-pair concordances for chronic alcoholism consistent with a very high heritability and no evidence of family environment. Use of a broader definition (one or more TBR) resulted in significant contributions of both genetic and family environmental sources.

Gurling et al. (19) studied twin pairs identified through treatment for alcoholism at the Maudsley Hospital in London. Cotwins were contacted for diagnostic interviews and prevalences of alcoholism among the cotwins were similar for

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Table 2 Twin Studies of Alcoholism Using Proband Ascertainment with Cotwin Follow-up

Authors	Sample	Diagnostic definition	Assessment method	Birth cohort	# pairs MM	# pairs FF	# pairs MF	Estimates[a] M	Estimates[a] F
Kaij, 1960	Sweden	1: Chronic alcoholism 2: ≥ 1 TBR	TBR plus clinical interviews on most	[b]	48 MZ 126 DZ	—	—	1: h^2 = .98[c] c^2 = .01 2: h^2 = .42 c^2 = .43	—
Gurling et al., 1984	Psychiatric service of London hospital	WHO alcohol dependence syndrome	Clinical interview	[d]	15 MZ 20 DZ	13 MZ 8 DZ	—	h^2 = .00 c^2 = .44[a]	
Pickens et al., 1991	Twins entering treatment in Minnesota, US (overlapping sample)	1: DSM-III AD 2: AAD	Personal interview	[f]	50 MZ 64 DZ	31 MZ 24 DZ	—	1: h^2 = .60 c^2 = .17 2: h^2 = .36 c^2 = .51	1: h^2 = .42 c^2 = .00 2: h^2 = .26 c^2 = .29
McGue et al., 1992		DSM-III AAD	Questionnaire		85 MZ 96 DZ	44 MZ 43 DZ	88	h^2 = .54 c^2 = .33	h^2 = .00 c^2 = .63
Caldwell & Gottesman, 1991	Missouri, U.S., inpatient and outpatient psychiatric settings	1: DSM-III-R AD 2: AAD	Personal interview	1905–1968[g]	27 MZ 26 DA	16 MZ 24 DZ	55	1: h^2 = .49 c^2 = .00 2: h^2 = .70 c^2 = .10	1: h^2 = .10 c^2 = .44 2: h^2 = .08 c^2 = .67

M = male; F = female; MM = male-male twin pairs; FF = female-female pairs; MF = male-female pairs; TBR = Temperance Board Registration; AD = alcohol dependence; AA = alcohol abuse; AAD = alcohol abuse and/or dependence.

[a] Variation due to specific environment is the remainder [1 − (h^2 + c^2)]. 1 and 2 indicate estimates for definitions 1 and 2.

[b] Birth years not reported.

[c] Estimates reported in (3).

[d] Birth years not reported; sample is patients ascertained since 1948.

[e] Estimates calculated from data reported, sexes combined due to small sample sizes and unavailability of prevalences separately for the sexes.

[f] Not reported, age mean ± sd = 34.5 ± 12.0.

[g] Approximate, birth years not reported.

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Table 3 Twin Studies of Alcoholism Using Archival Records with Population-Based Twin Registries

Authors	Sample	Diagnostic definition	Assessment method	Birth cohort	# Pairs			% affected		Estimates	
					MM	FF	MF	M	F	M	F
Koskenvuo et al., 1984	Finland, national twin registry	ICD-8 alcohol-related diagnoses	Hospital records 1972–79	<1958	1587 MZ 3753 DZ		—	1.9	0.2	h^2 = .35 c^2 = .19	h^2 = .00 c^2 = .00
Romanov et al., 1991			Records through 1985			—	—	2.5	—	h^2 = .58 c^2 = .00	—
Allgulander et al., 1991	Sweden, merger of psychiatric and twin registers	ICD-8 Alcoholism	Discharge diagnoses from 1969 to 1983	1926–1958	2293 MZ 3691 DZ	2736 MZ 4164DZ	—	1.7	0.5	h^2 = .08[a] c^2 = .32	h^2 = .20[a] c^2 = .42
Hrubec & Omenn, 1981	NAS-NRC Registry (US military)	ICD-8 Alcoholism	Military medical records through 1978	1917–1927	5932 MZ 7554 DZ	—	—	3.0	—	h^2 = .53[b] c^2 = .07	—
Reed et al., 1996		1: ICD-8 Alcoholism; 2: Alcoholism + alcohol-related diseases	Medical records through 1994			—	—	1: 3.9 2: 5.3	—	1: h^2 = .58 c^2 = .00 2: h^2 = .59 c^2 = .00	—
Kendler et al., 1997	Sweden, national twin registry	TBR	TBR	1902–1949	3185 MZ 5750 DZ	—	—	14.1	—	h^2 = .54 c^2 = .14	—

[a] Estimates calculated from data reported; original paper reported estimates for both sexes combined.
[b] Estimates from (3).

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Table 4 Twin Studies of Alcoholism Using Clinical Assessment with Population-Based Twin Registries

Authors	Sample	Diagnostic definition	Assessment method	Birth cohort	# pairs			% affected		Estimates	
					MM	FF	MF	M	F	M	F
Kendler et al., 1992	Virginia, U.S.; population-based twin registry	DSM-III-R AD	In-person interview	1934–1975	—	590 MZ 440 DZ	—	—	9.0	—	$h^2 = .56$ $c^2 = .00$
Prescott et al., 1999[a]		1: DSM-IV AD 2: AAD	Telephone interview		863 MZ 654 DZ	683 MZ 474 DZ	1423	24.9	11.4	1: $h^2 = .52$ $c^2 = .00$ 2: $h^2 = .56$ $c^2 = .00$	1: $h^2 = .59$ $c^2 = .00$ 2: $h^2 = .66$ $c^2 = .00$
True et al., 1996	U.S. military	DSM-III-R AD	Telephone interview	1939–1957	1864 MZ 1492 DZ	—	—	38.3	—	$h^2 = .55$ $c^2 = .00$	—
Heath et al., 1997	Australia twin registry	DSM-III-R AD	Telephone interview	1893–1964	396 MZ 231 DZ	932 MZ 534 DZ	592	23.5	5.6	$h^2 = .64$[b] $c^2 = .01$	

[a] FF sample overlaps with that from Kendler et al., 1992.
[b] No significant sex differences in estimates.

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MZ and DZ pairs. However, the small sample limits the power to test for sources of familial resemblance.

The samples studied by Pickens, McGue, and colleagues (20,21) were identified from records of alcoholism treatment facilities in the state of Minnesota in the United States. Treatment records, personal interviews, and questionnaires were used to identify twins and cotwins who met criteria for DSM-III alcohol dependence (AD) and alcohol abuse and/or dependence (AAD). Pickens et al. (20) reported results for same-sex twin pairs who were clinically interviewed. Twin-pair similarity was high for both diagnostic definitions among men, with higher heritability estimates for AD and stronger evidence for family environment for AAD. A similar pattern was reported for female twins, although the magnitude of estimates was lower than those reported for men.

The data reported by McGue et al. (21) included the earlier sample (20) plus opposite-sex pairs and pairs whose cotwins were assessed only via questionnaire. Based on this information, MZ and DZ female pairs had similar resemblance for AAD, yielding evidence for common environmental but not genetic contributions to the familiality of alcoholism in women. Results for male twins were similar to those found previously (20).

The fourth treatment-based interview study was conducted by Gottesman and colleagues and based on sequential ascertainment of twins treated at several psychiatric and alcohol treatment facilities in St. Louis, Missouri. Probands and cotwins were interviewed and received diagnoses of DSM-III-R AD and AAD. The ascertainment differs from other studies in that many probands had a primary diagnosis other than alcoholism. The results based on alcoholic probands have not yet been published but were presented by Caldwell and Gottesman (22) and are reported in Ref. 23. As with the other treatment-based studies, heritability of alcoholism among men was substantial, but in this study there was no evidence for common environmental contributions. Among women, pair resemblance was also substantial and similar for MZ and DZ pairs, yielding near-zero heritability estimates but significant contributions of shared environment. However, based on the relatively small sample sizes, these sex differences were not statistically significant.

B. Studies Using Archival Records Matched Against Twin Registries

Four studies have matched twin registries against archival information on TBR, military, or medical records to identify twin pairs concordant, discordant, and unaffected for alcoholism. These studies are summarized in Table 3.

Koskenvuo et al. (24) reported results from merging Finnish psychiatric records against the national twin registry to identify twins who received alcohol-

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related discharge diagnoses through 1979. Substantial heritability was reported for men, but owing to very low prevalences in women, no concordant female cases were observed. A follow-up to this study published by Romanov et al. (25) included information from hospital records through 1985 for male pairs, yielding more complete ascertainment and resulting in higher heritability estimates.

Allgulander et al. (26) used similar procedures with data from the Swedish Twin Registry to identify twins who received alcohol-related discharge diagnoses. For both male and female twin pairs, DZ pairs were nearly as similar as MZ pairs, yielding low heritability estimates and evidence for moderate common environmental effects. The prevalence of alcoholism in the sample was quite low, suggesting severe underascertainment of cases.

The National Academy of Sciences–National Research Council twin sample includes U.S. military veterans born between 1917 and 1927. Hrubec and Omenn (27) published twin-pair resemblance for ICD-8 alcoholism based on military medical records. MZ pairs were more similar than DZ pairs, resulting in moderate heritability. Reed et al. (28) published a follow-up of this sample using medical records through 1994, and obtained similar estimates.

Kendler et al. (29) reported results from merging Swedish TBR records with the Swedish twin registry for twins born between 1902 and 1949. MZ twin pairs were substantially more similar for TBR than DZ pairs, yielding moderate evidence of genetic influences plus evidence of some common environmental effects.

C. Studies Based on Interviews of Population and Volunteer Twin Registries

Table 4 summarizes the results of three studies that used unselected samples of twins and attempted to personally assess all participants for history of alcoholism.

True et al. (30) reported results from telephone interviews with male twin pairs who are members of the Vietnam Era Twin (VET) registry. Subjects were eligible for inclusion in the registry if they were born between 1939 and 1957 and both twins served in the U.S. military. Of over 10,000 eligible twins, 79% were located and interviewed. MZ twins were significantly more similar than DZ twins, producing a heritability estimate of .55 with no evidence for common environmental influences.

Heath et al. (31) reported results from a telephone interview assessment of members of the Australian National Health and Medical Research Council twin registry. Participants were eligible based on prior research participation, and 86% of those eligible were interviewed. Extensive analyses were conducted to test for biases due to sampling and attrition, but these had little effect on the obtained estimates. This study found strong evidence for genetic influences on the develop-

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ment of alcoholism for both sexes, while the evidence for common environmental effects was negligible.

1. The MCV Stress and Coping Study

The Medical College of Virginia Stress and Coping Study was begun in 1988 by Kenneth Kendler and colleagues at the Medical College of Virginia of Virginia Commonwealth University. The goals are to study environmental risk factors and genetic influences on the development of psychiatric and substance-use disorders. Subjects are Caucasian twins born between 1934 and 1975 and recruited from a birth registry formed from multiple births in the state of Virginia since 1918. The first component of this project was a study of adult female-female (FF) twin pairs. The study now includes four waves of interviews plus interviews of parents. A parallel study of male-male (MM) and male-female (MF) twin pairs, begun in 1993, consists of two completed waves of interviews. A third wave of interviews with MM pairs is underway. Interviews were completed on 92% of eligible FF twins and 73% of eligible twins from MM and MF pairs.

In 1992 Kendler et al. (32) published the results for twin resemblance for alcoholism based on the first wave of interviews with the FF sample. For several definitions of alcoholism, heritability estimates were substantial, ranging from .50 to .61. There was evidence for a small influence of common environment for DSM-III-R AD.

We recently published an extension of this work based on the new samples of male and opposite-sex pairs and an extensive reevaluation of the FF sample (33). Results were similar for all definitions studied, with heritability estimates ranging from .51 to .66 and of similar magnitude for men and women. We found little evidence of common environmental contributions to twin-pair similarity.

VI. ARE THERE SEX DIFFERENCES IN THE HERITABILITY OF ALCOHOLISM?

The results from published twin and adoption studies present a consistent picture of moderate to strong familiality of alcoholism in both men and women. For men, the majority of this is attributed to genetic factors. Among women, the sources for familiality vary across studies. One explanation for these differences is limited statistical power. The estimates of genetic and common environmental effects are highly correlated in twin models, so distinguishing between them is difficult in small samples. The fact that Pickens et al. report a heritability of .42 for women for AD based on a subset of the sample found by McGue et al. to have a heritability of .00 indicates the sensitivity of these analyses to small samples.

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Reanalyses by Heath et al. (34) indicate some reported sex differences are not statistically significant.

A. Etiological Heterogeneity

Another explanation for finding sex differences in genetic influences on alcoholism is etiological heterogeneity. As noted previously, Cloninger et al. reported greater genetic influences for early-onset men than for women or men with later onset (13). McGue et al. also reported evidence for etiological heterogeneity. Among 52 MZ and 44 DZ MM pairs in which the proband's symptoms began by age 20, the estimated heritability for AAD was $h^2 = .73$ compared to $h^2 = .30$ among MM pairs with later onset (21). The results among women were less marked but in the same direction, with greater similarity among pairs with early onset. The St Louis twin study (23) had low power to test for heterogeneity, but the results were not consistent with higher heritability for earlier onset. The prevalence of AAD among MZ male cotwins was higher among pairs with *later* onset (.90) than among those with earlier onset (.56), while DZ prevalences did not differ by onset age. In the Swedish TBR twin study (29), age at first TBR predicted cotwin risk equally well in MZ and DZ cotwins, suggesting early onset did not index genetic vulnerability.

Table 5 summarizes the results of preliminary analyses in the MCV twin sample of the association between onset age and familial risk. Probands were classified as early onset if their first symptom began prior to age 20. Rates of AD and AAD were tabulated for the cotwins of early- and later-onset twins. Among same-sex pairs, there is no evidence that early onset is associated with increased risk of alcoholism in a cotwin. There is some suggestion of increased risk to male cotwins of early-onset women, but this is not significant even in this large sample.

Even if early symptom onset were associated with increased risk to relatives, this does not necessarily indicate distinct etiologies. Rather, early onset could index greater liability in these probands, which would be expected to be associated with greater illness in relatives. Attempts to find etiologically distinct subtypes of alcoholism in genetically informative samples have identified types distinguished primarily by severity rather than by distinct patterns of clinical features (35,36).

Data from twin pairs can be used to test whether early- and later-onset alcoholism represent distinct subtypes versus different levels on a single dimension of severity. Data from our MCV twin pairs were organized into 3-by-3 tables for the three-category classification of unaffected, later onset, and early onset. The maximum-likelihood goodness of fit chi-square represents the adequacy of a single dimension to account for the twin-pair patterns in the data. Chi-squares for each zygosity group were : MZM = 12.6, DZM = 6.9, MZF = 4.0,

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Table 5 Probandwise Concordances for DSM-III-R Alcohol Dependence and Alcohol Abuse plus Dependence by Onset Age of Proband Among Twins from the MCV Stress and Coping Twin Study

	Prevalences among male cotwins			Prevalences among female cotwins		
	MZM	DZM	DZmF	MZF	DZF	DZfM
DSM-III-R alcohol dependence						
Proband with early onset (< age 20)	.50 (226)	.38 (195)	.45 (101)	.39 (44)	.07 (31)	.19 (227)
Proband with later onset (≥ age 20)	.46 (231)	.40 (168)	.31 (89)[a]	.40 (43)	.16 (38)	.15 (199)
DSM-III-R alcohol abuse and/or dependence						
Proband with early onset (< age 20)	.59 (284)	.50 (250)	.50 (133)	.33 (58)	.18 (44)	.22 (277)
Proband with later onset (≥ age 20)	.53 (289)	.51 (227)	.40 (111)[b]	.38 (56)	.14 (42)	.18 (270)

Values are prevalences in cotwins of affected probands and (*N* probands). Prevalences are for cotwin disorder, regardless of the age at which the affected cotwin's symptoms began. Mean age of symptom onset for DSM-III-R AAD: males = 20.9 (SD = 5.6, median = 19); females = 21.3 (SD = 6.1, median = 20). DZmF = male cotwin of affected female; DZfM = female cotwin of affected male. Two-tailed test of differences in proportions of early- and later-onset pairs.

[a] $p < .06$.

[b] $p < .12$.

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DZF = 1.3, DZO = 3.6 (all with 3 degrees of freedom). Only the value for MZM is significant at the $p < .05$ level. An examination of the discrepancies between observed and expected values for MZM pairs indicated that the misfit was due to excess pairs where both had later onset and fewer than expected pairs in which one twin had early and the other later onset. These results are consistent with there being some specificity for using onset as a marker of type rather than severity, but this is an indirect test and needs to be substantiated with other methods.

Another approach to this issue has been to estimate the heritability of individual diagnostic criteria and attempt to combine them to define a more heritable type of alcoholism (37). However, this method has been criticized on methodological grounds and was not replicated in a larger sample (38).

B. Study Ascertainment

A third explanation for apparent sex differences in genetic influences on alcoholism is study differences in ascertainment strategy. The four studies that report lower heritability of alcoholism in women were all are based on treatment-ascertained samples. Studies using archival data or treatment samples are likely to include more severe cases and be selected for excess comorbidity (39) or be unrepresentative in other ways, particularly for women (40). For example, in the Minnesota sample, 61% of the female probands had been treated for depression, compared with 16% of male probands (21). In the St. Louis twin sample, half the female probands had abuse or dependence on substances in addition to alcohol and 28% were diagnosed with antisocial personality disorder (23). This suggests that the women in these studies differ from affected women identified in population-based samples. The other two studies had methodological limitations of small sample size (19) and underascertainment of cases (26).

In the VET sample, treatment entry was influenced both by factors underlying the liability to alcoholism and by novel genetic and environmental influences on treatment seeking (30). Although this did not lead to large biases in the heritability estimates of alcoholism in that study, these results do suggest that treatment seeking is not a simple function of liability. If the processes responsible for women entering treatment are not simply a function of liability for alcoholism, the use of treatment ascertainment could misrepresent the etiological processes and yield biased heritability estimates (41). For example, assume that treatment entry of one twin increases the probability of the second twin seeking treatment or admitting her own symptoms, and this process affects MZ and DZ twin pairs to a similar degree. Common environmental processes would be responsible for treatment entry and would appear to contribute to the etiology of alcoholism as estimated from this study, even if familiality of the disorder was due to genetic factors.

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We investigated the role of treatment ascertainment on heritability estimates by applying a ''simulated'' treatment-ascertainment strategy to data collected in the population-based MCV study (42). Twins who met criteria for AD or AAD and who reported they obtained treatment for their alcohol disorders were designated as ''probands'' and we tabulated the prevalence of alcoholism in their cotwins. Results based on two definitions of treatment (inpatient or outpatient alcohol treatment, and any treatment) were compared to results from the random ascertainment method originally used in the sample.

Among individuals who were diagnosed as alcoholic, men were twice as likely as women to enter alcohol treatment. This difference was not accounted for by sex differences in clinical severity or course, suggesting that there were sex-related differences in treatment entry. Among men, heritability estimates were similar across sampling methods. For DSM-IV AD, the variance proportions estimated from identifying probands from alcoholism treatment ascertainment were: $h^2 = .56$, $c^2 = .25$, $e^2 = .19$, compared with $h^2 = .51$, $c^2 = .00$, $e^2 = .49$, for random ascertainment. The treatment ascertainment methods yielded higher estimates of common environmental influences, a finding similar to the results of twin studies that employed archival and treatment-based ascertainment.

Among women, heritability estimates based on the broad definition of treatment were similar to those obtained using the random ascertainment design, but estimates based on alcoholism treatment were (nonsignificantly) lower. Variance proportions for DSM-IV AD for alcohol treatment ascertainment were: $h^2 = .22$, $c^2 = .00$, $e^2 = .78$, compared to $h^2 = .62$, $c^2 = .00$, $e^2 = .38$, for random ascertainment. These results provide partial support for the hypothesis that differences in sampling method may account for differences among studies in heritability estimates.

VII. ARE THERE SEX DIFFERENCES IN THE TRANSMISSION OF ALCOHOLISM?

The key questions about sex differences in the transmission of alcoholism require information on resemblance in male-female relative pairs. Table 6 summarizes the four twin studies and two adoption studies that included information on resemblance for all types of relative pairs. Also shown are results from a meta-analysis by McGue and Slutske (43) of seven family studies that included data from such pairings.

These data were used to evaluate hypotheses about the transmission of alcoholism within and across sexes. The first analysis tested the multiple-threshold model described by Cloninger et al. (6), which predicts that affected women have higher liability than affected men. A relative risk (RR) greater than 1.0 indicates higher prevalences of alcoholism among relatives of women, consistent

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Table 6 Probandwise Concordances and Relative Risks for Sex Differences in Alcoholism Transmission from Studies that Included Same-Sex and Opposite-Sex Pairs of First-Degree Relatives

Study	Diagnosis	1° Male relative of an alcoholic		1° Female relative of an alcoholic:		Relative risk associated with female vs. male affected relative	Relative risk for same-sex vs. opposite-sex transmission
		Male (mM)	Female (mF)	Male (fM)	Female (fF)		
Family studies[a]	Varies	.27	.33	.06	.10	1.58	0.88
Twin studies							
McGue et al., 1992	DSM-III AAD	.54	.78	.31	.42	1.49*	1.17
Caldwell and Gottesman, 1991	DSM-III AD	.13	.57	.15	.25	2.11**	1.54
	DSM-III AAD	.46	.58	.33	.42	1.24	1.02
Heath et al., 1997[b,c]	DSM-III-R AD	.33	.60	.14	.17	1.17*	0.85
Prescott et al., 1999 and	DSM-III-R AAD	.50	.46	.20	.16	0.95	1.08
Prescott and Kendler, 2000[c,d]	DSM-III-R AD	.39	.38	.17	.12	0.95	1.00
	DSM-IV AD	.33	.34	.15	.10	0.96	0.99
Adoption studies							
Bohman et al., 1981	Temperance	.22	.26	.04	.10	1.40	0.79
Sigvardsson et al., 1996	Board	.23	.32	.01	.00	—[f]	—[f]
Combined samples[e]	Registration	.23	.29	.03	.08	1.45	0.82

* $p < .05$; ** $p < .01$.

AAD = alcohol abuse and/or dependence; AD = alcohol dependence.

Relative risks adjusted for sample sizes and sex differences in prevalences using log-linear analyses.

[a] Based on data presented in McGue and Slutske (43); sample sizes and significance levels not available.

[b] RRs based on approximate *N* based on data presented in Heath et al., (31).

[c] Prevalences are probandwise concordances but RRs based on full pairwise data, including information about unaffected pairs.

[d] Probandwise concordances for opposite-sex twin pairs are previously unpublished.

[e] Prevalences from Sigvardsson et al. (15). subjects with two alcoholic biological parents excluded.

[f] Not estimated because no affected females with affected mother.

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with this model's predictions. Values lower than 1.0 indicate greater prevalences among relatives of male alcoholics. The second test was for sex-specific transmission; i.e., the risk to relatives is greater if they are the same sex as the proband. An RR greater than 1.0 indicates evidence for sex-specific transmission. For each hypothesis, log-linear analysis was used to obtain relative risks, adjusting for sex-specific prevalences and sample sizes of the types of relative pairs. In reviewing these results, the emphasis will be on direction of effects and consistency across studies rather than on statistical significance, as the number of affected women in these samples is often too small to provide much statistical power.

The data from family, adoption, and the twin studies based on treatment samples all show evidence for stronger risk associated with an affected female relative. The magnitude of effect from these studies is about 1.4, suggesting a 40% increase in risk of alcoholism from being related to a female alcoholic rather than to a male alcoholic. The magnitude of effect is less strong (though significantly greater than 1.0) in the Heath et al. study, and does not differ from 1.0 in the Prescott et al. study. This may reflect differences in severity or etiology for women in treatment samples compared to affected women in the general population.

The evidence regarding sex-specific transmission is more variable, although none of the studies obtained an RR significantly different from 1.0. This may be in part because the tests are not independent, but partially competing. A strong sex of proband effect could obscure a weak effect of sex-specific transmission. These studies include many more affected men than women, so the results are weighted toward those pairings.

Another approach to investigating sex-specific transmission used in twin studies of population-based samples is to test whether twin-pair similarity (as indexed by a tetrachoric correlation) is lower among opposite-sex (MF) twin pairs than would be expected from the similarity of same-sex DZ pairs. (In the absence of sex-specific transmission, the MF correlation is expected to be the product of the square roots of the MM and FF correlations.) In the Australian twin study, the MF tetrachoric correlation was .43 compared to the expected value of .37 under no sex-specific transmission (31). In contrast, in the MCV study, the evidence was consistent with sex-specific transmission. For DSM-IV AAD the observed correlation of .14 was significantly lower than the expected value of .33. The estimated overlap in genetic liability between the sexes was 44% (95% CI = 12–76%) (33).

In summary, there is consistent evidence that relatives of women treated for alcoholism have higher risk for alcoholism than relatives of treated men. This suggests that women in treatment tend to have higher liability than their male counterparts. The results for untreated female alcoholics are less clear. The evidence regarding sex-specific transmission varies across studies, providing no consensus as to whether different sets of genetic factors influence the development

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of alcoholism in men and women. Some evidence from molecular genetic studies supports the existence of sex-specific loci (44), and a definitive answer to this issue will probably come from molecular rather than epidemiological studies.

VIII. LIMITATIONS OF AVAILABLE RESEARCH

The ability to detect sex differences in the etiology of alcoholism is affected by a variety of methodological limitations. The most serious problem is limited power due to the low prevalences of alcoholism in women. It is difficult to overcome this issue as twin and adoption studies have attempted complete ascertainment of affected individuals for entire countries or regions. It is unlikely that future studies using twin or adoption designs will have greatly increased power.

Some limitations arise from incomplete ascertainment based on treatment samples, particularly if there are sex differences in the processes underlying treatment entry or detection. Studies using hospital diagnoses may include only a small fraction of individuals with alcoholism residing in the community. Two twin studies that had later follow-up produced higher estimates of heritability and decreased evidence of common environment (25,28), suggesting the earlier results were biased by incomplete ascertainment. Use of TBR records also underestimates prevalences, but there is some evidence that this may not introduce sampling bias, at least for male alcoholics (18).

Studies using clinical interviews have better detection rates of positive cases, but suffer from incomplete ascertainment due to not all relatives being through the risk period for the development of alcoholism. The lower pair resemblance for later-onset alcoholism in some studies might increase with subsequent follow-up of currently unaffected individuals. Studies that tested for differences in twin-pair similarity by age group have typically not found that older pairs have greater similarity (29,31,33), as would be the case if incomplete ascertainment were a problem, but these tests have limited power.

Another limitation is that nearly all subjects studied are of northern and western European ancestry, so almost nothing is known about the etiology of alcoholism in non-Caucasian groups. Only the St. Louis twin sample had more than 10% of subjects who were nonwhite. Ethnic group differences in etiology could arise from differences in frequencies of genes influencing alcohol metabolism as well as from different environmental influences.

Estimation of genetic and common environmental influences from twin and adoption studies assumes homogeneity of etiology. The existence of different forms of alcoholism with different degrees of genetic influence may be obscured by combining all individuals who have a lifetime diagnosis, regardless of symptom severity, course, or family history.

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IX. BEYOND HERITABILITY: APPLICATIONS OF GENETIC EPIDEMIOLOGICAL RESEARCH

The goal of current twin, adoption, and high-risk research is to understand how genetically transmitted liability develops into alcoholism. This is being done through multiple lines of research, including studying genetic influences at different stages of alcohol involvement, identifying high-risk (or protective) environments that may interact with genetic liability, studying whether genetic influences are specific to alcoholism or also contribute to risk for other disorders, and identifying subclinical characteristics that may index genetic risk among vulnerable but unaffected individuals.

A. Genetic Influences at Different Stages of Alcohol Involvement

Epidemiological data (45) indicate that the proportion of heavy drinkers who become alcoholic is comparable for men and women. This suggests that sex differences arise prior to the development of heavy drinking. Population-based twin studies have found strong influences of common environmental factors on drinking initiation for both men and women. However, among drinkers, genetic factors appear to be more important than familial environment for influencing quantity and frequency of consumption (6). This suggests different genetic and environmental processes influence different stages of alcohol involvement.

We recently reported an overlap in the genetic factors underlying early first use of alcohol and later development of alcoholism (46). In addition to replicating the finding that early drinking was associated with increased risk for later alcohol dependence, we found early drinking was associated with *cotwins'* alcoholism. This association was stronger in MZ than DZ pairs, consistent with overlapping genetic influences for early use and alcoholism liability. In another example of this approach, Heath et al. (47) found genetic factors influencing alcoholism overlapped with those influencing physiological and subjective responses to ethanol during an alcohol challenge procedure.

B. Environmental Factors that Interact with Genetic Liability for Alcoholism

Several investigators have reported aspects of the environment that appear to interact with genetic liability for alcoholism. In the Swedish and Iowa adoption studies, having an adoptive family member with alcohol problems increased risk for alcohol abuse among children adopted away from alcoholic parents, but not among adoptees whose biological parents were not alcoholic (15–17). In the Iowa sample this effect was most pronounced among adoptees in rural communities.

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Studies of Finnish twins have found pairs who live in the same geographic region are more similar for alcoholism and alcohol consumption than those who live further apart, particularly if both live in a rural area (25,48). These results suggest family environment is less salient in more diverse, urban settings. Among Australian female twins, pairs who were married were less similar in their alcohol consumption than unmarried twins (49), consistent with an important influence of romantic partners on women's drinking. However, the interpretation of these findings is somewhat problematic, as pairs who differ in liability may be more likely to move apart or differ in marital status. Resolution of this issue awaits the results of a number of longitudinal studies of alcohol use, currently underway (e.g., 48,50).

Onset of alcohol use and level of alcohol consumption have been associated with age of menarche among female adolescent twins (51). This suggests hormonal status may alter expression of genetic liability and provides another way in which sex differences could arise. Alternatively, menarche could be an environmental modifier, as girls who mature early may have older peers and greater access to alcohol.

C. Factors that Mediate Genetic Liability for Alcoholism

A variety of characteristics have been suggested as mediators for genetic influences on alcoholism. Alcoholism is associated with increased risk for most psychiatric conditions, and some familial resemblance for alcoholism may arise indirectly through genetic predisposition to other disorders (2). Twin studies of alcoholism and other substance dependence support the existence of genetic influences that predispose to addiction to multiple substances as well as genetic factors specific to alcoholism (51–53). Sex differences in patterns of comorbidity with alcoholism provide a promising avenue for identification of etiologically distinct subtypes.

Another line of research is the use of genetically informative samples to identify subclinical characteristics that may be markers of genetic risk for alcoholism. Proposed *endophenotypes* include event-related potentials (54), psychophysiological measures (55), and subjective response to ethanol (56).

X. SUMMARY

Twin and adoption studies have established the importance of genetic influences in the etiology of alcoholism in men. The evidence for women is less consistent but newer studies suggest a similar degree of genetic influence. Women treated for alcoholism appear to have greater liability than treated men, but it is unclear whether this is true for untreated alcoholics. Results from some, but not all, stud-

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ies suggest the existence of sex-specific transmission of genetic liability for alcoholism.

Recent research has changed from an emphasis on estimating heritability to studying the factors that modify or mediate genetic liability. These applications, in conjunction with the use of measured genotypes from molecular genetic studies, identification of endophenotypes, and the longitudinal study of at-risk populations, offer promise for advancing our understanding of the mechanisms by which genetic vulnerability develops into alcoholism.

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8

The Dopamine D2 Receptor Gene and Alcoholism

Association Studies

Ulrich Finckh
University Hospital Hamburg–Eppendorf, University of Hamburg, Hamburg, Germany

I. INTRODUCTION

Genetic and nongenetic factors determine individual drug response profiles and psychopathology, both contributing at variable amounts to the risk of developing alcoholism. These traits are quantitatively rather than dichotomously distributed. Some candidate genes possibly involved in predisposition to alcoholism code for components of neuronal signal transmission in mesolimbic projections that form a key substrate for the reinforcing actions of all major drugs of abuse. Numerous case-control association studies in humans have already been performed by genotyping polymorphic candidate genes expressed in the mesolimbic areas. One of the genes most frequently assessed in alcoholism and various psychopathological conditions is the dopamine D2 receptor gene (*DRD2*). The results are highly conflicting, and multiple replication studies, mostly based on a single extragenic or intragenic *DRD2* polymorphism, do not reveal a clear trend. Genetic association studies in alcoholism have limitations due to phenotypic or genetic heterogeneity of study samples and a lack of functional correlates of the genotyped polymorphisms. Furthermore, association studies may not be sensitive enough to detect small effects attributable to a single gene in a complex phenotype. A single candidate gene may be related rather to specific traits than to a complex disorder in general. We genotyped simultaneously three polymorphisms (promoter −141C

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Table 1 Examples of Mouse Models with Homozygous Deficiencies (−/−) of Dopamine Receptors D1, D2, D3, D4, Dopamine Synthesis (Tyrosine Hydroxylase, TH), and Dopamine Transport (DAT1)

	D1 −/−	D2 −/−	D3 −/−	D4 −/−	TH −/−	DAT1 −/−
	Deficiencies in movement initiation and reactivity to external stimuli	Bradykinesia, catalepsy, Parkinson-like (dep. on genetic background)	Reduced anxiety-associated behaviour	Supersensitivity to locomotor activating effects of ethanol and psychostimulants	Lethal if not substituted with L-DOPA	Impaired spatial cognition
Phenotype characteristics (selected examples)	Lack of late phase of LTP in hippocampus	Lack of oplate reward and prepulse inhibition through amphetamine	Hypertension through elevated renin expression	Reduced exploration of novel stimuli		Lack of response to cocaine or amphetamine
	No locomotor activation through cocaine or amphetamine	Body weight ↓ (10–15%), body temperature ↓ (0.7°C)				Extracellular dopamine ↑
	However: after cocaine conditioning almost normal drug preference	Reduced fertility, hyperprolactinemia, pituitary lactotroph adenomas				
	Dynorphin and substance P mRNA ↓	Abnormal synaptic plasticity in striatum (mRNA: GDNF, NT4 ↓; enkephalin ↑)				

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		DAT function ↓, lack of autoreceptor mediated inhibitory control of DA release Postnatally compensatory upregulation of D3 expression			
Baseline activity	↓	? (May be masked through motor dysfunction) ↑	↓	↓	↑
Ethanol	Preference ↓	Preference ↓	Supersensitivity		
Food intake	↓			↓↓	
Human pathogenic mutation		Autosomal dominant myoclonus dystonia; affective disorders (53)	Autosomal recessive autonomous dysfunction (80)	Autosomal recessive Segawa syndrome, infantile parkinsonism (81–83)	

For review, see Online Mendelian Inheritance in Man (OMIM) database, http://www.ncbi.nlm.nih.gov/omim.

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Ins/Del; Exon 8 A/G; downstream *Taq*I A1/A2) spanning the entire coding region of *DRD2* and calculated linkage disequilibria in a clinically well-defined German sample of 292 alcoholics and 192 nonalcoholic control subjects. Our data suggest a predominant influence of the *DRD2* 3′-region over the 5′-region of the gene on various neuropsychiatric traits in alcoholics. There was an allele dosage-dependent effect of the haplotype *Ins*-G-A2 on anxiety, depressiveness, suicidality, and response to the dopaminergic agonist apomorphine. The data also suggest an increased probability of a clinical subtype of alcoholism in association with homozygosity of *DRD2* haplotype *Ins*-G-A2. However, to date, the existence and nature of functional genomic elements in the 3′-region of *DRD2* are unknown. Replication studies will be required for confirmation of these preliminary results.

From a genetic point of view, alcoholism may be considered a complex, multifactorial, and common disease. Individual biography, psychopathology, short- and long-lasting drug effects contribute to the clinical complexity of alcoholism. Both nongenetic and perhaps multiple genetic factors contribute to the multifactorial etiology, and social environment has a strong influence on the incidence of alcoholism.

In mendelian disorders, a rapidly growing number of disease genes and pathogenic mutations have been identified. The detection of susceptibility genes contributing to complex and multifactorial diseases still awaits such achievements (1). Alzheimer's disease is one of the few exceptions where a common susceptibility allele (APOE ε4) has been unambiguously identified in various human populations. In addition, three disease genes are known with mutations leading to autosomal dominant familial Alzheimer's disease. Unlike in Alzheimer's disease there are no such monogenic forms nor is there a clear morphological correlate of the pathological process known in alcoholism. Alcoholism is associated rather with quantitative traits like drug response profile, behavior, and psychopathology. Over 20 chromosomal regions linked to quantitative traits related to alcohol and drug responses have been mapped in recombinant inbred mouse strains (2). Several candidate genes coding for neurotransmitter receptors or enzymes involved in neurotransmission or neurotransmitter metabolism map to these quantitative trait loci (QTL). Some of these genes may be good candidates for association studies in alcoholism, particularly those expressed in mesolimbic projections of the basal forebrain that form a key substrate for the reinforcing actions of all major drugs of abuse. Knockout mouse models of several candidate genes exist. In addition to other behavioral, cognitive, and somatic traits, their phenotypes suggest a functional involvement of the respective gene products in alcohol sensitivity or preference (examples are listed in Table 1). These animals may be good starting points for knockins of naturally occurring (yet to be identified) or artificially constructed variants of the respective gene. Such experiments could resolve the question whether an increased function/expression or, rather, a lower func-

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tional level/disruption of the targeted gene contributes significantly to the quantitative trait of interest.

II. ASSOCIATION STUDIES

Numerous case-control association studies in humans have already been performed by genotyping polymorphic candidate genes expressed in the mesolimbic areas. Generally, these association studies in alcoholism and other psychiatric disorders suffer from contradictory results, lack of replicability, lack of morphological or biochemical correlates associated with the genotyped polymorphisms, and inconsistencies of clinical diagnoses. In contrast to the murine strains used for QTL mapping, no such highly inbred human population exists with pure predominance or absence of few genetic variants related to addictive behavior. Even in ethnical and geographic isolates significant allelic heterogeneity (risk alleles in many different genes) may exist if the founder population expanded from perhaps 1000 individuals approximately 100 generations ago. Therefore, whole genome-scanning studies by linkage or linkage disequilibrium mapping may not yield significant genomic linkage of a quantitative trait (see ref. 3 and references therein). Linkage studies have suggested various susceptibility loci for alcoholism with some of the linkage results depending on the diagnostic criteria used for definition of the phenotype (see also Hesselbrock et al., this volume). Only one of these chromosomal regions, on the long arm of chromosome 4, was identified in more than one study (for review see ref. 4). This chromosomal region was linked to protection against alcoholism and harbors the alcohol dehydrogenase (ADH) gene family. Association studies with population-based large study groups may be more suitable for identification of susceptibility alleles in heterogeneous disorders (5).

Perhaps several quantitative traits are involved in alcoholism. Thus, the number of susceptibility alleles from multiple genetic loci may be exceedingly high in alcoholism, and the complex disease per se may not be a sensitive indicator of the particular process conferring susceptibility (see also ref. 3). Different brain systems have been proposed to underlie various personality dimensions involved in different types of alcoholism (6). One of these traits (harm avoidance) has been recently mapped to a major locus on chromosome 8, which explained 38% of the variance of this trait (7). The data also suggested additional minor loci on other chromosomes. This example illustrates the possible heterogeneity of a single trait.

The phenotypic effect attributable to a single gene may be very small owing to allelic heterogeneity possibly underlying quantitative traits. Therefore, association studies based on single nucleotide polymorphisms (SNPs) within or near candidate genes may not necessarily achieve the power to identify genetic deter-

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minants of a quantitative trait in random samples. After choosing a candidate gene for association studies in alcoholism, several strategies may be used to enhance enrichment of the susceptibility alleles. Case groups may be selected from a population with reduced environmental risk, or stratified for disease severity, family history, age at disease onset, gender, clinical subtypes, personality traits (e.g., harm avoidance, reward dependence, novelty seeking), psychometric scorings (e.g., depressiveness, anxiety, well-being), sensitivity to alcohol, comorbidity of psychiatric disorder, electrophysiological parameters, response to pharmacological drug challenges, or other traits. Control groups may be population-based, disease-free, selected for extremes of a quantitative trait that are not associated with the disease, selected for negative family history, and matched to the case group by ethnic origin, age, and gender. The design of control groups has been the subject of intense discussion (see, e.g., ref. 8). Population admixture, migration, genetic drift, and other demographic factors may lead to unrecognized population stratification within or among study groups. It may be important to assess the genetic background of case and control groups to allow an interpretation of the results of association studies. This may be done by genotyping polymorphisms not linked with the candidate gene of interest. Pritchard and Rosenberg (9) proposed to genotype $\geq$15–20 unlinked microsatellite polymorphisms in both cases and controls, and to test for stratification.

Many human SNPs are of ancestral origin and exist at variable frequencies in genetically distant populations (10,11). Others may have evolved more recently and may be identified in descendants of distinct populations. To allow comparability of independent association studies in common diseases with a worldwide incidence, it may be favorable to include ancestral SNPs in addition to SNPs of specific interest in a given study population. Multiple combinations of allelic variants (i.e., haplotypes) at neighboring SNPs may have evolved through more recent mutations and recombinations in different populations (Fig. 1). Therefore, it is not possible to reliably compare independent association studies based on a single SNP without the knowledge of underlying haplotypes. The construction of unambiguous haplotypes based on SNP data from genomic DNA of unrelated subjects requires homozygosity for at least n-1 of the n genotyped SNPs in at least one subject. Rare haplotypes may be observed in subjects heterozygous for one of the more frequent haplotypes. The determination of rare haplotypes is very sensitive to genotyping errors. Some of the *DRD2* haplotypes previously described (12) could not be confirmed when repeating the genotyping (13). To determine the phase of neighboring SNPs in double or multiple heterozygotes, samples from parents or children of probands may be genotyped. Alternatively, individual haplotypes may be determined by genotyping allelic genomic fragments amplified through allele-specific PCR products (14) or by cloning.

The most interesting SNPs lead to amino acid changes in the corresponding gene product or affect gene expression through variation in regulatory genomic

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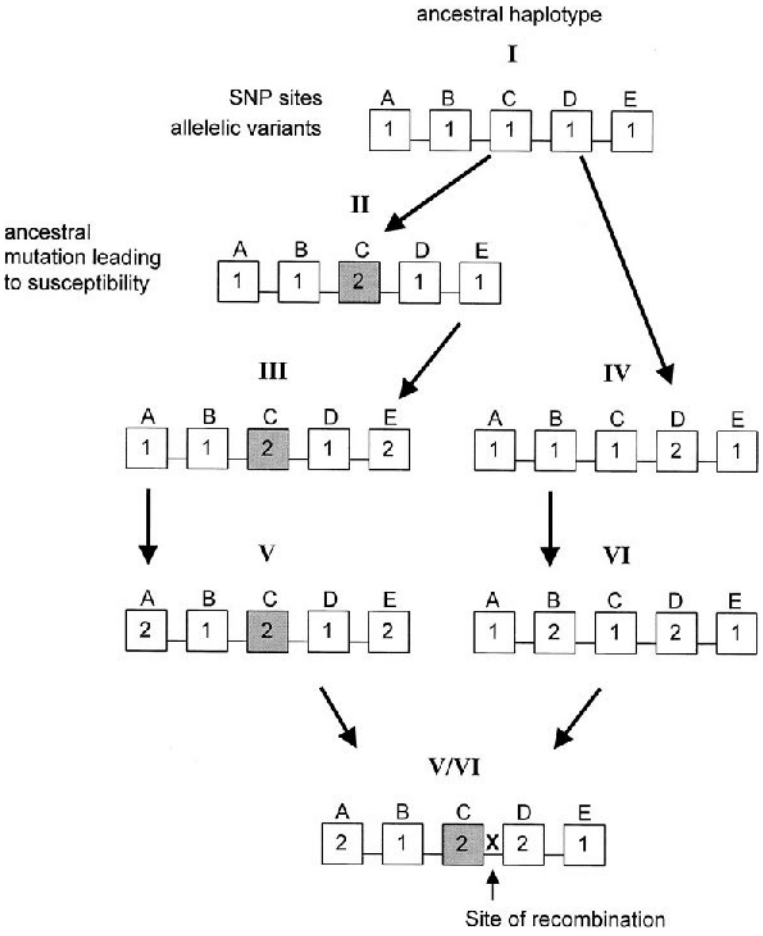

Figure 1 Model for evolution of haplotypes at a hypothetical genomic locus. Five SNPs (A–E) evolved independently at different time points of expansion of the human population. Each mutation from ancestral (1) to descendant (2) allelic variant was assumed to have occurred only once (indicated by arrows). Meiotic recombination (one example shown) leads to additional haplotypes thereby reducing linkage disequilibria between SNPs. An ancestral mutation at (hypothetically) functional site C introduced allelic variant 2 (shaded), which increases disease susceptibility. Susceptibility to alcoholism may not have counteracted reproduction or did not manifest over an expanded period of population expansion. The absence of selective pressure, or even a selective advantage, or genetic drift (see also ref. 3) may have influenced global or regional frequencies of SNPs and haplotypes. In populations with high prevalence of haplotypes III–VI an association between allele 2 of SNP E and susceptibility to the disease may be found through linkage disequilibrium between SNP E and SNP C. If only SNP E is genotyped, such an association will be overlooked in other populations with a high prevalence of haplotypes IV, VI, and V/VI or II. Even a low proportion of patients or controls with haplotypes V/VI or II may mask the association through the erroneous pooling of the haplotypes carrying the mutation (C 2) with nonmutated ones (C 1).

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Table 2 Polymorphisms in *DRD2*

DRD2 region[a]	Common name[b]	Restriction enzyme[c]	Ref.[d]	Reference sequence[e]	GenBank[f]	Ref.[g]	Effect[h]
Promoter	A-241G		39	A86	X53502	76	P
				A6100	AF148806	77	
Promoter	−141C *Ins/Del*	*Bst*NI (Ins)	39	Ins: C177	X53502	76	FP
				Ins: C6199	AF148806	77	
Intron 1	*Taq*I B B1/B2	*Taq*I (B2)	24	R7423	AF050737	23	P
Exon 2	L44L (CT**C** → CT**T**)		36	C8467	AF050737	23	P
Intron 2	*Taq*I D D1/D2	*Taq*I (D2)	34	Y12121	AF050737	23	P
Intron 2	STRP $CA_{(12-20)}$		24	G35 $CA_{(16)}$	X54392	24	P
				A4942 $TG_{(15)}$	X85812	21	
				A13514 $TG_{(16)}$	AF050737	23	
Exon 3	V96A (G**T**G → G**C**G)		36	T14858	AF050737	23	FP
Exon 4	L141L (CT**G** → CT**A**)		73	G16021	AF050737	23	P
Exon 4	V154I (**G**TC → **A**TC)		53	G16058	AF050737	23	M
Intron 4	T . . . G/C . . . A		22, 32	Y1125 . . . R1136	Z29558	22	P
				T17214 . . . G17225	AF050737	23	
Intron 5	15 T/G		22	K665	Z29559	22	P
				T18719	AF050737	23	
Intron 6	16 *Ins/Del* C		22	M571	Z29566	22	P
				A19128 G19129	AF050737	23	
Exon 7	P310S (**C**CG → **T**CG)		36	C20225	AF050737	23	FP

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Exon 7	S311C (T**C**C → T**G**C)		36, 37, 73	C20229	AF050737	23	FP
Exon 7	H313H (CA**C** → CA**T**) E7 T/C	*Nco*l (T)		Y20236 T T T C C	AF050737 M29066 S62137 S69899 X51362 M30625	23 20 78 79 72 71	P
Exon 7	P319P (CC**C** → CC**T**)		36	Y20254 C C C T T	AF050737 M29066 S62137 S69899 X51362 M30625	23 20 78 79 72 71	P
Exon 7	R361R (AG**G** → AG**A**)			G20380 G G G A A	AF050737 M29066 S62137 S69899 X51362 M30625	23 20 78 79 72 71	P
Exon 8	L441L (CT**C** → CT**T**)			C22255 C C C T T	AF050737 M29066 S62137 S69899 X51362 M30625	23 20 78 79 72 71	P

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Table 2 Continued

DRD2 region[a]	Common name[b]	Restriction enzyme[c]	Ref.[d]	Reference sequence[e]	GenBank[f]	Ref.[g]	Effect[h]
Exon 8	E8 A/G	*Hp*all (G)	33, 55, 58	R22316	AF050737	23	FP?
3′UTR				G	M29066	20	
				G	S69899	79	
				A	S62137	78	
				A	X51362	72	
				A	M30625	71	
Exon 8	Exon 8		74	C22640	AF050737	23	P
3′UTR	SSCP			C	M29066	20	
				G	S62137	78	
				C	S69899	79	
				C	X51362	72	
Exon 8	*Bso*Fl A/B	*BsoF*l (B)	75	B: G22989	AF050737	23	P
3′UTR				G	M29066	20	
				A: T	S62137	78	
				G	S69899	79	
				G	X51362	72	
Downstream	*Taq*l A A1/A2	*Taq*l (A2)	18	A2: C179	L22303	84	P
				C32806	AF050737	23	

[a] 3′UTR, 3′-untranslated region.
[b] Nucleotide changes in amino acid codons are shown in bold; STRP, short tandem repeat polymorphism; SSCP, single-strand conformation polymorphism.
[c] Allele cleaved by restriction endonuclease denoted in parentheses.
[d] References describing polymorphism.
[e] Nucleotide positions of alleleic variants in reference sequences; UPAC code: R, A + G; Y, C + T; K, G + T; M, C + A.
[f] GenBank accession numbers of reference sequences.
[g] Citations of reference sequences.
[h] P, neutral polymorphism; FP, functional polymorphism; M, mutation.

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elements. These functional polymorphisms show a tendency toward lower frequencies, higher diversities among populations, and a more recent evolution (10,11). The polymorphisms in alcohol and acetic aldehyde metabolizing enzymes (see chapters by Agarwal, Yin, and Whitfield, this volume) are good examples of functionally relevant sequence variants with significant phenotypic associations, particularly in populations with high frequencies of the alleles conferring altered enzymatic activity.

Most noncoding genomic regions with functional relevance for regulation of gene expression are not characterized sufficiently. Statistical analyses of large-scale sequencing and genotyping data revealed a significant action of selection against nonsynonymous (i.e., leading to an amino acid substitution) mutations as well as mutations in noncoding genomic regions neighboring protein coding elements (10,11,15). This suggests an important functional role of conserved noncoding elements. Noncoding elements involved in regulation of gene expression may play a critical role in candidate genes for quantitative traits involved in alcoholism. The genes for neurotransmitter receptors show a high degree of evolutionary conservation at the amino acid level. There are only few known examples of nonsynonymous mutations (see Tables 1 and 2). For most SNPs used in association studies there is no information on the functional relevance or the physical distance to functional genomic elements. Therefore, it is possible that association studies based on a single SNP cannot be replicated or may even lead to contradictory results (see legend of Fig. 1).

III. THE DOPAMINE D2 RECEPTOR GENE (DRD2)

The common neuronal correlate for reinforcement and reward seems to be the mesolimbic dopaminergic system. Drugs from different classes lead to activation of mesolimbic dopaminergic neurons (16). There is also evidence that activation of these dopaminergic neurons underlies learning processes involved in recognition of rewarding stimuli or aversive events (17). Among the above-mentioned candidate genes for alcoholism, the gene for the dopamine D2 receptor (*DRD2*) was the most frequently analyzed in association studies by genotyping the well-known *Taq*I A restriction site polymorphism with the allelic variants A1 and A2 (18).

DRD2 is localized on the long arm of human chromosome 11 at bands 11q22.3 and 11q23.1 (18,19). The coding region of *DRD2* is distributed across exons 2–8 and covers 13,925 bp of the gene (Fig. 2). For details see EMBL/GenBank accession numbers M29066 (mRNA; ref. 20), X85812 (intron 2; ref. 21), Z29564, Z29558, Z29559, Z29566, Z29557 (introns 3–7; ref. 22), AF050737 (downstream region; ref. 23). Intron 1 contains approximately 250 kb of unknown sequence (19). Owing to a polymorphic dinucleotide repeat element in intron 2

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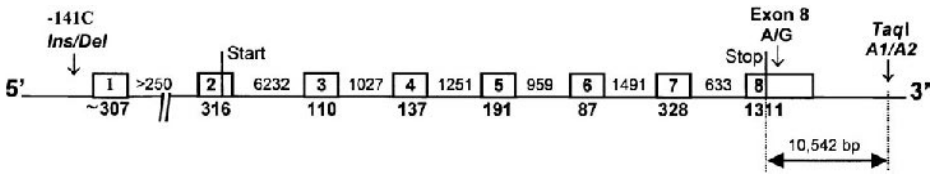


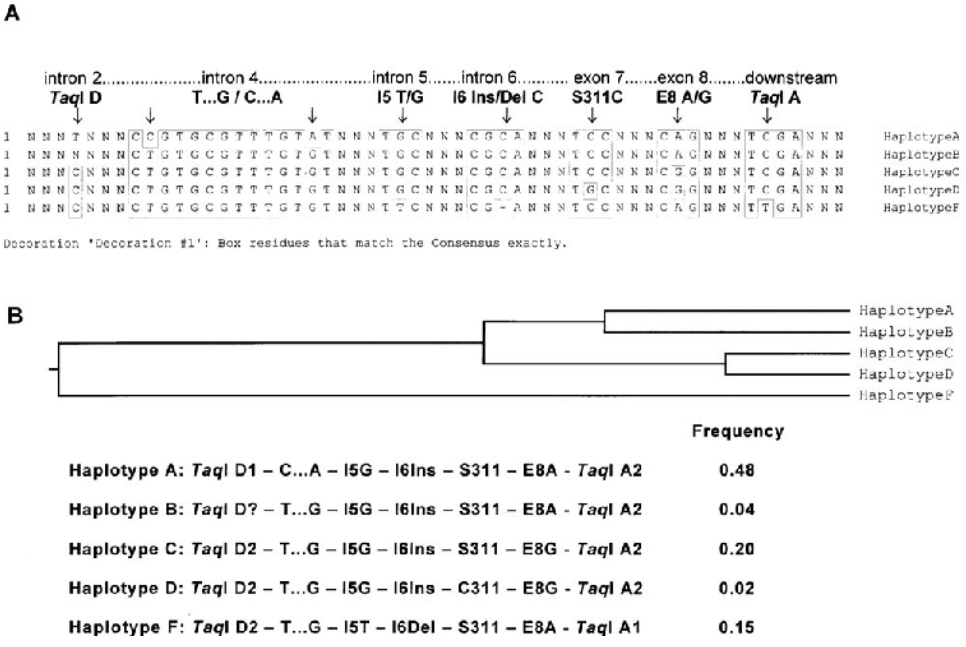
Figure 2 *DRD2* locus with exon numbers (in boxes), exon sizes in nucleotide base pairs (bold numbers, lower line), and intron sizes (nonbold numbers, upper line). Translation start and stop sites are shown. Positions of the three analyzed polymorphisms (58) are indicated by vertical arrows.

(24) and a 1-bp insertion/deletion polymorphism in intron 6 (22) there is a slight variability in length of the primary transcript. In addition to the polymorphic dinucleotide microsatellite in intron 2 (24), many SNPs have been described in *DRD2* (Table 2).

The *Taq*I A site is located approximately 10 kb downstream of the gene (Table 2, Fig. 2; ref. 23). There are significant differences in the frequency of the A1 allele between populations (23,25–27). Noble (28) reviewed 15 association studies on *Taq*I A and alcoholism. To avoid ethnic stratification, he selected studies from European Caucasians only. These studies contained a total of 1015 alcoholics and 898 controls with an overall statistically significant higher frequency of the A1 allele (f_{A1}) in the alcoholics (21.6%) compared to the controls (15.8%). As already discussed by others (29,30), this meta-analysis again gave the impression that the variability in allele frequencies was higher in the controls than in the alcoholics. Some studies with small control groups had particular low A1-allele frequencies in the controls, and there was a significant correlation between sample size and f_{A1} in the control samples (Spearman $r = 0.543$, $p = 0.037$) but not in the alcoholics samples (Spearman $r = -0.054$, $p = 0.85$). This suggests that some association studies suffer from genetic inhomogeneities and that some associations may have depended rather on the genotype frequency in control samples than in the patient samples. The human A1 allelic variant represents the phylogenetically ancestral sequence (TTGA), which is identical in primates (23,26,31; K. Kidd, personal communication, 2000). With the aid of known polymorphisms in and around *DRD2*, haplotypes have been constructed and their frequencies in various populations analyzed, along with various phylogenetic models (12,13,23,26,32,33). Together with a careful sampling strategy, genotyping of haplotypes instead of only one SNP may help to avoid population stratification and improve comparability of association studies in alcoholism (30).

The majority of *DRD2* association studies in Caucasian alcoholics were based on the *Taq*I A or *Taq*I B polymorphisms (Table 2), which show similar frequencies in most populations outside Africa with a high predominance of B1-

A1 and B2-A2 haplotypes in Europeans (23) and, therefore, may not differ significantly in their results. Another *Taq*I polymorphism, *Taq*I D, in *DRD2* intron 2 (34) has also been genotyped in various studies. *Taq*I B probably evolved on an ancient *Taq*I A1 allele (23). Both *Taq*I D and the A to G substitution polymorphism in *DRD2* exon 8 (E8 A/G), 52 bp downstream of the stop codon, seem to have evolved independently on *Taq*I A2 alleles later in human evolution (23,33; Fig. 3). In our samples E8 A/G subdivides the *Taq*I A2-containing haplotypes, and *Taq*I A1 is found almost exclusively in coupling with E8 A (Fig. 3, Table 3). Therefore, *DRD2* haplotype frequencies based on E8 A/G at least differ



	Frequency
Haplotype A: *Taq*I D1 – C...A – I5G – I6Ins – S311 – E8A - *Taq*I A2	**0.48**
Haplotype B: *Taq*I D? – T...G – I5G – I6Ins – S311 – E8A - *Taq*I A2	**0.04**
Haplotype C: *Taq*I D2 – T...G – I5G – I6Ins – S311 – E8G - *Taq*I A2	**0.20**
Haplotype D: *Taq*I D2 – T...G – I5G – I6Ins – C311 – E8G - *Taq*I A2	**0.02**
Haplotype F: *Taq*I D2 – T...G – I5T – I6Del – S311 – E8A - *Taq*I A1	**0.15**

Figure 3 (A) Virtual alignment of five *DRD2* haplotypes defined by genotype strings of seven polymorphisms (Jotun Hein method; LASERGENE software DNASTAR version 5.0, program MegAlign, DNASTAR Inc.). The physical distance between the polymorphisms was neglected here and may be derived from Table 2 (GenBank sequence AF050737). All haplotypes have been observed in at least one subject homozygous for at least six of the seven polymorphisms. (B) Tree based on the alignment and constructed by the same computer program. The tree suggests an ancestral origin of haplotype F containing *Taq*I A1 and later evolution of *Taq*I A2-containing haplotypes, which corresponds to evolutionary relationships published (23). Haplotype frequencies in the German population are estimated based on previous data (32).

Table 3 Combined Genotype Counts and Frequencies (%) of Three Different Polymorphisms that Span the Entire *DRD2* Coding Region.

		Patients (n = 292)			Controls (n = 192)		
	Exon 8:	A/A	A/G	G/G	A/A	A/G	G/G
	Ins/Ins	131 (44.7)	90 (30.8)	26 (8.9)	97 (50.5)	57 (29.7)	5 (2.6)
−141C	*Ins/Del*	7 (2.4)	24 (8.2)	10 (3.4)	7 (3.6)	17 (8.9)	5 (2.6)
	Del/Del	1 (0.3)	1 (0.3)	2 (0.7)	2 (1)	0	2 (1)
$f_{-141CDel}$[a]			0.084			0.096	
	*Taq*l A:	A2/A2	A2/A1	A1/A1	A2/A2	A2/A1	A1/A1
	A/A	76 (26)	51 (17.5)	12 (4.1)	75 (39.1)	27 (14.1)	4 (2.1)
Exon 8	A/G	87 (29.8)	27 (9.2)	1 (0.3)	50 (26)	23 (12)	1 (0.5)
	G/G	38 (13)	0	0	11 (5.7)	1 (0.5)	0
$f_{E8\ G}$[a]			0.327			0.255	
	−141C:	*Ins/Ins*	*Ins/Del*	*Del/Del*	*Ins/Ins*	*Ins/Del*	*Del/Del*
	A2/A2	159 (54.5)	38 (13)	4 (1.4)	112 (58.3)	20 (10.4)	4 (2.1)
*Taq*l A	A2/A1	75 (25.7)	3 (1)	0	43 (22.4)	8 (4.2)	0
	A1/A1	13 (4.5)	0	0	4 (2.1)	1 (0.5)	0
$f_{Taql\ A1}$[a]			0.178			0.159	

[a] $f_{-141CDel}$, $f_{E8\ G}$, $f_{Taql\ A1}$, frequencies of the respective rarer alleles of the three polymorphisms.

significantly from those based on the *Taq*I A and B systems in Germans (Caucasians) (Table 3).

Several polymorphisms in *DRD2* have been identified for which functional consequences have been demonstrated in vitro (Table 2). Cravchik et al. (35) analyzed ligand binding and signal transduction properties of the polymorphic variants Ala96, Ser310, and Cys311 (36,37) in transfected mammalian cells stably expressing these variants. The affinities of Ala96 and Cys311 for dopamine were significantly lower than the affinities of the Ser310 variant and D2 wild type. The binding of the antagonist [^{3}H]methylspiperone was not altered by these variants. The Ser310 and Cys311 variants (but not Ala96) also showed an impaired ability to inhibit forskolin-stimulated intracellular cAMP levels by dopamine. This effect was not due to differences in G-protein coupling but rather to conformational changes in the receptor protein with a reduced efficiency in activating the alpha subunit of the G-protein heterotrimer (35). In addition, several neuroleptics commonly used in the treatment of psychotic disorders had different binding affinities and potencies for these receptor variants (38). Arinami et al. (39) described a 1-bp insertion/deletion polymorphism (−141C *Ins/Del*) in the *DRD2* promoter region with a reduced reporter gene expression in association with −141C *Del* in transfected cell lines.

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Currently, no clinical or behavioral trait has been shown to be consistently associated with these functional and structural variants. Association findings between Ser311Cys or −141C *Ins/Del* with schizophrenia or alcoholism in Japanese (39–43, and others) or Swedish patients with schizophrenia (44) were not replicated in several other studies, including a Japanese study (45–52, and others).

Recently, linkage has been reported in one family with autosomal-dominant, alcohol-responsive myoclonus dystonia to a missense mutation (V154I) in *DRD2* exon 4 (53). The mutation was in coupling with the allelic variant A at the third position of codon 141 leading to the silent polymorphism L141L (Table 2). In addition to dystonia, various psychiatric disorders including depression, manic-depression, anxiety, panic attacks, and obsessive-compulsive disorder were associated with the mutation in this family. However, in vitro analysis of V154I did not reveal functional receptor alterations (54).

IV. PHENOTYPIC ASSOCIATIONS OF THE 3′-REGION OF *DRD2*

We reported an association between the above-mentioned A/G polymorphism in *DRD2* exon 8 (E8 A/G) 52 bp downstream of the stop codon and the clinical outcome of alcoholism in Germans (55). All patients underwent detoxification treatment. A subgroup of the patients participated in a follow-up program with repeated psychometric and clinical assessments including analysis of the response to the dopaminergic agonist apomorphine (56,57). Homozygosity E8 A/A was associated with increased depressiveness, anxiety, suicidality, a trend toward higher withdrawal severity, and reduced growth hormone (GH) responses after an apomorphine challenge. Some of the phenotypic associations seemed to depend on the dosage of the E8 A/G alleles. Similar to the previously reported 6-month follow-up data (55; see also Table 4), after 12 months, there was again an association of increased self reported anxiety (SAS) and depressiveness (SDS) scores with E8 A/A homozygosity (58).

To analyze whether the functional polymorphism −141C *Ins/Del* (39) interacts with the *DRD2* 3′-region or whether it shows independent phenotypic associations, −141C *Ins/Del* together with E8 A/G and the *Taq*I A1/A2 RFLP were genotyped in 292 alcoholic patients and 192 control individuals. These samples included participants who were ascertained for an earlier (55) and another more recent study (58).

The genomic region flanking −141C *Ins/Del* was amplified with primers promF02, 5′CAACC CTGGCTTCTGAGTCC3′, and promR02, 5′GAGCTGTACCTCCTCGGCGATC3′, by touchdown PCR (containing 5% DMSO) with a minimum annealing temperature of 55°C. *Bst*NI restriction

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Table 4 Analysis of Associations Between *DRD2* Exon 8 A/G and Promoter −141C *Ins/Del* Polymorphisms and Apomorphine-Induced Growth Hormone (GH) Responses on the Day of Admission to Clinical Withdrawal Treatment, Mean Scores of Self-Reported Anxiety (SAS) and Depressiveness (SDS), and History of Suicide Attempts in Alcoholics

		−141C and exon 8 genotypes						Exon 8 genotypes in 141C *Ins/Ins* homozygotes		
		−141C			exon 8					
		Ins/Ins	*Ins/Del*	*Del/Del*	A/A	A/G	G/G	A/A	A/G	G/G
	n	88	11	1	47	37	16	44	32	12
GH[a]		14.4	8.4	0	6.4	17.6	25.3	6.7	20.3	27.1
(s.d.)		(26.5)	(28.9)		(21.7)	(26.4)	(34.9)	(22.2)	(27.4)	(31.4)
	p[b]					0.023			0.016	
	p[c]	0.368			0.011			0.006		
	n	80	10	0	43	35	12	40	32	8
SAS[d]		31.2	29.4		34.1	28.8	26.7	34.3	28.9	25.1
(s.d.)		(8.8)	(6.0)		(9.6)	(6.7)	(4.5)	(9.8)	(6.9)	(2.3)
	p[b]					0.003			0.003	
	p[c]	0.525			0.001			0.001		
SDS[d]		34.7	32.9		38.1	32.1	28.9	38.6	32.1	26.0
(s.d.)		(11.4)	(6.9)		(11.6)	(9.9)	(6.6)	(11.8)	(10.1)	(4.3)
	p[b]					0.007			0.003	
	p[c]	0.610			0.002			0.002		
	n	226	38	3	127	107	33	120	85	21
Suicide attempts										
	No	166	31	3[g]	87	84	29	82	65	19
	Yes	60	7	0	40	23	4	38	20	2
	(%)	(26.5)	(18.4)		(31.5)	(21.5)	(12.1)	(31.7)	(23.5)	(9.5)
	p[e]		0.340			0.040			0.077	
	p[f]		0.155			0.011			0.026	

[a] Area under curve of serial GH measurements in 0.5-hr intervals after apomorphine challenge as described (55).
[b] ANOVA, DF = 2.
[c] ANOVA, DF = 1; E8 A/A vs. A/G *or* G/G; −141C *Ins/Ins* vs. *Ins/Del* or *Del/Del*.
[d] Mean SAS and SDS from repeated scorings at 2, 4, 6, 8, 12 weeks after clinical detoxification treatment.
[e] 2 × 3 tables, χ^2-test, DF = 2.
[f] Mantel-Haenszel test for linear association.
[g] Two of these three *Del/Del* homozygotes were also E8GG, one was E8AG.

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cleavage of the 207-bp product (−141C *Ins* allele) yielded fragments of 177 bp and 30 bp; the −141C *Del* allele (206 bp) is not cleaved. E8 A/G was amplified with primers INTR7, 5′GCCGTGCCTCCCCGGCTCTG3′ and EX8, 5′GGCAGTGAGGAGCATGGAGCCAAG3′ and genotyped by *Hpa*II restriction analysis of the 405-bp fragment that contained the polymorphic and two constant *Hpa*II restriction sites. *Hpa*II restriction yields E8 A-allelic fragments of 348, 45, and 12 bp, and E8 G-allelic fragments of 283, 65, 45, and 12 bp, respectively. *Taq*I A1/A2 was genotyped as described previously (59).

Allele frequencies and all combined genotypes are shown in Table 3. None of the polymorphisms deviated significantly from Hardy Weinberg equilibrium (HWE). Statistical analysis (for method see ref. 33) of the pairwise linkage disequilibria between the three polymorphisms revealed linkage disequilibria between −141C *Ins/Del* and E8 A/G in the patients ($\Delta_{-141C\times E8} = 0.032$; $\chi^2 = 16.92$) and controls ($\Delta_{-141C\times E8} = 0.042$; $\chi^2 = 18.29$). There was also linkage disequilibrium between promoter and *Taq*I A ($\Delta_{-141C\times TaqA} = 0.024$; $\chi^2 = 14.17$) in the patients, but not in the controls ($\Delta_{-141C\times TaqA} = 0.005$; $\chi^2 = 0.30$). This discrepancy was paralleled by a strong linkage disequilibrium between E8 A/G and *Taq*I A in the patients ($\Delta_{E8\times TaqA} = 0.068$; $\chi^2 = 40.07$) that was virtually absent in the controls ($\Delta_{E8\times TaqA} = 0.011$; $\chi^2 = 0.83$). Through correction for multiple calculations of linkage disequilibria χ^2 is >6.99 for $p < 0.05$, and χ^2 is >9.90 for $p < 0.01$.

In contrast to the associations between E8 A/A homozygosity and increased SAS, SDS, suicidality, and reduced GH responses (55), the associations between these traits and the genotype at −141C *Ins/Del* did not reach statistical significance (Table 4). Regression analyses revealed significant effects of E8 A/G but not of −141C *Ins/Del* on all these traits (Table 5). Complete follow-up data were available for 10 carriers of −141C *Del* (including 1 homozygote *Del/Del*). Eight of the 10 (80%, including the *Del/Del* homozygote) relapsed within the first 3 months after withdrawal treatment. Therefore, at months 3 and 12 only one and two carriers of −141C *Del*, respectively, were available for analyses of GH response and SAS/SDS. This did not allow statistical analyses of the respective associations with −141C *Ins/Del*.

This reanalysis of the previously reported associations in alcoholics after withdrawal treatment by using polymorphisms spanning the entire *DRD2* transcribed region suggests a predominant influence of the *DRD2* 3′-region over the 5′-region of the gene on various neuropsychiatric traits in the German population. However, although regression analyses only revealed significant effects of E8 A/G, the frequency of the −141C *Del* allele appears to be too low in our samples to allow exclusion of a moderate influence of the 5′-region in a minority of the patients. There may be a counteracting influence of E8 G and −141C *Del* on *DRD2* expression or function. This was assumed due to the high GH responses in association with E8 G and the reduced reporter gene expression in association

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Table 5 Regression Analyses of Associations Between *DRD2* exon 8 (E8) A/G and promoter (−141C) *Ins/Del* genotypes and apomorphine-induced growth hormone (GH) responses on the Day of Hospital Admission for Withdrawal Treatment, Mean Scores of Self-Reported Anxiety (SAS) and Depressiveness (SDS) (Linear Regression, Stepwise Method, Constant in Equation, Missing Values Replaced with Mean), and History of Suicide Attempts in Alcoholics (Logistic Regression, Forward LR Method)

		Independent variables							
Dependent variable		E8	−141C	Gender	Age	Alcohol in blood (yes/no)	Smoking (yes/no)	Cases in test (*n*)	Adjusted R^2
GH	*p*	0.034	0.111	0.540	0.411	0.027	0.132	100	0.101
	Beta	0.212	−0.157	0.059	−0.079	−0.222	0.145		
SAS	*p*	0.001	0.790	0.044	0.757	—	—	90	0.139
	Beta	−0.336	−0.028	−0.202	−0.031	—	—		
SDS	*p*	0.002	0.852	0.035	0.285	—	—	90	0.130
	Beta	−0.317	−0.019	−0.212	−0.107	—	—		
Suicide attempt	*p*	0.012	0.365	0.088	0.028	—	—	267	
	p log LR	0.009			0.024				

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with −141C *Del* (39). There was a slightly stronger association with E8 A/G after exclusion of carriers of −141C *Del* (Table 4), and the linkage disequilibria between −141C *Ins/Del* and E8 A/G in the patients as well as in the controls were in part due to a significantly higher rate of unambiguous (with respect to their phase with −141C *Ins/Del*) E8 G-alleles (9.7%) in *cis* with −141C *Del* compared to E8 A (3.7%; $\chi^2 = 13.39$, $p = 0.00025$, DF = 1). This may explain the stronger associations of the traits with E8 A/G after exclusion of −141C *Del* carriers.

All but one −141C *Ins/Ins*/E8 G/G homozygotes listed in Table 4 were also homozygous *Taq*I A2/A2. In addition, among all possible combined genotype counts, homozygosity for the haplotype *Ins*-G-A2 showed the most prominent difference between the patients and controls 8.9% (26/292) of the patients and 2.1% (4/192) of the controls were homozygous *Ins*-G-A2 (odds ratio = 4.59, C.I. 1.58–13.38) with a nominal $p = 0.0023$, i.e., not corrected for multiple testing. Our data suggest an increased probability to develop a clinical subtype of alcoholism in association with homozygosity of *DRD2* haplotype *Ins*-G-A2. We assume that this subtype is in part due to a robust expression or function of the *Ins*-G-A2 haplotype (as measured by GH response to apomorphine) that may enhance reward and prevent depressiveness and anxiety (as measured by SAS and SDS).

*Taq*I A1 was associated with reduced dopaminergic binding or reduced metabolism in brain regions containing dopaminergic representations (60–64), whereas −141C *Del* was associated with a higher ligand-binding potential (64). It may be interesting to reevaluate these studies with respect to the phenotypic associations with E8 A/G and the underlying haplotypes. In addition to the above-mentioned high proportion of −141C *Del* in coupling with E8 G, *Taq*I A1 was almost exclusively in *cis* with E8 A with only three (1.25%) of the unambiguous *Taq*I A1 alleles ($n = 240$) being in coupling with E8 G.

The T/C polymorphism at the third position of codon His313 in *DRD2* exon 7 (E7 T/C; Table 2) seems to be in strong linkage disequilibrium with E8 A/G. Our sequencing data from 27 subjects (55) and cloned cDNA sequences (GenBank accession numbers M30625, X52362) show coupling of E7 C with E8 A. Peroutka et al. (65) reported increased anxiety and depressiveness in association with E7 C/C homozygosity in patients with migraine. Similar to our data, this may support the view that the variances of anxiety and depressiveness share a common *DRD2*-associated genetic determinant, irrespective of the clinical disorder. The view of a common genetic basis of anxiety and depressiveness and their frequent ''comorbidity'' is supported through twin data and clinical studies (for review see ref. 66). Available sequencing data from *DRD2* intron 6 (22,67) together with genotyping data from other samples (68–70) and sequences of the published clones (20,67,71,72) are compatible with the view that E8 G (i.e., haplotype C in Fig. 3) corresponds to haplotype ''4'' from a previously published

DRD2 haplotyping scheme (68). Haplotype ''4'' was described to be associated with lower peripheral prolactin concentrations (69). Prolactin secretion is under well-known strong negative dopaminergic control, suggesting that haplotype ''4'' is associated with high D2 receptor expression, which corresponds to our observation of a higher growth hormone response in association with the exon 8 G-allele.

To date, the existence and nature of functional genomic elements in the 3′-region of *DRD2* are unknown, and it remains open whether these are causative for the observed associations. Therefore, independent replication studies are required that include -141C *Ins/Del*, E8 A/G, and *Taq*I A. If the *DRD2* haplotype is indeed associated with anxiety, depressiveness, suicidality, the clinical outcome of alcoholism, and with the function of the dopaminergic system, this may have significant impact on prognosis and treatment of alcoholism and other neuropsychiatric disorders, at least in a subgroup of patients.

V. CONCLUDING REMARKS

In the majority of our samples E8 A/G was genotyped twice by two different methods, allele-specific PCR (55) and *Hpa*II restriction digest (58). There were a few cases with discrepant genotyping results. These subjects were excluded from our studies. Few or even a single mistyping may significantly influence the calculated linkage disequilibria. We did not repeat *Taq*I A genotyping, which should be kept in mind when viewing the statistical differences in linkage disequilibria in the *DRD2* 3′-region between the patients and controls. Frequencies of alleles, genotypes, or common haplotypes are more robust against single genotyping errors. Nevertheless, the high odds ratio described above in association with homozygosity for haplotype *Ins*-G-A2 does not allow the conclusion that the *DRD2* genotype is associated with alcoholism *in general*. It may also reflect a sampling bias, e.g., through collection of patients voluntarily participating in a detoxification treatment and follow-up program. In addition, the ascertainment of voluntary control probands might also have led to a sampling bias. The associations between E8 A/G and psychometric, historical, and pharmacological traits in the alcoholics suggest a possible design of future replication studies. Within-group analysis of phenotypic associations may not suffer from such an ascertainment bias as the case-control studies may do.

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9

Biological Markers of Alcohol Use and Abuse in Theory and Practice

Anders Helander
Karolinska Institute and Hospital, Stockholm, Sweden

I. INTRODUCTION

Drunkenness and alcohol dependence represent major health, social, legal, and economic problems to both the individual and society. Excess alcohol intake is, for example, a common contributing risk factor in sickness (e.g., cirrhosis, pancreatitis, and hypertension), birth defects (fetal alcohol syndrome), accidents, injury, and trauma, and a leading cause of premature deaths (1). Alcohol-related problems are also important predictors of property damage, personal violence, and criminal activity (2). The associated economic costs for health care, social welfare, insurance, police, and justice systems are very high in most countries, and there are also many indirect costs related to productivity losses (3). Activities attempted at early identification and subsequent rehabilitation of those individuals who have, or are at risk for, alcohol problems therefore can be advisable for several reasons. However, to justify use of alcohol screening in the general population (e.g., health-care settings and workplaces), it is essential to utilize valid and reliable methods that can gain acceptance among both physicians and patients. This chapter gives a survey of biochemical markers of alcohol use and abuse and highlights some applications in selected and unselected populations.

II. METHODS TO IDENTIFY AND ESTIMATE ALCOHOL USE AND ABUSE

The traditional objective way to test for recent alcohol consumption is to determine the presence of ethanol in breath or body fluids (4). However, ethanol is

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rapidly cleared from the body, so a person may consume substantial amounts of alcoholic beverages (at least one bottle of wine, or ~4–5 cans of beer, or ~5–6 drinks; corresponding to ~60–80 g ethanol) in the evening and still present a negative breath or blood test the next morning (5). Furthermore, finding a single sample positive for ethanol is not a reliable indication of the person's drinking habits and whether there might be underlying alcohol problems.

Clinical interviews, patient self-reports, and various alcohol-screening instruments (questionnaires or tests), such as the CAGE (6), MAST (7), and AUDIT (8), have been commonly employed to obtain data about a person's quantity and frequency of current and past alcohol consumption, as well as any alcohol-related social-medical problems. However, although quantity-frequency questions usually represent an important source of information and have a low rate of false-positive responses, the primary weakness is that people may not report their alcohol intake accurately (9). Well-controlled population surveys have, for example, accounted for less than half of the known sales volume of alcohol (10). There are also indications that persons who are alcohol dependent may deliberately deny relapse and underreport their true intake more than light drinkers (11,12). Therefore, as the clinical signs of heavy drinking are rather modest, at least in the early stages of misuse, underdiagnosis of alcohol abuse and dependence is not uncommon by these methods.

Excess alcohol may profoundly influence normal cell and organ functioning and various metabolic processes through direct and indirect mechanisms. Therefore, to assist physicians with diagnosing problem drinking in a more objective way, a large number of laboratory tests, or biological *state* markers, based on abnormal blood and urine chemistry and altered hematology, have become available, and new ones are continuously being developed (13,14). Besides the many applications in clinical practice such as to identify persons with elevated or harmful levels of alcohol consumption and monitor abstinence and relapse during outpatient treatment of alcohol dependent subjects, biological markers also have found uses in workplace testing (15), forensic medicine (16), and experimental alcohol research (17).

Examples of currently employed tests of acute and chronic alcohol consumption are given in Table 1, and they are also described in greater detail below. The biological alcohol markers furnish indirect ways to:

- Provide at least rough estimates of the amounts consumed and the duration of ingestion (e.g., acute or prolonged heavy drinking)
- Evaluate any harmful effects of alcohol on the human body (e.g., alcohol-induced liver injury)

For example, testing urine for a raised 5-hydroxytryptophol to 5-hydroxyindoleacetic acid ratio (5HTOL/5HIAA) provides a new method to reveal alcohol consumption for several hours after ethanol is no longer measurable in blood or

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Table 1 Details of Some Biochemical Tests Used to Monitor Acute and Chronic Alcohol Ingestion

Test	Body specimen	Duration after acute or chronic intake
Ethanol	Blood, breath, urine, saliva, sweat	≤10–12 hr after last drink depending on dose
Methanol	Blood, urine, breath	*Acute*: ≤10 hr after ethanol has been cleared *Chronic*: days
Ethyl glucuronide (EtG)	Serum, urine	*Acute*: ≤10 hr (serum) after ethanol has been cleared *Chronic*:?
5HTOL/5HIAA	Urine, serum	≤ 10–15 hr after ethanol has been cleared depending on dose
MCV	Whole blood	~1–3 months
GGT, AST, ALT	Serum, plasma	~2–5 weeks
CDT	Serum	~1–3 weeks

breath, thereby considerably increasing the possibility to detect any recent drinking (12). Additionally, measurement of carbohydrate-deficient transferrin (CDT) and γ-glutamyltransferase (GGT) in samples of blood are examples of complementary biochemical tests used as indicators of prolonged excessive alcohol consumption and associated liver affection, respectively (18).

III. SENSITIVITY, SPECIFICITY, AND PREDICTIVE VALUES

Being indirect measures, or indicators, of alcohol use or abuse, alcohol markers are usually evaluated in terms of sensitivity and specificity. In this connection, *sensitivity* refers to the ability of a test to detect individuals with a certain level and duration of alcohol consumption, whereas *specificity* refers to its ability to exclude those who drink less. Consequently, a marker with high sensitivity yields few false-negative results and one with high specificity few false-positives (Fig. 1a). The ideal marker should of course be both 100% sensitive and 100% specific, but this is never achieved because reference ranges for what is considered ''normal'' and ''abnormal'' values tend to overlap. Moreover, the biological response to a given dose of alcohol is known to show large interindividual variability, and some may thus be able to drink excessively without displaying abnormal test results, which implies a low sensitivity of the marker in these subjects. By con-

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a)

Alcohol use/abuse	Test result +	Test result −
+	True positive	False negative
−	False positive	True negative

$$\text{Sensitivity (\%)} = \frac{\text{True positive}}{\text{True positive} + \text{False negative}}$$

$$\text{Specificity (\%)} = \frac{\text{True negative}}{\text{True negative} + \text{False positive}}$$

b)

$$\text{Positive predictive value (PPV; \%)} = \frac{\text{True positive}}{\text{True} + \text{False positive}}$$

$$\text{Negative predictive value (NPV; \%)} = \frac{\text{True negative}}{\text{True} + \text{False negative}}$$

Figure 1 Methods used to calculate (a) the sensitivity and specificity and (b) the positive and negative predictive values of biochemical markers.

trast, some biological alcohol markers may yield positive results also in people suffering from non-alcohol-related liver problems, or after taking certain kinds of medication, which implies a low specificity.

A traditional method to calculate reference intervals for laboratory parameters is to use the mean $\pm 2 \times$ the standard deviation (SD) of the values in a healthy control population. It should be noted that this practice will always result

in a specificity of less than 100%, because assuming a Gaussian distribution, ~5% of the control values will be lying outside (half above and half below) the reference interval. However, in reality the distribution of control values is often asymmetrical and intervals have to be adjusted accordingly (19). There are also several factors that are rather unique when establishing reference intervals for alcohol use or abuse. First, most studies aimed at evaluating the sensitivity and specificity of biological alcohol markers have continued to rely on self-report data about alcohol consumption as the ''gold standard.'' Considering that many patients fail to provide an accurate history of their true intake, as discussed earlier, this of course creates a validity problem. Second, alcohol consumption patterns and ''social drinking'' norms may vary considerably between cultures and societies, and thereby also the constitution of ''control'' populations. Thus, it is not always possible to make direct comparisons between different alcohol studies.

Besides the sensitivity and specificity figures, if the frequency, or prevalence, of the impairment (in this case, alcohol use or abuse) in the population to be studied is known, it is also possible to estimate the likelihood of obtaining a correct classification (the predictive value) when using the marker in question. The *positive predictive value* gives the likelihood that a positive test result is truly positive, and the *negative predictive value* the likelihood that a negative test result is truly negative (Fig. 1b). However, even if the marker is both relatively sensitive (e.g., 90%) and specific (e.g., 95%), the risk for incorrect classification might still be quite high if the impairment occurs only infrequently (i.e., a low prevalence) in the population (see examples in Table 2).

It should be kept in mind that the sensitivity, specificity, and predictive values of biological markers are highly dependent upon the cutoff, or threshold limit, chosen to distinguish between normal and abnormal values. For example,

Table 2 Estimated Chance of a Correct Classification (Positive and Negative Predictive Values) When Using Alcohol Markers with Different Sensitivity and Specificity in Various Settings (Hypothetical)

Setting	Approximate prevalence of alcohol-related problems	Sensitivity/ specificity of a marker	Positive/negative predictive value (PPV/NPV)
General population	8%	90%/95%	61%/99%
		70%/70%	17%/96%
Intensive care unit	20%	90%/95%	82%/97%
		70%/70%	37%/90%
Drunk drivers	50%	90%/95%	95%/90%
		70%/70%	70%/70%

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the upper reference limit may be raised to obtain a higher specificity of the test, but at the same time the sensitivity becomes gradually reduced, and vice versa. For each marker, different cutoffs may also be utilized in different settings. If markers are to be used for early identification of hazardous or harmful drinking, or detection of relapse in connection with rehabilitation, this places high demands on the sensitivity. In contrast, if a positive test result may lead to any legal sanctions to the individual (e.g., loss of employment or revocation of driving license), this places especially high demands on the specificity, as reflected in a low risk for obtaining false-positive identifications.

In recent years, the use of receiver-operating characteristic (ROC) curves has become popular as a tool for comparing different laboratory tests and selecting an optimal cutoff point (20,21). By creating a plot showing the relation between sensitivity (i.e., the percentage of true positives) and specificity (i.e., the percentage of false positives) at different cutoffs between normal and abnormal values, the overall performance of the markers, or analytical methods, can be illustrated graphically. However, it should be noted that ROC curves cover the entire sensitivity and specificity ranges (from 0 to 100% each), most of which are of no or limited use in clinical practice.

IV. LABORATORY TESTS I: ETHANOL AND METABOLITES

A. Ethanol

The most obvious way to prove intake of alcoholic beverages (the true ''gold standard'') is by demonstrating the presence of ethanol in body fluids or breath (4). Ethanol is rapidly absorbed from the stomach and small intestine into the circulatory system, and it then distributes in all body fluids and tissues in proportion to the water content. Because of this, different body fluid specimens collected at the same time may vary somewhat in their ethanol concentration (see example for urine and blood fractions in Table 3). The presence of ethanol in the body discloses recent drinking, but the amounts consumed and duration of drinking cannot be ascertained owing to a fairly rapid clearance of ethanol from the body at a rate of ~0.1 g/kg/hr (which corresponds to ~0.15–0.20 g/L/hr in the blood). As a consequence, a person may consume substantial amounts of alcoholic beverages in the evening (~60–80 g) and still present a negative ethanol test in the next morning (5). Indeed, there are indications that individuals undergoing outpatient treatment for their alcohol problems often continue to drink without admitting to, but they regulate the amount consumed and also the time they stop drinking to be able to present a negative breath or blood result at the time for testing (12). Compared with blood and breath, ethanol can be detected for some hours longer in urine, owing to retention of urine in the bladder (22).

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Table 3 Differences in Ethanol Concentrations Between Various Blood Fractions and Urine

Sample	Relative ethanol concentration	Range	Refs.
Whole blood	1.00	—	167
Plasma/serum	~1.15	~1.05–1.25	167
Erythrocytes	~0.85	~0.65–1.00	167
Urine	~1.30	~0.20–2.65[a]	168

[a] Very large variation in urine/blood ethanol concentration ratio if subjects are in the absorptive or postabsorptive state.

A positive ethanol test may or may not have any relationship to chronic abuse, because a low concentration of ethanol may result from recent intake of a small alcohol dose as well as from the late stages of eliminating a much larger intoxicating dose. Nevertheless, finding a high blood-ethanol concentration (BEC) during daytime clearly indicates advanced drinking habits. Moreover, showing little or no signs of intoxication (e.g., while driving a car) even at a fairly high BEC of >1.5 g/L (>0.15%) indicates that the person has become tolerant to the behavioral effects of ethanol as a result of prolonged excessive exposure. Checking for a positive concentration of ethanol is therefore always advisable before more detailed examinations are made.

Determination of ethanol in blood or urine is a relatively simple task, and valid and reliable results can be obtained with the aid of gas chromatographic (GC) or enzymatic (alcohol dehydrogenase, ADH) methods (23). In routine clinical and forensic applications, the limit of quantitation of the various laboratory methods is about 0.1 g/L. For some field applications (e.g., emergency departments, traffic medicine, and workplace testing), noninvasive sampling methods may be feasible to monitor alcohol exposure such as analyzing saliva with dipsticks or expired air with hand-held breathalyzers (24,25). However, the limit of quantitation of these on-site assays is similar to that for measuring ethanol in blood or urine.

A small amount (<1%) of the ingested ethanol is excreted through the skin by passive diffusion and through the sweat glands. This route of excretion has found applications in clinical practice and research as a way to monitor alcohol consumption over longer time periods (26). A tamper-resistant and waterproof pad (sweat patch) positioned on the body collects the fluid excreted from the skin at a steady rate for a period up to ~1 week. The concentration of ethanol is then determined by GC and the result obtained provides a cumulative index of alcohol exposure (i.e., roughly the average BEC) during the collection period. However, the threshold sensitivity for measuring ethanol in this way is approximately a

BEC in excess of 0.2 g/L (27,28). A miniaturized wearable, electronic alcohol sensor and recorder is also available (29).

B. Metabolites of Ethanol Oxidation—Acetic Acid and Acetaldehyde

Most of the ingested alcohol (95–98%) is metabolized primarily in the liver and only a small part (2–5%) is excreted in breath and urine (Fig. 2). Ethanol is metabolized in a two-stage oxidation process; first to acetaldehyde by the action of ADH, and to a low extent also by microsomal ethanol-oxidizing systems and catalase, and this primary metabolite is rapidly converted into acetic acid (acetate) by aldehyde dehydrogenase (ALDH). The oxidation of ethanol to acetaldehyde takes place in the cytosolic compartment of the hepatocyte and the acetaldehyde formed is rapidly and irreversibly oxidized to acetic acid mainly by the mitochondrial *ALDH2* isozyme. The acetic acid formed from oxidation of acetaldehyde is either oxidized further to yield energy (the end-products being CO_2 and H_2O), or channeled into biosynthetic pathways, or released into the circulation.

Raised concentrations of the intermediary products of ethanol oxidation

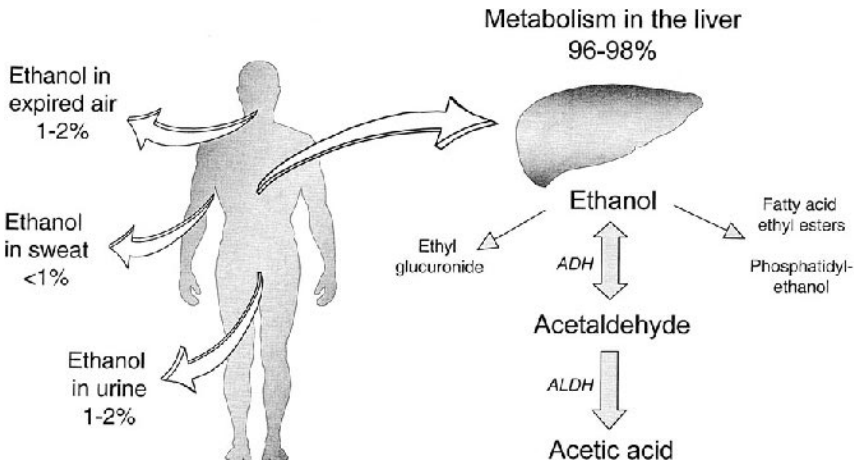

Figure 2 Major and minor routes of elimination of ingested ethanol in the human body. Most of the ingested alcohol is metabolized primarily in the liver and only a small part is excreted in breath, sweat, and urine. Ethanol is metabolized in a two-stage oxidation process, first to acetaldehyde mainly by the action of alcohol dehydrogenase (ADH), and this primary metabolite is rapidly converted into acetic acid (acetate) by aldehyde dehydrogenase (ALDH).

have been proposed as ways to confirm recent alcohol consumption. However, measuring acetaldehyde is not very practical because of the extremely low concentrations present under normal conditions (<1% of the ethanol concentration). Acetaldehyde is rapidly converted to acetic acid, and the concentration of free acetaldehyde in peripheral blood is further reduced owing to a more or less specific interaction with various endogenous molecules (see below). The analytical procedures are also much more challenging than those for the analysis of alcohol (30). An additional problem arises when the blood sample contains ethanol, because a substantial amount of acetaldehyde may then be formed artifactually during the analytical process (30,31). Measuring acetaldehyde in breath instead of blood has been suggested as an alternative approach, but there are analytical problems also with breath testing (32).

In alcohol-dependent patients undergoing treatment with disulfiram (Antabuse), a potent inhibitor of ALDH (33), and also in Asian individuals who produce an inactive variant of the *ALDH2* isozyme (34), circulating levels of acetaldehyde will, however, be very high after alcohol ingestion. The associated aversive reactions (e.g., headache, nausea, and flushing) should deter disulfiram patients from drinking and may also protect Asian populations against the development of excessive or problematic drinking (35,36).

Aldehydes are highly reactive substances that may combine with biomolecules such as DNA (37), phospholipids (38), and several proteins to form stable and unstable adducts (39,40). The interaction of acetaldehyde with hepatocellular macromolecules may also trigger an immunological attack and this might participate in the immune response associated with alcohol-induced liver disease (41,42). Measurement of acetaldehyde-hemoglobin adducts, or antibodies that recognize acetaldehyde-modified structures, have also emerged as possible biochemical markers of excessive drinking (43–45). Although some of these methods have shown promise as complementary tests to the conventional markers (46), and were even more sensitive for the detection of chronic excessive alcohol consumption (47), additional work is still necessary to determine the reliability and diagnostic potential of these tests for the identification of excessive drinking in unselected populations.

The concentration of acetic acid in blood depends on the rate of hepatic ethanol oxidation and its utilization by peripheral tissues. The concentration appears to be independent of the BEC, and instead increases with the development of metabolic tolerance to ethanol (i.e., rate of ethanol elimination) (48). Measuring blood acetic acid has therefore been suggested as a marker of chronic alcohol abuse rather than acute intake (49,50), and as such the sensitivity and specificity of this test was significantly higher than for GGT. It should be emphasized, however, that the circulating concentration of acetic acid remains increased only as long as ethanol is being metabolized and, moreover, that rates of ethanol metabolism exhibit large interindividual variations even in moderate drinkers.

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C. Nonoxidative Metabolites of Ethanol—Ethyl Glucuronide, Fatty Acid Ethyl Esters, and Phosphatidylethanol

A very small fraction of the ingested ethanol (<1%) undergoes conjugation with UDP-glucuronic acid to produce ethyl glucuronide (EtG) (Fig. 2), a water-soluble metabolite that is excreted in the urine (51). As for methanol and the 5HTOL/5HIAA ratio (see below), the washout constant for EtG is much longer than for ethanol (52). Accordingly, a positive finding of EtG in blood or urine provides a strong indication that the person was recently drinking alcohol, even if the ethanol itself is no longer present (53). During recent years much interest has focused on EtG as a test for recent alcohol ingestion, and sensitive and specific quantitative methods based on GC-MS and LC-MS technique have been developed (54–56). However, there are also indications that EtG may accumulate in body fluids during prolonged daily drinking but this has not yet been examined in detail.

Fatty acid ethyl esters (FAEE) are esterification products of fatty acids and ethanol synthesized through the action of the enzyme FAEE synthase (Fig. 2), and these end-products have been proposed as sensitive and specific markers of alcohol intake (57–59). Increasing evidence also indicates that FAEE are toxic metabolites and possibly mediators of ethanol-induced organ damage (60). After alcohol intake, the serum concentration of FAEE initially closely parallels that of ethanol (e.g., similar time for peak concentrations), but because of a very slow terminal elimination phase, FAEE persist in the blood for some time after ethanol is no longer detectable (59). Accordingly, in one experiment, all subjects who reached a peak BEC higher than 1.5 g/L showed low but detectable FAEE concentrations in their blood for at least 24 hr after alcohol intake was completed. Additionally, all blood samples with trace concentrations of ethanol were found to be positive for FAEE. A disadvantage of this test is that the analytical method is quite complex for routine use; it requires an initial isolation of FAEE from serum specimens by solid-phase extraction prior to quantitation by GC (61) or GC-MS (62). Furthermore, if plasma samples are used instead of serum, FAEE values have to be corrected (59).

Phosphatidylethanol is an abnormal phospholipid formed in cell membranes in the presence of ethanol (Fig. 2) (63,64). Phosphatidylethanol has been proposed as a highly specific marker of chronic heavy drinking within the previous week(s). After alcoholic patients, with a self-reported intake of 60–300 g ethanol daily for a period of ≥1 week, were admitted to a detoxification program, phosphatidylethanol remained detectable in their blood of for up to 2 weeks (65). Moreover, the sensitivity of this test was reported to be greater than, or at least equal to, that of CDT (66). The analytical procedure used for quantitation of phosphatidylethanol has been improved over the years but is still rather complex

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for routine use; it requires extraction of the lipids from whole-blood specimens followed by quantitation using an HPLC system equipped with an evaporative light-scattering detector (66,67).

V. LABORATORY TESTS II: METABOLIC INTERACTIONS

A. Methanol

Methanol and ethanol occur naturally in body fluids at low concentrations of about 1.0 mg/L, even without drinking any alcoholic beverages (68–70). These endogenous alcohols are probably derived from dietary sources (e.g., fruits) or from fermentation of carbohydrates or other enzymatic processes during the course of normal metabolism (71). Methanol and ethanol share the same enzyme system for detoxification, the class I ADH. However, class I ADH shows a ~10-fold higher affinity for oxidation of ethanol than methanol, which means that during a period of heavy drinking when ethanol is being metabolized, the concentration of methanol in body fluids successively increases (72,73). This difference in affinity for ADH is also the basis for the use of ethanol administration during treatment of methanol poisoning (74,75).

Methanol is not cleared from the blood until the ethanol concentration sinks below ~0.2 g/L, which means that an elevated concentration will persist for some hours after ethanol can no longer be detected (5,22,76,77). However, methanol is also present as a congener in alcoholic beverages, which augments the endogenous sources of this alcohol. It should also be pointed out that after continuous heavy drinking, methanol accumulates and levels might then exceed 10 mg/L; this threshold is used to indicate alcohol problems among drunk driving offenders (78,79). Accordingly, a problem when using methanol as alcohol marker is that an abnormally high concentration in samples of blood or urine could reflect recent as well as long-term continuous drinking. Methanol is determined by conventional head-space GC and the sensitivity of this assay can be increased by a salting-out procedure (80).

B. Ratio of 5-Hydroxytryptophol to 5-Hydroxyindole-3-Acetic Acid (5HTOL/5HIAA)

Ethanol ingestion perturbs the metabolism of serotonin (5-hydroxytryptamine) and this interaction can be utilized for detection of recent alcohol intake. 5HTOL is normally a minor (<1%) metabolite of serotonin but after intake of alcohol, the formation of 5HTOL increases dramatically in a dose-dependent manner, whereas 5HIAA, the major metabolite under normal conditions, is correspondingly decreased (81). This shift in serotonin metabolism occurs because of competitive inhibition of ALDH by the ethanol-derived acetaldehyde, and also the

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alteration of the NADH/NAD^+ ratio to a more reduced potential during ethanol metabolism (82). The urinary output of 5HTOL does not recover to baseline levels until several hours after ethanol is no longer measurable (83). Accordingly, the 5HTOL/5HIAA ratio is a much more sensitive test than measuring ethanol alone and can detect consumption of even moderate amounts of alcohol within the preceding ~24 hr (5,12,18).

To improve the accuracy of this test in routine clinical use, 5HTOL is expressed as a ratio to 5HIAA, because this compensates for variations in the concentration of 5HTOL caused by urine dilution as well as dietary sources of serotonin (e.g., high amounts in banana and pineapple) (84). Apart from consumption of alcohol, treatment with potent ALDH inhibitors like disulfiram (Antabuse) represent the only known cause of a raised 5HTOL/5HIAA ratio (85). In contrast to methanol (and EtG?), the baseline value for 5HTOL/5HIAA is not increased after prolonged alcohol abuse and the test can therefore be used to identify recent drinking in both moderate and chronic consumers. Urinary 5HIAA can, for example, be determined by HPLC with electrochemical detection using direct sample injection (86). 5HTOL, which is excreted mainly in conjugated form, can be determined by GC-MS following enzymatic hydrolysis (87,88), or directly using LC-MS technique. However, an immunoassay for 5HTOL is underway and this will clearly improve the utility of this test in routine clinical use.

C. Carbohydrate-Deficient Transferrin (CDT)

CDT, which refers to an abnormal microheterogeneity of the iron transport protein transferrin in serum, is a relatively new alcohol marker (the first commercial test kit was introduced in 1992) (89,90). In normal transferrin C phenotype serum, the most abundant isoform contains two *N*-linked biantennary carbohydrate chains with a total of four terminal sialic acid residues (named tetrasialotransferrin). However, intake of approximately 50–80 g of alcohol per day during at least 1–2 weeks prior to sampling often results in an elevated CDT level, usually defined as increased concentrations of transferrin molecules that lack one (disialotransferrin) or both (asialotransferrin) of their carbohydrate chains (91,92). The absence of the negatively charged sialic acid residues results in a higher isoelectric point than for normal transferrin, and most analytical procedures therefore utilize charge-based separation of CDT molecules prior to quantitation (93). The biological mechanism by which alcohol causes elevation of CDT has not yet been identified in detail, but may involve acetaldehyde-mediated inhibition of the enzymes responsible for glycosyl transfer (94,95). When drinking is discontinued, the serum CDT level normalizes with a half-life of ~1.5–2 weeks (89,96). A drawback with CDT is that different procedures for quantitation are, or have until recently been, in routine use, and this often hampers the direct comparison of results between published studies.

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Although many studies have indicated that CDT values become elevated substantially earlier in response to prolonged excessive drinking than conventional markers like GGT and MCV, its major asset is the higher specificity (89). Only a few causes of false-positive CDT results have been identified so far, and these include rare genetic transferrin variants (97), primary biliary cirrhosis (affects predominantly women) (98), chronic viral hepatitis (99), and hepatocellular carcinoma (100). Unusually high CDT values are also common in patients with extremely rare, inherited neurological disorders known as carbohydrate-deficient glycoprotein syndromes (CDGS) (101). Although CDGS patients represent no overall problem for the clinical utility of CDT, their parents often show abnormally high CDT values and the transferrin isoform pattern as observed by HPLC is also indistinguishable from that observed after excessive drinking (manuscript in preparation).

What is less recognized is that the risk for obtaining erroneous CDT results largely depends upon the procedure used for quantitation; most of the causes listed above refer to studies using the original CDTect immunoassay test kit, which measures CDT as an absolute amount. Because at least some causes for false-positives relate to an abnormally high serum transferrin concentration, instead measuring the ratio of CDT to the total transferrin concentration (i.e., % CDT) may improve diagnostic performance in these cases (93,102). Nevertheless, recent results have shown that irrespective of whether the CDT result is given as an absolute or relative amount, rare genetic transferrin variants may cause false-positive (transferrin D) but also false-negative (transferrin B) results when the commercial immunoassays currently available on the market are used (manuscript in preparation). However, these and other transferrin variants show unique isoform patterns that are readily identified by isoelectric focusing and HPLC (Fig. 3) techniques, although even then reliable quantitation of CDT is not always possible.

D. Mean Corpuscular Volume of Erythrocytes (MCV)

MCV is often measured as part of a routine blood count and indicates the mean size of the red blood cells (erythrocytes). An elevated MCV is often observed in alcoholic patients, and this hematological parameter has been widely used as a marker of excessive alcohol consumption (103). The underlying cause of swelling of the erythrocytes is unknown but may be a direct toxic effect of ethanol, or related to changes in folic acid or vitamin B metabolism (104). The sensitivity of MCV is much too low to motivate its use as a single indicator (105–107), and there are also several conditions besides heavy drinking (e.g., nonalcoholic liver diseases, smoking, and certain medications) that may lead to elevated values (108). However, MCV often shows higher specificity compared with the other standard biochemical tests (109,110). MCV may also be used to detect evidence

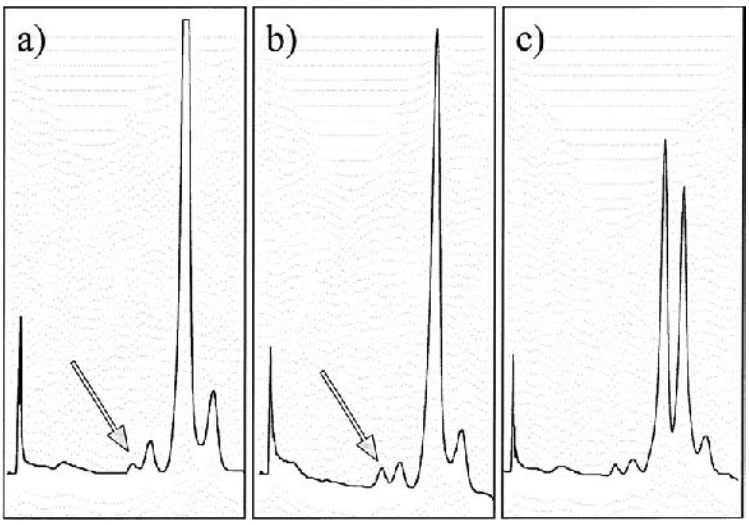

Figure 3 Human transferrin isoform patterns as observed by HPLC-UV technique. The chromatograms represent (a) a normal serum sample, (b) a serum sample with increased concentration of disialotransferrin (CDT; indicated by arrow) collected from an alcohol-dependent subject, and (c) a rare genetic transferrin variant (transferrin CD), which may cause false-positive results with the immunoassay methods.

of earlier heavy drinking after a longer period (months) of abstinence, because it takes relatively long to recover to normal values (the life span of erythrocytes in the circulation is ~120 days).

VI. LABORATORY TESTS III: TISSUE AND ORGAN DAMAGE

A. γ-Glutamyltransferase (GGT), Aspartate Aminotransferase (AST), and Alanine Aminotransferase (ALT)

Other traditional biochemical tests being used to identify prolonged alcohol misuse include the enzymes GGT, AST, and ALT. They are measured in samples of blood and are among the standard diagnostic tools used in clinical medicine (blood-chemistry profiles) to indicate nonspecific liver dysfunction. However, these tests will predominantly identify persons who have already been drinking large amounts of alcohol for a considerable period of time (months to years). The ALT/AST ratio (111), and the ratio of mitochondrial to total AST (112),

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have been proposed as improved means to discriminate alcoholic from nonalcoholic liver disease, but this was not confirmed in studies on unselected populations (113,114). Another main disadvantage is that raised levels of these enzymes may result from several other causes besides heavy drinking, including commonly prescribed drugs such as barbiturates and antiepileptics, conditions such as obesity and pregnancy, and most liver disorders of nonalcoholic origin (115,116). Interestingly, coffee intake may inhibit the induction of serum liver enzyme levels caused by alcohol consumption, and could thereby protect against liver damage due to alcohol (117–119).

Although the conventional alcohol markers may have limited utility in screening for potentially hazardous consumption in the general population, they are useful for follow-up of patients with already established alcoholic liver disease. Besides, most causes of false-positive test results are well known and can therefore be corrected for in the clinical situation.

VII. APPLICATION OF ALCOHOL MARKERS IN DIFFERENT SETTINGS

A. Unselected Populations

Primary care physicians and workplace health services need to have simple yet reliable methods to intervene with hazardous and harmful drinkers during regular visits. Several studies have provided evidence that alcohol screening with the use of instruments like the AUDIT and brief advice and counseling delivered as part of routine primary care may significantly reduce alcohol consumption by these patients (120,121), although the overall efficiency of brief intervention has also been questioned by others (122).

Alcohol screening using laboratory parameters has mainly employed the indicators of organ and tissue damage included in ordinary blood-chemistry profiles (e.g., GGT, AST, and ALT). However, because these tests are nonspecific for alcohol, they have limited utility for identifying high-risk drinkers in the general population. Recently, the more alcohol-specific CDT test has also been evaluated for the same purpose. Here it again needs to be pointed out that even when using a fairly sensitive and specific test, the risk for incorrect classification might be quite high if the prevalence of the impairment is low (Table 2). Accordingly, most studies have found CDT superior to the conventional markers in this context, but still of restricted value in random screening for potentially hazardous alcohol consumption in general medical or community settings (107,123–125).

There are, however, a number of issues that should be considered to improve the usefulness of any biochemical marker in screening for excessive alcohol consumption in unselected populations:

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First, the level of the test result obviously needs to be taken into account. Although the response of biological parameters to a given quantity and frequency of alcohol intake may vary from one individual to another, a highly increased test value is generally much more likely to result from heavy drinking than a value just outside the reference interval.

Second, the time delay between drinking and sampling is an important issue. Because the various biochemical markers have different life spans, the time since last intake should always be considered (126).

Third, it may always be advisable to collect a second sample for comparison, preferably within 1–2 weeks, to see if values have normalized or remain unchanged.

Fourth, if an increased CDT value, as determined by any of the immunoassay methods, is not corroborated by a clinical suspicion of problem drinking (e.g., other laboratory tests, or recognition of alcohol-related medical disorders), it should be validated by HPLC technique to rule out the risk of false-positives due to genetic transferrin variants (96,127).

Finally, combining CDT with other biochemical (128–130) or self-report screening measures (15) may improve sensitivity.

One way to gain better acceptance for the use of alcohol markers in primary care and workplace settings is to focus on personal health rather than identification of alcohol abuse (15). Excess alcohol intake is a common contributing risk factor in sickness and the desire to reduce alcohol-related injuries resulting in absenteeism and costly rehabilitation can well justify screening in the workplace (131,132). In certain workplaces, tests of acute alcohol consumption such as the urinary 5HTOL/5HIAA ratio may be used upon return to safety-sensitive duties (133), because it is well known that the aftereffects of drinking (i.e., hangover symptoms) can impair behavioral functions and thereby increase the risk of becoming involved in accidents.

Screening for potentially harmful drinking may also be incorporated into the context of other longitudinal prevention programs, such as cancers, high cholesterol, and hypertension, during routine health examination (134). In helping patients to decrease their alcohol consumption, giving biofeedback with biochemical markers may also be a powerful motivating factor (135).

B. Clinical Populations

Many persons admitted to general hospitals (e.g., intensive care and psychiatric emergency departments) have undiagnosed alcohol problems and are, at least partly, treated for the consequences of their drinking (136–138). For example, based on the presence of positive ethanol tests, the estimated prevalence of alcohol involvement among trauma patients usually ranges from 15 to 25% (139), and it may be even higher (~30–40%) during evening and late-night hours (140).

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Identification of patients with underlying alcohol-related problems may have important health and economic implications. For example, surgical patients who are dependent on alcohol have an increased risk for postoperative morbidity (the most frequent complications being sepsis, pneumonia, and bleeding episodes) and mortality (141,142), and detoxification of these patients prior to surgery can reduce the incidence of postoperative complications with need for extended hospitalization. Recent studies have found the CDT test suitable for this purpose (143,144). Additionally, because chronic drinkers often continue to consume alcohol even on the evening prior to surgery, combining CDT with a sensitive short-term marker like 5HTOL/5HIAA could improve further the possibility of identifying these high-risk patients (143).

C. Rehabilitation of Alcohol-Dependent Subjects

Biochemical alcohol markers have been found very useful as objective tools in monitoring of compliance and early detection of relapse during outpatient treatment of alcohol-dependent subjects (18,145,146). However, the response of various markers to excessive drinking may vary between individuals; for example, some individuals (~10–30% of the population) will not show a marked increase in CDT (non- or low responders) (18,147,148).

The following issues should be considered to improve the usefulness of biochemical markers during follow-up of an alcohol-dependent outpatient:

First, by monitoring of the changes in a set of complementary markers of excessive drinking, preferably CDT and GGT (18,149), during a 2–4-week period of alcohol withdrawal (e.g., hospitalization or disulfiram treatment) (150), the most sensitive single marker can be identified. If values normalize on withdrawal, this also confirms that alcohol was most likely the cause of the elevated test result.

Second, after discharge from inpatient treatment, testing should be continued for monitoring excessive drinking on a routine or random basis in connection with return visits to the clinic. Giving patients feedback about the test results may also improve self-report and treatment outcome (135).

Third, using serial testing, a rising CDT value can detect return to heavy drinking before the relapse is admitted by the patient (151,152). The possibility of detecting relapse can be improved further by introducing individualized cutoff limits between normal and elevated levels of any alcohol marker (153,154). Furthermore, because some people have baseline values outside the reference interval even without prior excessive drinking, this strategy also improves the reliability of the test results yielding a higher specificity.

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Fourth, a test to monitor acute drinking should always be employed in parallel. The 5HTOL/5HIAA marker has proven valuable for this purpose during outpatient treatment, and was much more sensitive than conventional breath ethanol testing (12). Moreover, because 5HTOL/5HIAA is performed on urine samples, it may easily be combined with testing of illicit drugs.

D. Traffic Medicine

Alcohol is involved in a large number of fatal road traffic injuries and deaths (155), and to deter people from driving under the influence of alcohol, statutory limits have been introduced in most countries. However, although a large proportion of the drunk drivers are indeed problem drinkers and many also repeat offenders (156,157), finding a punishable ethanol concentration at the time for arrest is not a reliable indication of the actual drinking habits and whether there might be underlying alcohol problems.

There are several obvious and potential applications of alcohol markers in traffic medicine. One example is in identification of alcohol-dependent subjects among drunk drivers, because these individuals may require medical treatment as a complement to punishment (79,158). A study of all deaths in Stockholm in 1987 revealed that over 80% of those ever convicted for driving under the influence had a suspected alcohol-related death (159). CDT, in combination with GGT, has also proven effective in confirmation of abstinence and detection of relapse in connection with rehabilitation of convicted drunk drivers and regranting of driving licenses, and may reduce recidivism rates after relicensing (160). Alcohol markers may also find uses in identification of alcohol-dependent individuals among those involved in serious traffic accidents, because they are at increased risk for postoperative complications but may be unable, or unwilling, to provide verbal information.

E. Forensic Applications

There are also important applications of biochemical alcohol markers in forensic toxicology. For example, testing for 5HTOL/5HIAA or ethyl glucuronide can be used to settle whether the ethanol identified in a blood or urine specimen originates from alcohol ingestion prior to death or has been generated artifactually (16,56). It is well known that very high concentrations of ethanol may be formed by microbial action (i.e., by yeast or bacteria) between time of death and autopsy, or upon incorrect storage of unpreserved biological specimens prior to analysis (161). These tests have also found uses in connection with the prosecution of drunk drivers, because challenging the results of forensic alcohol analysis (e.g.,

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due to contamination or artifactual formation prior to analysis) is not uncommon (162).

Markers of prolonged excessive drinking may be used to detect chronic alcohol use in medical examiner cases. Deaths from the effects of alcohol intoxication are routinely encountered in forensic practice, and especially CDT has shown promise as a postmortem marker of antemortem alcohol use (163–166).

VIII. CONCLUSIONS

The field of biochemical alcohol markers has expanded greatly and both clinical and forensic applications are now well documented. When used properly, alcohol markers are useful as objective measures in early identification of individuals who have or may develop an alcohol problem, and they also help to monitor abstinence and relapse in response to treatment. Although no single marker covering all forms of alcohol use and abuse is likely to be found, combining two or more complementary tests of acute and/or chronic drinking can be very informative. A number of alcohol markers with good sensitivity and specificity are already in routine use, and a few others are in sight. Nonetheless, filling the gap between testing for acute alcohol consumption (e.g., ethanol and 5HTOL/5HIAA) on the one hand and markers of excessive long-term drinking (e.g., CDT and GGT) on the other still stands as an important goal in future marker research.

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10

Neurobiology of Alcoholism

Recent Findings and Possible Implications for Treatment

Michael Soyka and Christian G. Schütz
Psychiatric Hospital, University of Munich, Munich, Germany

I. INTRODUCTION

Unlike decades ago, alcoholism today is believed to be associated with distinct neurobiological changes in the brain. Biological mechanisms probably associated with alcohol craving and relapse have been examined at the neurochemical level (for review see ref. 1) and at the molecular level (2). Brain structures such as the mesolombic system and the prefrontal cortex have clearly been identified as key structures in the development and perpetuation of addiction. Furthermore, the essential role of genetics in alcoholism has been addressed in a number of ambitious studies including the COGA project (see Hesselbrock, this volume). With respect to treatment, the role of certain neurotransmitters and receptors mediating positive or negative reinforcement, among others, are of special relevance. Pharmacotherapy of drug abuse/dependence disorders, including alcoholism, is a new and interesting research area (for review see refs. 3–6). What are the neurobiological mechanisms that are ''teaching the brain to take drugs?'' as *Science* asked in 1999. There is no single ''alcohol receptor'' in the brain but a number of neurotransmitters, including GABA, serotonin, norepinephrine, glutamate, dopamine, and the opioid-endorphin system, have been found to be implicated in the development of alcohol intake, reward, tolerance, withdrawal, or more complex phenomena such as craving. There is a close functional interaction between them. The dopamine release in the mesolimbic system is modulated by glutamatergic neurons and indirectly by opioidergic mechanisms. Since to date

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glutamatergic and dopaminergic drugs as well as opioid antagonists are of special relevance for treatment of alcoholism this review will focus on these neurotransmitter systems.

II. DOPAMINE AND ALCOHOLISM

The mesolimbic dopamine system is believed to be a key structure in mediating rewarding psychotropic effects of most drugs with abuse potential, including alcohol. Stimulation of the mesolimbic dopamine system has repeatedly been shown to be associated with (positive) reinforcement in drugs of abuse (for review see refs. 7–11). The interaction of the opioid and dopamine system in the ventral tegmental area and the nucleus accumbens is essential for the development of addictive behavior (for review see refs. 12,13). Two different mechanisms have been discussed as being essential for the development of addiction. The ''psychomotor stimulant theory of drug dependence'' (10) says that the addictive properties of substances are mediated via the activation of dopaminergic reinforcement pathways especially in the median forebrain bundle. The ''incentive-sensitization theory'' (14) states that drugs of abuse enhance the mesotelencephalic dopamine neurotransmission. Persisting sensitization-related neuroadaptations of the mesoaccumbens dopamine system may result in addicts hyperactively reacting to the effects of drugs even after long-term abstinence.

A hyperresponsiveness of midbrain dopaminergic neurons to various stimuli may underlie behavioral sensitization to drugs of abuse (11). The mesolimbic dopamine system is essential for facilitating the translation of the motivational influence of drug-associated cues into behavioral output (15). Dopaminergic neurotransmission has also been suggested to be associated with alcohol withdrawal, craving, and psychopathological symptoms such as depression and anhedonia (16–20). The blockade of mesolimbic dopaminergic neurotransmission may therefore affect alcohol intake.

A. Data from Animal Studies

In animal studies decreased brain dopamine levels after cessation of alcohol administration have repeatedly been shown (16,17,21,22). A low density of D2 receptors has been associated with high alcohol preference in mice (23) and rats (24,25). Alterations in alcohol-induced dopamine metabolism have been described in AA rats (26). Alcohol-naive AA rats, however, did not appear to have changes in dopamine D1 or D2 receptor-binding characteristics (27).

Effects of dopaminergic drugs on alcohol (and drug) intake in animals, preferrably rats, have repeatedly been studied. In many studies the DA antagonist

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flupenthixol was used (for review see ref. 28). Acute administration of 0.1–1 mg/kg (*cis*)-flupenthixol resulted in a dose-dependent and pronounced reduction of alcohol consumption. The alcohol-intake-reducing effect of flupenthixol was considered to be moderately selective, as food intake was also affected, but nonspecific, as effects on alcohol intake did not coincide with effects on alcohol preference. While short-term application of flupenthixol resulted in reduced alcohol intake, more chronic treatment resulted in an increased alcohol consumption (unpublished data). This example demonstrates how careful effects of drugs on alcohol consumption have to be studied before clinical conclusions can be drawn.

Interestingly, the D2 receptor antagonist sulpiride was shown to increase alcohol intake in alcohol-preferring rats (29) while the dopamine agonist bromocriptine was found to decrease both alcohol consumption and the development of acute tolerance (30).

B. Studies in Humans

Function of the brain dopamine system can be studied in different ways. Dopamine metabolites such as homovanillic acid (HVA) can be measured in the cerebrospinal fluid. It was shown that there is an excessive DA turnover and increased HVA concentration during alcohol withdrawal, especially in patients with severe withdrawal including psychotic symptomatology. Other studies have suggested low levels of HVA in abstinent alcoholics (31).

C. Neuroimaging Studies

The function of dopamine receptors can also be studied by means of neuroimaging techniques (for review, see Heinz, this volume). Neuroimaging data also support a role of the dopaminergic system in alcoholism and drug abuse/dependence. A number of single photon emission computed tomography (SPECT) and positron emission tomography (PET) studies have focused on the dopamine system in alcoholics. Hietala et al. (32) demonstrated that striatal dopamine D2 receptors were affected in alcoholics who were abstinent for 1–68 weeks. A decreased dopamine D2 receptor binding and a normal striatal DA transporter binding was shown by Volkow et al. (33). Normal DA transporter binding was also reported in violent early-onset alcoholics (34). SPECT data suggest a reduced dopamine transporter availability during and an increase after alcohol withdrawal (19,20).

D. Neuroendocrinological Studies

Neuroendocrinological studies also suggest a dopaminergic dysfunction in alcoholics. In most neuroendocrinological challenge tests the DA receptor agonist

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apomorphine was used to study function of the DA system. A blunted growth hormone response following apomorphine treatment was associated with a reduced dopaminergic neurotransmission. This was shown in relapsing alcoholics in the initial phase of abstinence (35,36) but also after 3 months (37) and even 78 months (38).

E. Polymorphism of Dopamine Receptors and Alcoholism

Alcoholism has also been associated with a particular polymorphism in the A1 allele of the dopamine D2 receptor (*DRD2*) gene (39,40). This finding had attracted significant attention in the scientific community and resulted in a broad number of replication studies (for review see ref. 41 and Hesselbrock, this volume). Most of the subsequent studies have failed to replicate the initial finding (42), or questioned the specificity of this finding for alcoholism (43). Many recent studies have shown negative results (41,44,45).

III. GLUTAMATE AND ALCOHOL

Glutamate is the most widespread excitatory neurotramitter in the central nervous system (CNS). It is believed that approximately 70% of the excitatory CNS synapses utilize glutamate as a transmitter in the brain (46). Glutamate activates three major ionotropic receptor subtypes, namely the AMPA, kainate, and NMDA receptor, and a number of metabotropic receptors. NMDA-sensitive glutamate receptors are believed to be of special relevance for the pathophysiology of a number of neuropsychiatric disorders. NMDA receptors are coupled to high-conductance cationic channels permeable for K^+, Na^+, and Ca^{2+}. Ca^{2+} flux is essential for neuronal excitability. The receptor is modulated positively by polyamines (spermine and spermidine) and glycine, which binds to a specific binding site. Glycine functions as a coagonist. The NMDA receptor is blocked in a dose- and voltage-dependent manner by Mg^{2+}. NMDA receptors are believed to play a major role in mediating neural plasticity, learning processes, and development. Two major subunit families, $NMDAR_1$ and $NMDAR_2$, have been identified and cloned. Glutamate has a physiological role in many processes but an overactivation of this system may also lead to excitotoxic effects. Glutamate then may function as an endogenous neurotoxin.

There are numerous glutamatergic neurons and pathways in the CNS with maximum densities in the cerebral cortex, hippocampus, striatum, septum, and amygdala. There are glutamatergic pathways from the cerebral cortex to the striatum and to and from the subthalamic nucleus. Furthermore, all excitatory projection pathways from the hippocampus utilize glutamate as a transmitter. The hip-

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pocamus is a major target zone for studies involving memory and learning but also for alcohol and schizophrenia research. There is also a close functional relationship between glutamatergic and dopaminergic neurotransmission. Glutamate modulates dopamine release in the nucleus accumbens.

Glutamate receptor subtypes in alcoholics, especially the *N*-methyl-*D*-aspartate (NMDA) receptor, have been shown to play a major role in discriminative effects, locomotor stimulation, and reinforcing effects of alcohol (47–50). Preclinical studies suggest that NMDA receptors are essential for behavioral sensitization to alcohol (51). Changes in the glutamatergic neurotransmission are postulated to be responsible for a number of alcohol-related neuropsychiatric disorders such as seizures or Wernicke-Korsakoff syndrome (52). Alcohol itself was found to inhibit the activity of the NMDA receptor subtype and to reduce NMDA stimulated in currents in a concentration-dependent fashion. In abstinent alcoholics, a dysfunction of the glutamatergic neurotransmission and NMDA-receptor function with increased activity of voltage-gated Ca^{2+}-channels is suggested to be the basis of hyperexcitability in alcoholics. Long-term alcohol intake increases the levels of NMDA receptor subunits, up-regulates NMDA receptor-related binding, and produces cross-tolerance with other noncompetitive NMDA antagonists (46,53,54).

A. Neuropharmalogical Models

Studies using NMDA antagonists to examine alcohol-like effects are of special interest to study the glutamatergic basis of alcoholism. The high-affinity NMDA antagonist ketamine was shown to produce ethanol-like subjective effects in detoxified alcoholics in subanesthetic doses (54). In a similar model the low-affinity NMDA antagonist dextrometorphan was used to study the subjective effects of ethanol in humans (55,56). Dextromethorphan has been used as an over-the-counter antitussive medication for more than 40 years. The main reason for its use in this experiment was its extremely favorable safety profile. Dextromethorphan (+)−3 methoxy-*N*-methylmorphinan is a specific noncompetitive antagonist with a binding affinity at the ion channel of the NMDA receptor complex of Ki (nM) 3500. Its metabolite dextrorphan has a Ki (nM) of 222 (in comparison: MK-801 15, PCP 42, Memantine 540, Amantadine 10500).

Results indicated that dextrometorphan but not placebo can induce alcohol-like subjective effects but also craving in alcoholics. We were able to show ethanol-like effects in both detoxified alcoholics and healthy controls, and mild increases in the serum levels of the endocrinological parameters cortisol and prolactin in healthy controls as well as in the alcohol dependents were found. In contrast to the study of Krystal et al. (54), a mild craving could be induced in patients. For dextromethorphan the results of this study give further evidence to

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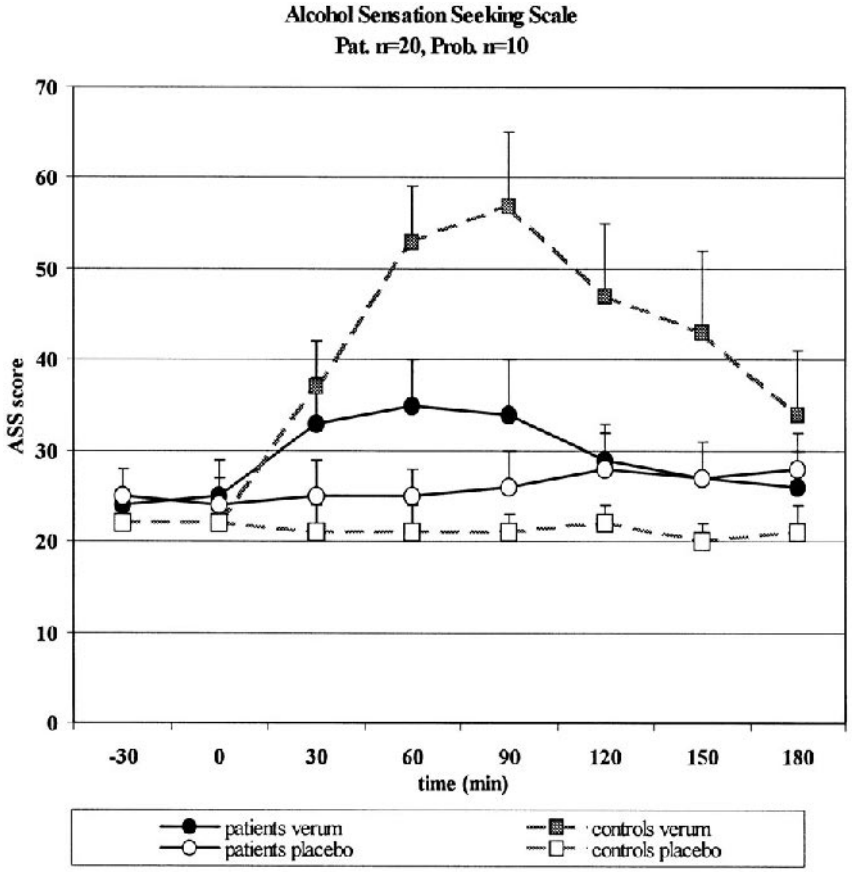

Figure 1 Effects of dextromethorphan 2mg/kg or placebo on Alcohol Sensation Seeking Scale Scores in recently detoxified alcoholics (n = 20) and age-matched healthy controls (n = 10). Values are expressed as mean ±SEM. See ''Subjects and Methods'' and ''Results'' sections for explanations of statistical analysis (From Ref. 56).

previous findings that NMDA receptors are involved in mediating much of ethanol's effects. (See Figs. 1 and 2.)

Results from animal models suggest that both noncompetitive (e.g., dizocilpine) and competitive (e.g., CGP 40116) antagonists are capable of substituting for ethanol. Findings further suggest that the NMDA receptor and GABA-A receptor are essential for ethanol stimulus effects.

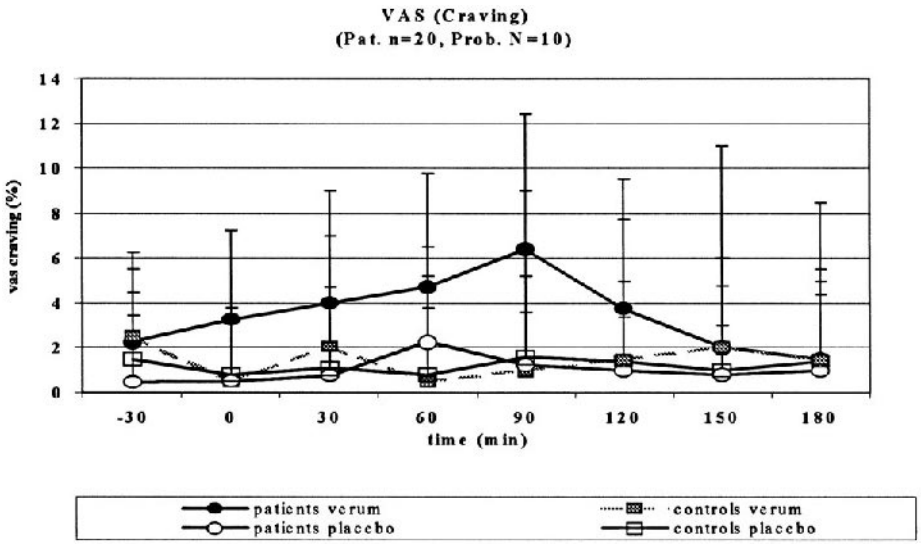

Figure 2 Effects of dextromethorphan 2mg/kg or placebo on self rated ''craving'' in recently detoxified alcoholics (n = 20) and age-matched healthy controls (n = 10). Values are expressed as mean ±SEM. See ''Subjects and Methods'' and ''Results'' sections for explanations of statistical analysis (From Ref. 56).

IV. OPIOID-ENDORPHIN SYSTEM AND ALCOHOLISM

Recently the effectiveness of naltrexone, an unspecific opioid antagonist, in decreasing alcohol consumption of alcohol-dependent patients has been demonstrated (57,58). A breakthrough in the understanding of the endogenous opioid system in 1973 was the experimental demonstration of opioid binding sites in the brain (59–61). This is considered a key step in modern neuropsychopharmacology and in the neurobiology of addition. The studies on the endogenous opioid system supposedly have retained something like a role model even today. However, notwithstanding the huge increase in knowledge of the endogenous opioid system, so far the predicted linkage to a vulnerability in the form of a predisposition (e.g., preexisting functional deficiency) or adaptive changes (e.g., reduction in opioid peptide synthesis or down-regulation of opioid receptors) has not been convincingly demonstrated (62).

The opioid system has to be distinguished from the neurotransmitters described above in that the transmitter is a peptide. Peptides are under the direct control of the cell nucleus. They are produced by transcription and translation

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forming inactive prehormons or precursors from the DNA template and are actively transported in ribosomes to the synapsis. There is no reuptake.

The main groups of opioid peptides are the β-endorphin (precursor pro-opiomelanocortin), the Met^5 und Leu^5-enkephalin (precursor pro-enkephalin), and dynorphin A und B (precursor pro-dynorphin). It is important to note that in opposition to the earlier one-cell, one-transmitter theory it is now accepted that peptides such as opioids are colocalized with other transmitters (e.g., enkephalin and serotonin in the raphe nucleus). Receptors can similarly be divided into μ-receptors (from the Logan morphine) δ-receptors (from the ligand, and κ-receptors. Characteristic pharmacological profiles can be attributed to the activation of the specific receptor types. The profiles show similarity in the case of μ receptors and δ receptors, whereas the activation of the κ receptors results in a rather different pharmacological profile (63). This is of particular interest in view of their differing motivational effects (64).

The functions of the endogenous opioid system have been described to consist in a number of ''housekeeping roles'' including a modulatory role of gastrointestinal, endocrine, and autonomic functions; a sensory role, particularly prominent in inhibiting responses to noxious stimuli; an emotional role, evident in the powerful rewarding and addicting properties of the endogenous and exogenous opioids; and a cognitive role, manifested in the opioid modulation of learning and memory (65).

The specific participation of the endogenous opioid system in mediating effects of alcoholic consumption behavior and alcohol dependence have been investigated in numerous studies. In vitro and in vivo experiments have demonstrated changes in opioid peptide release and changes of opioid receptor binding after acute and chronic alcohol application (66–68). Results have not always been consistent and mechanisms mediating the direct effect of alcohol on peptide and receptor expression and peptide release are far from being well understood (69). Hypotheses proposed include the ''opioid surfeit hypothesis,'' which proposes excessive alcohol consumption to be due to an ''overactivity of the endogenous opioid system,'' (70) and the ''opioid compensation hypothesis,'' which proposes an underactivity of the opioid system to induce excessive alcohol consumption (71). The existence of two relatively crude and contrary hypotheses indicates that, even today, very basic questions remain regarding the involvement of the endogenous opioid system in mediating the effects of alcohol consumption. Research into the field of increased vulnerability associated with the opioid system has been carried out by Gianoulakis and her group. She identified an oversensitivity of pituitary beta-endorphin response to drinking in a genetically high-risk population (sons of alcoholics) (72).

One of the earlier works on the mechanisms involved in alcohol mediating its effects with the help of the opioid system has been the tetraisoquinoline hypothesis suggested 30 years ago (73,74). Condensation of acetaldehyde and dopa-

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mine results in the formation of tetrahydropapaveroline and tetrahydropaveroline. These substances are psychoactive, increasing voluntary alcohol intake (75,76), but amounts produced are so small that this pathway currently is considered irrelevant (77). Given the current central role of the dopaminergic mesolimbic system in the hypothesized model of substance dependence including alcohol dependence, it is not surprising that a hypothetical model has been developed to explain the effects of alcohol on the mesolimbic system mediated by the opioid system. Increase in dopamine release is induced by activation of the μ-receptors in the ventral tegemental area, whereas activation of κ-receptors in the nucleus accumbens inhibits dopamine release (64).

Indirect links between the opioid system and alcohol consumption have been suggested to be due to the ''stress'' factor. Stress may be followed by a rebound defiency in β-endorphin, followed by induction of opioid release by alcohol consumption (78).

The disadvantage of all these studies is that the endogenous opioid system had to be studied in animal models or utilizing peripheral measurements of β-endorphin released from the pituitary. In both cases it is not clear how far the necessary extension of the model to the endogenous opioid system of human alcohol-dependent patients holds up. New technologies in neuroimaging have made it possible to measure in vivo changes in brain function, including the opioid system. We investigated central opioid receptor binding in eight alcohol-dependent men (DSM-IV) and 11 matched healthy controls using dynamic ^{11}C-diprenorphin PET scans. Alcoholics were scanned immediately before detoxification and 4 weeks into abstinence. Care was taken to confirm absence of effects due to atrophy and partial volume effect. A parallel version of spectral analysis was applied on a voxel level to extract delivery and retention of ^{11}C-diprenorphine. No significant regional differences in tracer delivery were observed between the patient group and the controls. Findings indicate successful transformation for both groups. For pixelwise comparisons of relative values, SPM99 routines were applied after proportional scaling. Comparing whole-brain global values in alcohol-dependent men before detoxification (mean IRF_{60}: 0.129) and controls (mean IRF_{60}: 0.109) we found nonsignificantly increased diprenorphine binding in alcoholics ($p = 0.11$). After detoxification whole-brain global detoxification values (mean IRF_{60}: 0.109) were identical in the two groups.

Increased relative binding could be demonstrated in extended cortical regions, specifically the frontal cortex. Clusters did not reach significance when corrected for multiple analysis. Significant decreased binding ($p_{corrected}$: 0.003) could be detected in the midbrain region, medial posterior part of the thalamus, and subthalamus (li $>$ re). Four weeks of abstinence did not induce significant overall changes in binding, but measured changes do indicate a trend toward normalization of the regional tracer retention. The results must be considered preliminary, but these findings indicate differentiated persistent altered endoge-

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nous opioid binding in chronic alcohol-dependent men, with increased binding in cortical regions and decreased binding in subcortical regions associated with the limbic system, specifically involving the ventral tegmental area.

V. OTHER MECHANISMS

These will be addressed only briefly. A dysfunctional serotonin (5-HT) system has been implicated in alcoholism (78). A number of preclinical data from animal studies point in that direction (79,80) but clinical studies in noncomorbid (nondepressed, nonsuicidal) alcoholics have shown disappointing clinical results (for review see ref. 87). This is true for a number of SSRIs, with the possible exception of citalopram and 5-HT_2 receptor antagonists, such as ritanserin (preclinical data: refs. 79, 81–83; clinical data: ref. 84).

VI. CLINICAL IMPLICATIONS

Pharmacology-based relapse prevention is a new and promising research area. Methodological problems of studies using so-called anticraving drugs have been discussed by Kranzler et al. (85), Lehert (86), and Moncrieff and Drummond (86a) among others. A number of possible alcohol-intake-reducing agents, so-called anticraving drugs, have been studied for relapse prevention of alcoholism. The glutamate modulator acamprosate and opioid antagonists such as naltrexone and nalmefene have produced the most favorable results (for review see refs. 4, 5, 87).

Both dopamine agonists and antagonists have been tested as possible anticraving drugs, including bromocriptine, lisuride, tiapride, and flupenthixol (for review see ref. 87). Results in clinical studies using the dopamine agonist lisuride were negative. Lisuride was reported to increase relapse rates in detoxified alcoholics in two placebo-controlled, double-blind studies (88). The dopamine agonist bromocriptine in rats decreased acute alcohol tolerance and voluntary alcohol consumption (30). Clinical findings in humans are not entirely clear. Lawford et al. (89) linked treatment outcome with bromocriptine in alcoholics to the D2 dopamine receptor A1 allele.

Positive effects of flupenthixol on alcohol intake as well as other drug intake have been reported in a recent uncontrolled study on 20 patients who received flupenthixol on a short time basis (0.5–1 mg/day) (90). Unfortunately the drugs positive effects on alcohol intake obtained in the animal could not be seen in a recently finished, unpublished placebo-controlled, double-blind study (n = 272) (91). After 6 months of treatment, abstinence rates of the placebo group

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were even superior to those of the flupenthixol group (Böning and Wiesbeck, personal communication). More positive effects have been reported for flupenthixol decanoate in dual-diagnosis schizophrenics (92–94). The possible anticraving effect of flupenthixol is currently under investigation in an open study of alcohol-abusing schizophrenics in Germany ($n = 20$).

Although there are some promising preclinical studies using other glutamatergic compounds such as the NMDA antagonist memantine (95), the only glutamatergic drug clinically used for treatment of alcoholism so far is the homotaurinate derivative calcium acetylhomotaurinate (acamprosate) (96). Although its mechanism of action is not entinely clear (97), many findings suggest that it probably acts via interaction with the NMDA receptor (98–100). Acamprosate is an antagonist of the excitatory amino acid's stimulatory effect. Acamprosate was found to enhance NMDA-receptor-mediated neurotransmission (100a) and to reduce Ca^{2+} fluxes and the postsynaptic efficacy of excitatory amino acid neurotransmitters such as L-glutamate, (especially NMDA subtype), thus lowering the neuronal excitability (101,102).

Acamprosate proved to be efficient in the reduction of alcohol intake in both animal models (103–106) and a number of large placebo-controlled, double-blind studies in Europe (107–109; for review see refs. 3, 5). The drug is currently under investigation in the United States.

Other NMDA antagonists or modulators might be of interest for future research in that area.

The most realistic alternative to acamprosate treatment is the use of opioid antagonists like naltrexone and nalmefene. The initial studies in 1992 by Volpicelli et al. (57) and O'Malley et al. (58) had shown naltrexone to decrease alcohol consumption, not so much abstinence rates, in alcoholics. While other clinical trials have supported these positive findings (110–113) and suggested a positive safety profile (114), more recent studies in Europe, basically unpublished, have shown negative results (4,87). To date treatment with opioid antagonists has shown most promising results in combination with behavioral therapy (112). Other studies are necessary to examine interaction of different psychotherapeutic and pharmacological approaches in relapse prevention.

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11

Neurodegeneration and Neuroadaptation in Alcoholism

Andreas Heinz and Karl Mann
Central Institute of Mental Health, Mannheim, Germany

I. CEREBRAL ATROPHY AND REGENERATION

A majority of alcohol-dependent patients display ventricular enlargement and increased sulci compared to age-matched control subjects (1–3). The reason for these indicators of cortical atrophy is unclear, nor is it known whether the reconstitution of cortical atrophy during abstinence is due to rehydration or a regeneration of neuronal cell compartments (4,5). Alcohol-associated brain atrophy is specifically pronounced in the gray and white matter of the frontal cortex (6). Brain atrophy has also been observed in the anterior hippocampus and cerebellum of alcoholics who did not suffer from Wernicke-Korsakoff's syndrome and who did not show specific memory deficits (7,8). In a 5-year study, Pfefferbaum and co-workers (9) observed an increased reduction of gray matter in the anterior temporal lobe of alcoholics who continued to consume alcohol compared with abstinent patients and age-matched control subjects. Several studies indicated that women might be more vulnerable than men toward the neurotoxic effects of alcohol intake (10). When comparable amounts of alcohol were consumed, women showed an increased atrophy in the left hippocampus (11) and the splenium (12) compared to alcohol-dependent men and healthy control subjects.

Relaxation times in magnetic resonance imaging (MRI) can be used to assess whether the recovery of alcohol-related brain atrophy during abstinence is associated with rehydration (13–16). So far, these studies have led to contradictory results. Besson and co-workers (13,17) and Smith et al. (18,19) observed an increase in T1-weighted relaxation times during early abstinence, which may be due to rehydratation. An autopsy study (20), on the other hand, found no

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indications that brain atrophy in alcoholics is associated with changes in water content. In accordance with this observation, two further studies observed no difference in T1- and T2-weighted relaxation times, which were repeatedly measured during the first weeks of abstinence (4,21), when a significant reduction of brain atrophy can be observed (8,22). These studies do not support the hypothesis that the recovery of brain atrophy during early abstinence is simply due to rehydration and suggest that neuroregeneration may be involved in this process.

Neuroregeneration can be assessed with magnetic resonance spectroscopy (MRS) (23). ^{1}H-MRI spectroscopy is a noninvasive method that assesses brain metabolites by measuring the concentrations of substances like *N*-acetyl-aspartate (NAA), an unspecific marker of neuronal integrity (24,25). NAA concentrations are usually standardized to creatinine (Cr) concentrations; i.e., the ratio of NAA/Cr is measured, because Cr concentrations are usually unchanged in different neuropsychiatric diseases (26,27). Spectroscopy studies observed a reduced NAA/Cr ratio in the frontal cortex (23) and cerebellum (28,29) of alcohol-dependent patients. In a follow-up study, Martin and co-workers (30) observed a significant increase in the choline-to-NAA ratio during early abstinence and suggested that this finding was due to an increase of the concentration of choline in neuronal membranes.

Frontal brain atrophy and regeneration may be specifically important for the pathogenesis and recovery from cognitive deficits and impulsive alcohol intake (31–34). Frontal neuronal degeneration may affect the executive behavior control and working memory functions (35) and may result in a deficit of long-term behavior planning and behavior disinhibition (36,37). In a volumetric study, frontal brain atrophy was associated with motivational deficits and other so-called negative symptoms among alcoholics (38). In 1979, Jenkins and Parsons (39) observed reversible working memory deficits among alcoholics as assessed with the Wisconsin Card Sorting Test, which were correlated with a reduced prefrontal glucose utilization in a PET study (40). So far, no studies have assessed the potential association between frontal brain atrophy and chronic alcohol effects on neurotransmitter systems that may specifically affect impulsive and compulsive alcohol intake.

II. NEURODEGENERATION AND NEUROADAPTATION OF MONOAMINERGIC NEUROTRANSMITTER SYSTEMS

Chronic alcohol intake interacts with a multitude of central neurotransmitter systems. Effects on monoaminergic neurotransmitter systems are specifically interesting, as they may directly cause or maintain addictive behavior (41,42). The so-called dopaminergic reward system, ascending from the midbrain to the ventral and partially also to the dorsal striatum, has long been a focus of addiction

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research, as different drugs of abuse, including alcohol, stimulate dopaminergic neurotransmission and thus reinforce drug intake (43–46). Conversely, serotonergic stimulation of the behavior inhibition system was supposed to inhibit ongoing behavior (47), hypothetically because of unpleasant feelings of punishment that are encoded by this neurotransmitter system (48). It was suggested that a dysfunction of this ''punishment system'' due to a serotonergic deficit predisposes toward disinhibited, impulsive drug intake and antisocial behavior (49,50). Among early-onset alcoholics, several studies observed a dysfunction of central serotonergic neurotransmission as measured by low concentrations of the serotonin metabolite 5-hydroxyindoleactic acid in the cerebrospinal fluid (5-HIAA in CSF) (51,52). In recent years, it has been observed that selective serotonin reuptake inhibitors or drugs of abuse such as ''ecstasy'' (3,4-methylenedioxymetamphetamine; MDMA) that increase synaptic serotonin concentrations are associated with a *decrease* in negative mood states (53,54). In this view, feelings of anxiety, depression, and threat perception, so-called negative mood states, may be the primary correlate of serotonergic dysfunction, while clinical depression or impulsive aggression may develop under the influence of additional factors including social learning or isolation stress (55–57).

A. Serotonergic Dysfunction and the Predisposition Toward Excessive Alcohol Intake

With regard to the question whether a serotonergic dysfunction is associated with impulsivity relevant for the pathogenesis of alcoholism, Cloninger (50) suggested that there is a so-called ''type 2'' alcoholism that is characterized by early disease onset, impulsivity, and antisocial personality traits. However, it is unclear whether low serotonin turnover in alcoholism is a cause for or a consequence of chronic alcohol intake (51,58). Therefore, it is important to study predispositional factors of excessive alcohol intake in animal models that provide the opportunity to assess serotonergic neurotransmission under socially defined conditions before chronic alcohol intake has occurred (40). In humans and nonhuman primates, serotonin transporter availability can be measured with SPECT and the radioligand β-CIT in the dorsal brainstem (raphe) area, the site of origin of subcortical and cortical projections (59–61). In this area, β-CIT is exclusively displaced by selective serotonin reuptake inhibitors but not by substances such as GBR, which selectively bind to dopamine transporters (59). In a brain-imaging study with β-CIT SPECT, adult rhesus monkeys were examined that were predisposed to excessive alcohol intake due to early social separation stress (60). These primates had been separated from their mothers directly after birth; during early social separation stress, CSF 5-HIAA concentrations decreased and remained on a lower level compared with serotonin turnover rates in mother-reared primates even during adulthood (40,60). During social separation, the primates displayed increased

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anxiety-like behaviors (62). Especially the male primates displayed increased aggressiveness during adulthood (40). In these primates, low CSF 5-HIAA concentrations were correlated with an increased availability of serotonin transporters in the raphe area, increased self-initiated aggressiveness, and a low response to a standardized alcohol dose (60). Both impulsive aggressiveness and a low range of unpleasant alcohol effects such as ataxia or sedation are factors that were associated with the risk of excessive alcohol intake and alcoholism in previous studies (47,63). In the reported primate study, both factors were correlated with a low serotonin turnover rate and an increased availability of serotonin transporters (60).

The increased availability of raphe serotonin transporters in primates with low CSF 5-HIAA concentrations may be due to a real increase in serotonin transporter density. An increase in striatal dopamine transporter density in monkeys predisposed to excessive alcohol intake was observed by Mash and others (42). In a combined microdialysis and β-CIT SPECT study, an increase in monoamine uptake sites was correlated with decreased extracellular neurotransmitter concentrations (64) and, conversely, loss or blockade of dopamine or serotonin transporters induces an increase in extracellular neurotransmitter concentrations (65,66), indicating that the availability of monoamine uptake sites regulates extracellular monoamine concentrations. Alternatively, a decreased serotonin concentration in the synapse may be correlated with increased β-CIT binding to serotonin uptake sites because of decreased competition with endogenous serotonin for binding at serotonin transporters. In accordance with the latter hypothesis, increases in serotonin production after serotonin depletion were associated with in vivo displacement of β-CIT from the serotonin transporter binding site (67). In any event, an increased availability of serotonin transporters as assessed with β-CIT SPECT indicates decreased concentrations of synaptic serotonin and was associated with low concentrations of 5-HIAA in the CSF (60).

A low response to alcohol was also increased in young men with a positive family history of alcoholism (63,68,69) and was influenced by genetic factors in twin studies (70,71). Interestingly, Schuckit and co-workers (72) observed a low response to acute alcohol effects among subjects who carried a certain genotype of the promoter for the serotonin transporter, which in vitro had been associated with an increased availability of serotonin transporters (73). In the reported study of nonhuman primates (60), no significant association was observed between the in vivo 5-HTT availability in the brain stem and the genetic constitution of the serotonin transporters. In these primates that underwent serious social separation stress, environmental stress may have been an overriding influence on serotonergic neurotransmission. This finding may indicate that an increased availability of serotonin transporters may be associated with a low acute alcohol response, independent of whether the increased serotonin transporter availability is predom-

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inantly the result of genotype effects or environmental factors such as early social separation stress.

The observed association of serotonergic dysfunction following social isolation is interesting in the light of a twin study by Johnson and co-workers (74), who found no genetic contribution to antisocial behavior among alcoholics. In an adoption study of Bohmann and others (75), children of alcohol-dependent parents were more frequently both alcohol-dependent and violent; however, there was no indication for a disposition toward violent behavior independent of alcoholism. Further adoption and twin studies also did not observe a significant genetic contribution to violent behavior (76,77). These studies seem to indicate that environmental factors may play a dominant role in the pathogenesis of human violence. One of these environmental factors predisposing toward alcoholism and violent behavior may be a dysfunction of serotonergic neurotransmission that has repeatedly been observed after early social isolation stress (40,78,79).

B. Serotonergic Dysfunction and the Maintenance of Excessive Alcohol Intake and Alcoholism

Neurotransmitter interactions with chronic alcohol intake may be modified by neurodegeneration, neuroadaptation, and genotype effects (80–83). Unlike neurodegeneration, neuroadaptation is defined as a nonpermanent functional change in neurotransmission, which often may represent a counteradaptive process that balances the effects of chronic alcohol intake on neurotransmitter systems (82). Due to important anatomical and functional differences in monoaminergic neurotransmission between rodents and primates, studies in humans and nonhuman primates are warranted to assess neuroadaptive and neurodegenerative processes and their interaction with genotype effects (84–86).

Male alcoholics who had abstained from alcohol for 4 weeks showed a reduction of raphe serotonin transporters compared to age-matched healthy control subjects (61). Loss of serotonin transporters in male alcoholics was more profound or persistent than reductions in striatal dopamine transporters, which at this time of abstinence did not differ from healthy control subjects (61,87). In accordance with this observation, Halliday et al. (80) observed a significant reduction in serotonergic cells in the brain stem of alcoholics. A substantial number of neurons that originate in the raphe area innervate other raphe nuclei (88), so a loss of serotonergic neurons would also affect the density of presynaptic serotonin transporters in this brain area. The reduction in serotonin transporters among male alcoholics was correlated with the amount of lifetime alcohol consumption and may represent a neurotoxic effect of chronic alcohol intake on serotonin neurons and transporters in the raphe area (61).

When the genetic constitution of the promoter for the serotonin transporter

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was assessed, a significant reduction of raphe serotonin transporters was observed only in a specific genetic subgroup, namely homozygote carriers of the long (''*l*'') allele of the promoter for the serotonin transporter gene (''*ll*-homozygotes'') (89). It has been suggested by Lesch and co-workers (73) that among healthy control subjects, this genotype is associated with an in vitro increase in serotonin transporter expression and functional capacity. In the central nervous system, subjects with this genotype displayed a similar increase in serotonin transporters in an autoradiographic study (90) and in vivo in a brain imaging study (89). As discussed earlier, this genotype was associated with low acute unpleasant effects of alcohol intake before chronic alcohol intake was started and with an increased risk to develop alcoholism in a prospective human study with a follow-up over 15 years (72) and in a nonhuman primate study (60). After chronic alcohol intake, this same genotype may render subjects more vulnerable to the toxic effects of chronic alcohol consumption and thus result in a pronounced loss of raphe serotonin transporters (89). If replicated, this finding would indicate an interesting genotype-environmental interaction, in which a specific genotype is associated with both an increased risk for excessive alcohol intake and an increased vulnerability to the neurotoxic effects of chronic alcohol intake. Further studies will have to assess genotype effects on monoaminergic neurodegeneration among alcoholics.

C. Neuroadaptation Versus Neurodegeneration in the Central Dopaminergic System

Alcohol intake stimulates the firing rate of central dopaminergic neurons that originate in the substantia nigra and ventral tegmentum (VTA) and project to the ventral and dorsal striatum (91,92). Several animal experiments in rodents showed that chronic alcohol intake was associated with a counteradaptive downregulation of postsynaptic striatal dopamine D2 receptors, which was not permanent but recovered within the first week of abstinence (93,94). PET studies in alcohol-dependent patients also reported a reduction in striatal dopamine D2 receptor availability (95,96). A reduction in the sensitivity of central dopamine D2 receptors was observed in neuroendocrinological challenge studies with the dopamine agonist apomorphine (97,98). Further studies in alcoholics confirmed that in accordance with animal experiments, the reduction of central D2 receptor sensitivity was not permanent and returned toward control levels within 1 week after detoxification. A prolonged reduction in central D2 receptor sensitivity was predictive of subsequent poor treatment outcome (32), and in some patients with early relapses, dopamine D2 receptor sensitivity did not reach control levels even when patients were followed for an observation period of up to 3 months after detoxification (99).

Patients with delayed recovery of central D2 receptor sensitivity and poor treatment outcome did not differ from treatment responders in the amount of

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lifetime alcohol intake or other clinical variables (98,99). Therefore, it was suggested that the genetic constitution of the dopamine D2 receptor may be associated with prolonged recovery from chronic alcohol intoxication. While an exon 8 polymorphism of the dopamine D2 receptor gene (*DRD2*) was found to interact with central dopaminergic sensitivity before detoxification, i.e., in the state of chronic alcohol intoxication (100), no such interactions with either the *DRD2* exon 8 or the *Taq*I A polymorphism were found during early abstinence (100,101).

Instead, several studies suggested that postsynaptic D2 receptor sensitivity may remain reduced during abstinence because presynaptic dopamine release is sensitized even in the absence of chronic alcohol intake (102–104). This hypothesis is in contrast to reports of an early decrease in synaptic dopamine release during the first hours of detoxification: When chronic ethanol intake is terminated, ethanol-induced dopamine release is abolished and synaptic dopamine concentrations are decreased during the first 24 h of abstinence (105). During the first weeks of abstinence, however, a phasic, stimulus-dependent increase in central dopamine release may occur due to smoking, in stress situations, and when conditioned stimuli (''cues'') are presented that have previously been associated with alcohol intake (44,106–108). A stimulus-dependent dopamine release is supposed to attribute incentive salience to the cue that indicates upcoming reward and may subjectively be experienced as craving for the drug reward that has previously been associated with the conditioned cue (109). Studies of presynaptic striatal dopamine synthesis with DOPA-PET and of peripheral and central concentrations of dopamine and its major metabolite, homovanillic acid (HVA), indicated that dopamine turnover is increased among abstinent alcoholics (102–104,108). In three of these studies, indices of increased dopamine turnover were associated with poor treatment outcome (101–103) and in one study with smoking (108). Postsynaptic D2 receptor down-regulation in the striatum may thus compensate for the sensitized conditioned presynaptic dopamine release or for additional effects of tobacco consumption.

The effects of increased presynaptic dopamine release may be potentiated by a reduced dopamine clearance from the synaptic cleft. This clearance is performed by dopamine transporters, which are responsible for presynaptic dopamine reuptake and therefore play a major role in the regulation of extracellular dopamine concentrations (64,66). In 1995, Tiihonen and co-workers (110) observed a reduction in dopamine transporter availability measured with β-CIT SPECT in the striatum of alcohol-dependent patients with late disease onset. In contrast to this observation, two further studies failed to confirm a reduction in dopamine transporters among alcoholics (61,96). The heterogeneous study results may be explained by the observation of Laine and co-workers (87) that striatal dopamine transporter availability was reduced among alcoholics who had been detoxified for 4 days and increased toward a normal range when the subjects

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were examined again after 4 weeks of abstinence. In the study of Tiihonen et al. (110), alcoholics showed a wide range in their duration of abstinence, while in the study of Heinz et al. (61), all patients had abstained for at least 4 weeks.

Another factor that may affect in vivo dopamine transporter (DAT) availability is DAT genotype. A polymorphism of the 3′ untranslated region of the DAT gene (SLC6A3) (111) has been associated with severity of withdrawal among alcoholics (112,113), with cocaine-induced paranoia (114), and the ease of smoking cessation (115). Each of these clinical associations may be due to genetic variations in the availability of the DAT protein and the respective effects on dopamine reuptake and synaptic dopamine concentrations (116). The variable number of tandem repeat (VNTR) polymorphisms in the 3′ region of SLC6A3 was genotyped and the availability of striatal DAT protein was measured in vivo with β-CIT SPECT in abstinent alcoholics and control subjects. Consistent with earlier studies, striatal dopamine transporter availability was not reduced among alcoholics after 4 weeks of abstinence (87,96) and alcoholism per se was not associated with DAT genotype. DAT genotype, however, was associated with the in vivo dopamine transporter availability in the putamen. Individuals with the 9-repeat/10-repeat genotype showed a significant reduction of DAT availability in the putamen compared with individuals who were homozygotes for the 10-repeat allele (116). This finding suggests that the VNTR polymorphism of the DAT gene affects DAT availability via translation of the DAT protein. The differences in DAT availability may be clinically silent unless rapid changes of dopamine release occur, e.g., during early withdrawal (32,105). This observation may explain the previously observed association between this DAT polymorphism and the severity of alcohol withdrawal and other clinical phenomena (112–115).

In summary, both alcohol and genotype effects seem to influence DAT availability during early abstinence. A reduction of striatal dopamine transporters during chronic alcohol intake and a rather rapid recovery during early abstinence was also observed in a study of nonhuman primates (42) and mirrors changes in other factors of dopaminergic neurotransmission such as dopamine receptor sensitivity (117). The observation of a normal DAT availability among alcoholics after a few weeks of abstinence (61,87,96) does not support the hypothesis that the reduction in DAT availability in recently detoxified alcoholics is due to neurodegeneration. Eshleman and co-workers (118) suggested that alcohol-induced dopamine release may be in part mediated by dopamine transporters that reverse their normal role and release dopamine. If this is the case, a down-regulation of presynaptic dopamine transporters during chronic alcohol intake may help to counteract alcohol effects on dopamine release. The down-regulation of striatal DAT among alcoholics would thus represent a neuroadaptive process similar to the down-regulation of postsynaptic D2 receptors, which may explain the similar duration of recovery during early abstinence (42,87,117).

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III. SUMMARY AND GENERAL IMPLICATIONS

This review of central neuroadaptation and neurodegeneration in alcoholism emphasizes several conclusions. First, it is necessary to assess and monitor the time course of changes in cerebral atrophy and changes in specific neurotransmitter systems such as dopaminergic and serotonergic neurotransmission (4). Neurobiological states that predispose toward excessive alcohol intake, such as a reduced dopamine and serotonin turnover rate (42,41,60), will differ fundamentally from the effects of chronic alcohol intake, which is known to stimulate dopamine and serotonin release (42,52,58,91,92) and which seems to induce secondary neuroadaptive changes in monoaminergic neurotransmission as well as neurotoxic brain damage (4,42,61,80,101). Both cerebral atrophy and neuroadaptive changes in monoaminergic neurotransmission seem to recover during early abstinence; however, subjects showed a wide interindividual variation that was associated with their relapse risk (32). Measurements of monoaminergic neurotransmission that include only one time point may therefore thus run the risk of mistaking a state of neuroadaptation for persistent neurodegeneration.

Second, it is important to assess several factors of monoaminergic neurotransmission simultaneously. In a primate study, increased phasic, stimulus-dependent dopamine release was associated with a—most likely adaptive—down-regulation of postsynaptic dopamine D2 receptors (64), and a reduction in dopamine or serotonin reuptake sites may be correlated with increased synaptic neurotransmitter concentrations (66,60,64). An observed reduction in striatal dopamine or raphe serotonin transporters during chronic alcohol intake can therefore not be interpreted as an indication of reduced monoaminergic neurotransmission, as it may even be associated with increased synaptic monoamine concentrations (62,64).

Third, genotype effects may modify neurodegeneration and neuroadaptation. The genetic constitution of the dopamine transporter gene was associated with in vivo transporter availability in the putamen (116) and may interact with the severity of alcohol withdrawal during the first days of abstinence (112,113), when dopamine transporters are still recovering from the effects of chronic alcohol consumption (42,87). A longer-lasting reduction in raphe serotonin transporters was found in a genetically defined subgroup of male alcoholics and was associated with the severity of clinical depression (61). Subjects with this genotype were predisposed to excessive alcohol intake (63) and may be specifically vulnerable to chronic alcohol intoxication (89). The reasons for this vulnerability may include alcohol-toxic effects on raphe neurons, effects of alcohol withdrawal, or stress-induced cortisol release (119). Further studies will be necessary to better characterize genotype-environmental interactions that affect neuroadaptation and neurodegeneration in alcoholism.

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12

Pathogenic Mechanisms in Alcoholic Myopathy

Victor R. Preedy and Timothy John Peters
King's College, University of London, London, England

Junko Adachi
Kobe University School of Medicine, Kobe, Japan

Sohail Ahmed and David Mantle
Newcastle General Hospital, Newcastle upon Tyne, England

Onni Niemela and Seppo Parkkila
University of Oulu, Oulu, and EP Central Hospital Laboratory, Seinäjoki, Finland

Simon Worrall
The University of Queensland, Brisbane, Queensland, Australia

I. INTRODUCTION

Skeletal muscle myopathy occurs as a consequence of prolonged ethanol misuse and is characterized by atrophy of type II fibers (particularly the type IIb subset); the type I fibres are relatively resilient though they may atrophy in the most serious cases. Subjects with alcoholic myopathy may have lost on average 20% of their entire musculature, though in some susceptible individuals up to 30% of muscle may be lost. Neither liver impairment, neuropathy, nor overt malnutrition is a major contributing factor. However, recent evidence using ELISA and immunohistochemical analysis suggests that there are significant levels of acetaldehyde-protein adducts within muscle, particularly in the subsarcolemmal region. This implicates impairment of membrane-associated processes (for example, abnormalities in contractile response or intracellular signalling) and the formation of neoantigens.

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Table 1 Prevalence of Skeletal Muscle Disorders

	Incidence per 1,000,000	Treatable/ reversible?
Becker muscular dystrophy	10	No
Duchenne muscular dystrophy	30	No
Chronic alcoholic myopathy	20,000	Yes

Source: Figures are adapted from ref. 7 and based on a number of sources.

Alcoholic myopathy is characterized by skeletal muscle weakness, with patients encountering frequent falls and difficulties in gait (1). Histological analysis of skeletal muscle biopsies shows that the diameter of type II (glycolytic, fast-twitch, anaerobic) fibers is preferentially reduced. Type I fibers may exhibit a mild hypertrophy in the initial stages of alcoholic exposure though in severe persistent alcoholism, type I fibers may also atrophy (2,3). A substantial amount of evidence shows that the reductions in fiber diameters are associated with concomitant decreases in muscle protein content. These data include reductions in protein/DNA ratios, myosin heavy chain, midarm circumference, urinary creatinine excretion, and the body mass index (1,4–6).

Between one-half and two-thirds of all alcohol misusers suffer from alcoholic myopathy (reviewed in ref. 7). Acute alcoholic myopathy, characterized by rhabdomyolysis, is comparatively rare and occurs in fewer than 5% of chronic alcohol misusers (1). Based on the relative extent of alcohol misuse in England, it is clear that the incidence of alcoholic myopathy is substantially greater than the more widely researched heritable disorders such as Becker or Duchenne muscular dystrophy (7) (Table 1). A similarly high prevalence of alcohol abuse in other European, North American, and other developed countries means that alcoholic myopathy is the most common form of skeletal muscle disease. However, the precise sequence of events that lead to alcoholic myopathy is unknown. In this chapter we describe some features of alcoholic myopathy suggesting that this disease is a multifaceted disorder encompassing a number of pathogenic mechanisms. These include reduced rates of muscle protein synthesis and breakdown, the generation of free radicals and endocrine abnormalities, as well as the formation of acetaldehyde adducts (2,3). Skeletal muscle disorders also occur in neuropathies (8,9), liver disease (10,11), endocrine dysfunction (12,13), and malnutrition (14–16). It is therefore relevant to determine whether they may also contribute to alcoholic myopathy. Addressing these issues may potentially lead to the identification of novel diagnostic criteria or treatment strategies and etiological mechanisms.

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II. ANIMAL MODEL OF ALCOHOLIC MYOPATHY

Although analysis of individual skeletal muscle fibers in clinical studies presents numerous practical problems, investigations into alcoholic myopathy have been facilitated by the development of a suitable animal model based on the application of the Lieber-DeCarli feeding regimen. Rats fed ethanol as 35% of their total energy intake for 6 weeks have reduced skeletal muscle mass, largely due to the preferential reduction in the weight of type II fiber–predominant muscles including the plantaris and gastrocnemius. Muscles containing predominantly type I fibers such as the soleus are usually unaffected by alcohol feeding though in some circumstances their weight may be reduced: the latter may reflect an extreme myopathic lesion. In a systematic investigation, we have shown that the animal model displays virtually all the features seen in its clinical counterpart (Table 2) (17).

III. ACETALDEHYDE ADDUCT FORMATION

The formation of protein adducts in alcoholic liver disease has received considerable attention. Likely consequences of adduct formation include the inducement of immunogenic mechanisms via the formation of neoantigens (for examples in alcoholic liver disease, see refs. 18–22). There is a counterpart in the well-investigated phenomenon of protein glycation in diabetes. There is some evidence to support the contention that adduct formation may also occur in muscle. For example, acetaldehyde covalently binds to skeletal muscle actin in vitro via lysyl residues under both reducing and nonreducing conditions (23). To determine whether adduct formation occurs in skeletal muscle in vivo we assayed acetaldehyde- (reduced and unreduced), malondialdehyde-, malondialdehyde-acetaldehyde-, and alpha-hydroxyethyl-protein adducts in plantaris muscles from rats fed alcohol for 6 weeks on the Lieber-DeCarli pair-feeding regimen (S Worrall, O Niemela, S Parkkila, TJ Peters, and VR Preedy, unpublished observations). Using ELISA we showed that, all the above-mentioned adducts were increased in the liver of ethanol-fed rats compared to pair-fed controls. In muscle, there were increased amounts of unreduced-acetaldehyde adducts in ethanol-fed animals when compared to pair-fed controls (Table 3). However, ELISA showed there was no evidence of increased reduced-acetaldehyde-, malondialdehyde-, malondialdehyde-acetaldehyde-, or alpha-hydroxyethyl-protein adducts in muscle of alcohol-fed rats. Immunohistochemical studies also confirmed the presence of acetaldehyde adducts (both reduced and unreduced) in the muscle of ethanol-fed rats. The immunohistological staining reactions were strongest in the subsarcolemma region (i.e., muscle membrane or surface) though positive intracellular signals were also seen (S Worrall, O Niemela, S Parkkila, TJ Peters, and VR Preedy, unpublished observations).

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Table 2 Comparison of Myopathy in Clinical and Experimental Studies

Feature	Clinical myopathy	Experimental myopathy
Incidence	Studies from Australia, USA, Denmark, India, China, Spain and the UK show that type II fiber atrophy occurs in 40–65% of alcoholics (different diagnostic criteria have been used including histology, electromyograms, fiber-diameter measurements, and muscle strength)	All rats appear to develop myopathy in experimental studies though there is some variability; in mature rats, the myopathy develops after 3 months though a more rapid response occurs in immature rats (i.e., 6 weeks) presumably due to the greater rate of protein synthesis in younger animals
Type I fibers	The diameters of type I fibers (with abundant mitochondria) are reduced in extreme alcoholism or are generally unaffected; these fibers may show hypertrophy in some circumstances	Type I fiber–predominant muscles such as the soleus show only moderate or no reductions in weight
Type II fibers	The diameters of type II fibers show marked reductions; in particular type IIb (with little or no mitochondria) fiber subsets are affected to a greater extent than type IIa (with a moderate number of mitochondria) fibers	Type II fiber–predominant muscles such as the plantaris show marked reductions in weight
Malnutrition	As a rule of thumb, approximately half of alcoholics have a nutritional deficiency of either one or more micro- or macronutrients; in a recent study 100% of all alcoholics were deficient in folate and vitamin E intakes while 85–95% were deficient in selenium and vitamin D intakes; 50–85% were deficient in calcium and zinc and vitamins A, B_1, B_2, B_6, and C intakes; apart from alpha-tocopherol, the following are similar in myopathic and nonmyopathic alcoholics: vitamin D, B_1, B_2, B_6, and B_{12}, folate, and general nutritional status as well as protein nutrition	There is no malnutrition; both control and alcoholic rats receive identical amounts of the same diet albeit differences for calories provided by alcohol (35%) in treated rats, which is replaced by isocaloric glucose in controls; however, consideration needs to be given to the possibility that gastrointestinal disturbances may contribute to malnutrition

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Neuropathy	Neuropathy occurs in some myopathic subjects; however, this is not a significant contributing factor as the incidence of neuropathy is no different in myopathic and nonmyopathic alcoholics; the incidence of neuropathy in alcoholics is about 15% compared to 40–60% who have myopathy	There is no histological evidence that the experimental myopathy is due to neurological damage
Liver disease	Liver disease frequently occurs in alcoholics; however, the incidence of liver disease (for example cirrhosis) is no different in myopathic and nonmyopathic alcoholics	Fatty liver develops in all alcohol-fed rats; experimental cirrhosis for 17 weeks does not cause myopathy; this contrasts with alcohol feeding for 6 weeks, which causes myopathy
Muscle apoptosis	Using the TUNEL assay, this is not a contributing factor; there is no difference in apoptosis in myopathic and nonmyopathic alcoholics	Using both the TUNEL assay and the DNA laddering technique, there is no evidence of apoptosis in alcoholic myopathy
Muscle RNA	Reduced muscle RNA is observed in alcoholic myopathy and correlates with increasing atrophy	Reduced muscle RNA is observed in experimental myopathy and RNA may contribute to impaired protein synthesis; however, the relative abundance of mRNAs encoding ribosomal proteins are not affected by alcohol feeding, whereas muscle RNase activities are increased
Muscle protein content	Decreased protein/DNA ratios occur with reduced type II diameters; Ca^{2+}-activated ATPase activities are decreased in myopathic subjects	Muscle protein content is reduced in type II fiber–predominant muscles from alcoholic rats
Muscle protein breakdown	Muscle proteolysis is reduced as reflected by decreased urinary 3-methylhistidine excretion; addition of alcohol and acetaldehyde to human muscle in vitro reduces protease activities	Rates of muscle protein breakdown are reduced by alcohol feeding; some muscle proteases activities are also reduced
Muscle protein synthesis	Muscle protein synthesis is reduced	Muscle protein synthesis is reduced in myopathic muscle in response to either acute or chronic alcohol

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Table 3 Acetaldehyde-Protein Adducts in Muscles in Response to Alcohol Feeding

	Protein adduct levels by ELISA (absorbance at 405 nm)		
	Control	Ethanol	*p*
Soleus	0.127 ± 0.014	0.208 ± 0.017	<0.025
Plantaris	0.132 ± 0.015	0.222 ± 0.016	<0.025
	Protein adduct staining score by immunohistochemistry (arbitrary Units)		
	Control	Ethanol	*p*
Soleus	0.667 ± 0.304	1.500 ± 0.312	<0.05
Plantaris	0.667 ± 0.304	1.583 ± 0.248	<0.025

Male Wistar rats were fed on a nutritionally complete liquid diet containing 35% of total calories as ethanol. Controls were pair-fed identical amounts of the same diet in which ethanol was replaced by isocaloric glucose. After 6 weeks rats were killed and the skeletal muscles (soleus: type I fiber–predominant; plantaris: type II fiber–predominant) were dissected out for analysis of unreduced adducts by ELISA or both reduced and unreduced adducts by immunohistochemistry. ELISA data are expressed as absorbency units at 405 nm for 10 μg tissue; mean ± SEM ($n = 6$ pairs in each group).
Source: S. Worrall, O Niemela, S Parkkila, TJ Peters, and VR Preedy, unpublished observations.

In the aforementioned studies we observed acetaldehyde adducts in muscle from control rats: this has been reported previously for control liver tissue (24). Endogenous acetaldehyde may be generated by bacteria in the intestine (25,26). In addition, serine hydroxy-methyltransferase catalyzes the interconversion of glycine from threonine and may generate acetaldehyde (24).

The immunohistochemical location of adducts in the sarcolemmal and subsarcolemmal regions of ethanol-fed rats may reflect a simple diffusion effect of acetaldehyde or possibly a localized generation of acetaldehyde. With regard to the latter, it has been suggested that skeletal muscle cytochrome P-450 is located in the sarcoplasmic reticulum (27). It is important to point out that human or rat skeletal muscle also contains alcohol and aldehyde dehydrogenase (28), aldehyde dehydrogenase (ALDH) isoforms 1, 2, and 5 in human muscle (29), and constitutive cytosolic ALDH (30).

We feel that acetaldehyde adduct formation will have important implications for skeletal muscle function. Adducts formed in the sarcolemmal and sub-

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sarcolemmal regions may interfere with intracellular signaling or post-receptor cascades. Alternatively, myofibrillary proteins located within the intracellular compartment of muscle may be subject to conformational and structural changes thereby leading to contractile defects and muscle weakness.

IV. NUTRITIONAL STATUS IN ALCOHOLIC MYOPATHY

Nutritional abnormalities in alcohol misusers are very common and may arise from either the socioeconomic limitations in obtaining adequate nutrition, the calorific displacement of micronutrients, or the defects in nutrient absorption, retention, and metabolism due to intestinal or hepatic abnormalities. For example, vitamin D deficiency occurs in alcoholism and it may itself induce myopathic features (31,32). However, a comprehensive study has shown that vitamin D deficiency per se does not contribute to the genesis of alcoholic myopathy (33). Similarly, there is a lack of a relationship between deficiencies of riboflavin, pyridoxine, thiamine, vitamin B_{12}, folate, and general nutrition in patients with alcoholic myopathy (5,33,34). The exception to the tentative conclusion that alcoholic myopathy occurs independently of overt malnutrition is the British study showing reduced serum concentrations of alpha-tocopherol and selenium in myopathic compared to nonmyopathic alcoholics (35). In contrast, a clinical investigation in Spanish alcoholics has failed to show a similar correlation with alpha-tocopherol deficiency and alcoholic myopathy, and this probably reflects overall nutritional differences between UK and Spanish alcoholics or other reasons (36). Moreover, in laboratory animal studies we have shown that experimental alpha-tocopherol supplementation does not prevent either the acute (reduced protein synthesis) or chronic (reduction in muscle mass, protein, and RNA contents) lesions due to alcohol dosage (37).

V. FREE RADICALS IN ALCOHOLIC MYOPATHY

Reactive oxygen free radical species (ROS) have been implicated in alcoholic liver disease (see reviews in refs. 38–40). Alcoholic myopathy may also be due to ROS (41). This is a reasonable hypothesis, since type II fibers have lower concentrations of antioxidants such as alpha-tocopherol, superoxide dismutase, catalase, and glutathione peroxidase than type I fibers (42–47). In contrast, the concentrations of the imidazole dipeptides (anserine, homocarnosine, and carnosine), which are important antioxidants, are higher in type II than type I fibers (48,49). However, the imidazole dipeptides in both type I and II fiber-rich muscle are unaltered or increased in response to chronic ethanol feeding (50).

More recently, we have assayed two cholesterol-derived hydroperoxides

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as novel markers of oxidative stress in muscle, namely 7α-hydroperoxycholest-5-en-3β-ol (7α-OOH) and 7β-hydroperoxycholest-5-en-3β-ol (7β-OOH) (51). In response to acute ethanol dosage (75 mmol/kg body weight: analysis after 24 hr) 7α-OOH and 7β-OOH were significantly elevated in both plantaris and soleus skeletal muscle of treated rats. In contrast, the concentration of muscle protein carbonyl was not significantly affected in these animals (51). Currently studies are being made on the effects of chronic alcohol feeding on markers of ROS-induced damage though for the present the role of ROS in the pathogenesis of alcoholic muscle disease remains uncertain. In general, the balance of research by other groups is in favor of ROS involvement in alcoholic liver disease.

VI. THE CONTRIBUTION OF HEPATIC, NEUROLOGICAL, AND ENDOCRINE IMPAIRMENT

Liver disease in general has a marked deleterious effect on skeletal muscle biochemistry and function (11,52–55). Thus, in cirrhosis, skeletal muscle protein synthesis is reduced though 3-methylhistidine efflux across the leg, a marker of protein degradation, is unaltered (54). This latter study contrast with reports showing increased whole-body 3-methylhistidine excretion in cirrhosis (52). It is therefore possible that the myopathy seen in alcoholics is the direct consequence of liver disease. However, systematic studies have shown that there is no direct relationship between the genesis of alcoholic myopathy and liver disease (1,56). These conclusions have been supported by animal studies (57).

Skeletal muscle damage may arise as a consequence of neurological disorders (58–62). However, comprehensive analyses in chronic alcoholics with skeletal muscle disease show that the entity alcoholic myopathy occurs independently of neurological damage (63).

Glucocorticoid excess, i.e., pseudo-Cushing's syndrome, is rare in alcoholism but theoretically may contribute to alcoholic myopathy (64–67). However, the contribution of excess plasma cortisol to the development of alcoholic myopathy has been excluded (68). Nevertheless, other endocrine abnormalities may contribute toward alcoholic myopathy and this is an area that merits further detailed investigation. For example, it has been shown that the response of smooth muscle to ethanol-induced changes in protein synthesis is modified by both adrenalectomy and thyroidectomy (69).

VII. DEFECTS IN PROTEIN TURNOVER

The decreases in muscle protein pool size must be due to changes in protein synthesis or protein breakdown (3,70). With regard to the effects of alcohol on

muscle protein breakdown, the overall excretion of urinary 3-methylhistidine (a marker of contractile protein degradation) in myopathic alcoholics decreases (71). In contrast, experimental chronic alcohol feeding studies have indicated that excretion of urinary 3-methylhistidine increases, though animals were not pair-fed, which may implicate an interaction between nutritional status and muscle metabolism (72). Nevertheless, the possibility that alcohol may decrease protein degradation in accordance with clinical studies (71) has been supported by two observations. First, the rate of protein degradation in rats subjected to pair feeding with the Lieber-DeCarli protocol is reduced, as calculated from the difference between the fractional rate of growth and degradation (73). Second, the addition of alcohol and acetaldehyde to human muscle preparations in vitro shows that both these analytes reduce the activities of a wide spectrum of lysosomal and nonlysosomal proteases (74). However, the inhibitory effects of alcohol and acetaldehyde in vitro are only achieved using very high levels of alcohol (74) (Table 4). Ethanol in concentrations of 17 and 170 mmol/L had no significant effect on any of the enzyme activities, whereas 1700 mmol/L was inhibitory. Acetaldehyde in concentrations of 1.7 mmol/L only affected arginyl aminopeptidase activities (activities reduced by 50%; $p < 0.05$) whereas there was no significant effect on other enzyme activities at this concentration (data not shown for brevity). When acetaldehyde concentrations were increased to 17 and 170 mmol, inhibitory effects were observed (74).

More recently we have assayed the activities of a variety of proteolytic enzymes including cathepsins B, D, H, and L and dipeptidyl aminopeptidases-I and -II, (lysosomal; Table 5) and alanyl aminopeptidase, arginyl aminopeptidase, leucyl aminopeptidase, dipeptidyl aminopeptidase IV, tripeptidyl aminopeptidase, and proline endopeptidase (cytoplasmic proteases; Table 5) as well as enzymes of the Ca^{2+}-dependent calpastatin-calpain system (S Ahmed, D Mantle, M Koll, and VR Preedy, unpublished observations; Table 6). These enzymes constitute the most active proteases in this tissue and represent an index of protein degradation capacity in skeletal muscle. However, none of the activities of these enzymes were significantly affected by chronic alcohol feeding (Tables 5 and 6). Although these data may appear to be at variance with the observation that the overall rate of protein degradation decreases, consideration needs to be given to the possibility that some other enzyme activities may have changed. It is also possible that acute transitions in alcohol concentrations will cause much more complex changes than can be assessed by measuring the activities of these enzymes under artificial conditions. Consideration also needs to be given to the fact that in vitro, artificial substrates (although widely used) are employed whereas in vivo the endogenous proteins or peptides constitute natural substrates for these enzymes. In addition, there may be complex interactions between alcohol and nutrition (see, for example, ref. 75), which is not reproduced in the rat study at present.

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Table 4 Cytoplasmic and Lysosomal Protease Activities in Human Muscle In Vitro in Response to Alcohol and Acetaldehyde

	Effect of ethanol on cytoplasmic and lysosomal protease activity (% change)	
	170 mmol/L	1700 mmol/L
Cytoplasmic		
Alanyl aminopeptidase	+1	−10
Arginyl aminopeptidase	+2	−31*
Leucyl aminopeptidase	−20	−58*
Diaminopeptidase IV	0	−48*
Triaminopeptidase	0	−49*
Proline endopeptidase	−11	−63*
Lysosomal		
Diaminopeptidase I	+3	+1
Diaminopeptidase II	0	−42*
Cathepsin B	+5	−58*
Cathepsin H	+6	−56*
Cathepsin L	+1	−12
Cathepsin D	−2	−55*
	Effect of acetaldehyde on cytoplasmic and lysosomal protease activity (% change)	
	17 mmol/L	170 mmol/L
Cytoplasmic		
Alanyl aminopeptidase	−63*	−81*
Arginyl aminopeptidase	−72*	−90*
Diaminopeptidase IV	−11	−44*
Triaminopeptidase	−39*	−71*
Proline endopeptidase	−1	−34*
Lysosomal		
Diaminopeptidase I	−39*	−74*
Diaminopeptidase II	−34*	−56*
Cathepsin B	0	+1
Cathepsin H	−64*	−79*
Cathepsin L	−1	−3

Human muscle homogenates were incubated with various concentration of alcohol or acetaldehyde in vitro. A minus prefix indicates a reduction in mean enzyme activity, where a positive prefix indicates a mean increase (although none of the latter achieved statically significance).

* $p < 0.05$ in comparison with control preparations without ethanol or acetaldehyde. Ethanol in concentrations of 17 mmol/L had no significance on any of the enzyme activities (data not shown). Acetaldehyde in concentrations of 1.7 mmol/L affected only arginyl aminopeptidase activities (activities reduced by 50%; $p < 0.05$) whereas there was no significant effect on other enzyme activities at this concentration (data not shown for brevity). Leucyl aminopeptidase and cathepsin D activities were assayed only in muscle from alcohol-exposed preparations.

Source: Adapted from ref. 74.

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Table 5 Cytoplasmic and Lysosomal Proteases in Gastrocnemius Muscle of Rats Fed Ethanol for 6 Weeks

	Cytoplasmic (nmol substrate/hr/mg protein)		
	Control	Ethanol	*p*
Alanyl aminopeptidase	874 ± 128	794 ± 69	>0.05
Arginyl aminopeptidase	972 ± 69	970 ± 114	>0.05
Leucyl aminopeptidase	64 ± 5	53 ± 6	>0.05
Diaminopeptidase IV	76 ± 2	78 ± 3	>0.05
Triaminopeptidase	95 ± 12	75 ± 6	>0.05
Proline endopeptidase	140 ± 13	155 ± 9	>0.05
	Lysosomal (nmol substrate/hr/mg protein)		
	Control	Ethanol	*p*
Diaminopeptidase I	307 ± 22	274 ± 34	>0.05
Diaminopeptidase II	77 ± 3	70 ± 4	>0.05
Cathepsin B	18 ± 4	23 ± 5	>0.05
Cathepsin L	144 ± 15	114 ± 7	>0.05
Cathepsin H	50 ± 2	43 ± 6	>0.05
Cathepsin D[a]	5 ± 1	5 ± 1	>0.05

Data are presented as mean ± SEM, $n = 6$ pairs in each group. Rats were fed liquid diets containing ethanol as 35% of total calories (treated) or pair-fed the same diet in which ethanol was replaced by isocaloric glucose (controls).

[a] Units for cathepsin D as units/hr/mg protein. Differences between mean were determined by Students *t*-test for paired samples.

$p > 0.05$, not significant (NS).

Source: S Ahmed, D Mantle, M Koll, and VR Preedy, unpublished observations.

It is possible that the formation of acetaldehyde-adducted proteins described here will influence the overall rate of skeletal muscle protein degradation. For example, carbonyl-modified proteins are more susceptible to protein degradation than the parent proteins (76). However, acetaldehyde-adducted proteins are more stable and reduce the rate of protein degradation (24). This decrease is compatible with the reduction in the overall rate of protein degradation described earlier.

Both human (77) and laboratory rat studies (78,79) show that chronic alcohol consumption reduces muscle protein synthesis in vivo (reviewed in refs. 2, 3, 70, 80, 81). The decline in muscle protein synthesis is a result, in part, of reductions in total mixed RNA, which is largely ribosomal (82). Reduced concentrations of muscle RNA may arise from increases in muscle RNases activity (83).

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Table 6 Calcium-Activated Proteases and Calpastatin Activities in Skeletal Muscle of Rats Fed Ethanol for 6 Weeks

	Control	Ethanol	*p*
Calpastatin (units/g)	669 ± 113	716 ± 117	>0.05
Microcalpain (units/g)	29 ± 9	25 ± 6	>0.05
Millicalpain (units/g)	392 ± 87	362 ± 51	>0.05
Calpastatin/microcalpain ratio	24.0 ± 2.6	27.7 ± 3.7	>0.05
Calpastatin/millicalpain ratio	1.99 ± 0.22	1.97 ± 0.15	>0.05

For details, see legend to Table 5. For the analysis of calpain and calpastatins, muscle from whole hind-limb musculature was analyzed as the analytical procedures necessitated at least 1 g of muscle. Previous studies have shown that the whole hind-limb musculature reflects changes in either the gastrocnemius or whole-body musculature. Data are presented as mean ± SEM of 5–6 pairs of observations.

$p > 0.05$, not significant (NS).

Source: S Ahmed, D Mantle, TJ Peters, and VR Preedy, unpublished observations.

In response to acute alcohol dosage regimens, skeletal muscle protein synthesis also declines. This effect is also seen when acetaldehyde is administered (84). More profound reductions in skeletal muscle protein synthesis occur when pathological levels of acetaldehyde are raised by cyanamide, an inhibitor of aldehyde dehydrogenase (84).

One might question how muscle wasting occurs when both protein synthesis and degradation rates in muscle are reduced by chronic alcohol exposure. The answer to this pertains to the fact that the decrease in protein synthesis is substantially greater than the decrease in protein degradation.

VIII. CONCLUSIONS

Chronic alcoholic myopathy is arguably the most common myopathy in the Western hemisphere. It arises as a direct result of excessive alcohol ingestion by processes that may involve free radicals and/or defects in antioxidant systems, acetaldehyde adduct formation, and decreases in protein synthesis. The pathogenic role of protein degradation, however, requires further clarification.

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13

The Alcoholic Patient in the Perioperative Period

Tim Neumann, Michael Martin, and Claudia Spies
University Hospital Charité, Humboldt University of Berlin, Berlin, Germany

I. INCIDENCE

Alcohol misuse is quite common in the general population and in patients in the hospital. Every fifth patient in a general hospital is an alcohol misuser (1,2). The risk of being admitted to the hospital increases with the amount of alcohol consumed (3). Chronic alcohol misuse is more common in surgical patients than in neurological or psychiatric patients (4). Among patients with cancer of the upper digestive tract the rate of alcohol misuse is more than 50% (5–8). Trauma is also associated with an increased incidence of alcohol misuse (9–12). Among multiple injured patients it is approximately 50% (12).

II. PERIOPERATIVE MORBIDITY

Alcohol misusers have a two- to fourfold risk of postoperative morbidity after surgery (14–16). Most of the recent studies defined a daily consumption of 60 g/day as alcohol misuse (8,12,17–20). Some studies also used criteria of the American Psychiatric Association, the *Diagnostic and Statistical Manual of Mental Disorders* (third edition–revised: DSM-III-R) (8,12,21), or the Michigan Alcoholism screening test (MAST) questionnaire (17,20,22).

The most common complications are infections, cardiopulmonary insufficiency, episodes of bleeding, and the alcohol withdrawal syndrome. The pathogenic mechanisms are probably preoperative immunosuppression, preclinical

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cardiac insufficiency, hemostatic imbalance, and an altered response to surgical stress (13–15). This leads to a prolonged hospital stay, an increased incidence of secondary surgery, and prolonged intensive-care therapy (13–15). Among patients with cancer of the upper digestive tract undergoing tumor resection and subsequent admission to the intensive care unit, the median difference regarding the duration of the intensive-care-unit stay between alcoholics and nonalcoholics was 8 days (8).

III. IMMUNE SYSTEM

Chronic ethanol administration alters the immune status and can increase host susceptibility to infections caused by bacterial and viral pathogens (23–32). Postoperative infections may be related to preoperative immunosuppression (33) and surgery or trauma adds to the ethanol-induced immunosuppression (34,35). T-cell-mediated response, delayed-type hypersensitivity, interleukin (IL)-2 expression, initial tumor necrosis factor (TNF), interferon production, and cytolytic activity are down-regulated by ethanol. This effect is enhanced by surgery or trauma (13–15,17,34,36,37). Antigen-presenting cells are important for the development of both Th1-helper and Th2-helper lymphocyte-regulated immune responses. A preferential induction of Th2 versus Th1 immune response has been suggested in chronic alcoholics. Alcohol impairs Th1-lymphocyte-regulated, cell-mediated immune responses. Antibody responses, regulated by Th2 lymphocytes, are either unimpaired or enhanced (34,38).

In a 1992 study by Tønnesen et al. (17) the skin response of delayed-type hypersensitivity of patients undergoing colon or rectum surgery was decreased after surgery in all patients. The stress response is responsible for this kind of immunosuppressive state in the postoperative period. But the reduction was to a significantly larger extent greater among alcoholics compared to nondrinkers. Delayed-type hypersensitivity requires 2 months to normalize after abstinence (39,40).

Ethanol consumption alters neuroendocrine and immune functions (41) in the same manner as surgery (35). An abnormal hypothalamic-pituitary-adrenal axis has been linked to the development of inflammation and infection in the animal model (42). The cortisol response to stress may be altered for more than 6 months after abstinence in alcoholic patients (43). Significantly decreased levels of proinflammatory cytokines TNF-α, IL-1, IL-6, and IL-8 in septic shock patients with a history of alcohol misuse were found by our study group (44).

In an investigation of patients with cancer of the upper digestive tract undergoing tumor resection with subsequent intensive-care-unit stay the rate of infectious complications in alcoholics was significantly increased: pneumonia oc-

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curred in 38% of the alcoholics compared to 7% of the nondrinkers. In this study sepsis occurred only in chronic alcoholics (8).

Not the microbiological agent, but the host determines the reaction of the organism and the degree of systemic response (45).

On the other hand, prolonged ventilatory support of patients with severe alcohol withdrawal syndrome requiring sedation therapy leads to pulmonary complications (46). Nicotine abuse, which is common among alcohol misusers, leads to more pulmonary complications and adds to morbidity (47). Also wound infections are more frequent among alcoholic patients compared to controls (15,48).

IV. CARDIOVASCULAR COMPLICATIONS

Alcoholics have more cardiac complications in the postoperative period, e.g., arrhythmias and cardiac insufficiency (12,14,15). A subclinical cardiomyopathy may be present in alcohol misusers (49,50). The central features are an altered protein synthesis. Both alcohol and its metabolite acetaldehyde may alter myocardial function (49,50). Preoperative left ventricular function expressed as ejection fraction is significantly reduced (14,15,17,51). After 1–3 months the dysfunction is at least partly reversible (51,52).

Hypokalemia, present in patients with increased sympatic tone in the postoperative period, especially if exaggerated by an alcohol withdrawal syndrome, increases the risk of cardiac arrhythmias.

Hypoxemia after major surgery may contribute to cardiac and wound complications (15,53). Sudden episodic hypoxemia in the patients who continued to drink may be due to the altered sleep physiology described in chronic alcohol abusers (54). Sleep deterioration with a high prevalence of apneic and hypopneic episodes may continue for 3–6 weeks in detoxified misusers (54,55).

Comorbidity (nicotine abuse, coronary artery disease, nutritional deficiencies, cancer) will additionally confuse the clinical picture (47).

V. BLEEDING COMPLICATIONS

Bleeding complications are more frequent among alcoholics and the need for transfusion is significantly higher (15,17,18,56). Bleeding time is prolonged. Responsible for these findings is not just a severe alcoholic liver disease that impairs the synthesis of hemostatic factors. Ethanol impairs thrombocyte function. Ethanol suppresses thrombopoiesis at the level of megakaryocyte maturation. Platelet count and mean volume are reduced. Platelet aggregation to all kinds of stimulus is impaired (15,57,58). Moderate alcohol consumption may affect several hemo-

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static factors, including fibrinogen concentration, platelet aggregability, and the fibrinolytic factors tissue-type plasminogen activator and plasminogen activator inhibitor (59).

The increased bleeding time seen in alcoholics normalizes after 1 week of abstinence (40).

VI. ALCOHOL WITHDRAWAL SYNDROME (AWS)

A potential life-threatening complication is AWS (13). The effect of chronic alcohol exposure on various neurotransmitter systems includes the glutamatergic, GABAergic, dopaminergic, serotoninergic, cholinergic, and opoidergic systems, including the hypothalamus-pituitary-adrenal axis with complex interactions (13), and may explain the clinical manifestation of AWS.

Within 6–24 hr after the last drink alcohol-dependent patients may develop autonomic hyperactivity through disinhibition of the sympathetic activity in the locus coereleus, including tremulousness, sweating, nausea, vomiting, anxiety, and agitation. The autonomic hyperactivity can lead to life-threatening cardiovascular complications. Epileptiform seizures can occur 12–24 hr after abstinence through changes of the glutamatergic and GABA-ergic system after withdrawal. The changes in the cholinergic system can produce cognitive disorders, clouding of consciousness, and confusion and impaired attention, whereas the changes in the dopaminergic system can produce productive psychotic symptoms such as auditory and visual hallucinations. A depressive and anxious mood is typical for patients in withdrawal. The serotonergic system seems to be involved here (13).

The incidence of AWS at the time of admission into the hospital was 8% among a representative sample of patients in an Australian study (60), whereas the incidence can be 16% among patients after surgery (8) and 31% after severe trauma (12). Among severely ill patients the differential diagnosis of AWS can be difficult. Most of the patients are intubated and ventilated, interfering with the diagnosis of cognitive disorders. Other centrally active medication given in the ICU setting will further complicate the clinical picture. Common complications such as bleeding, metabolic and electrolyte disorders, infections, hypoxia, pain, or focal neurological disorders should be excluded or treated before the diagnosis of AWS can be established (13).

VII. TREATMENT OF AWS

A meta-analysis published in 1997 in JAMA (61) suggests evidence for medication combinations, including a long-acting gabaergic medication, to treat acute

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alcohol withdrawal for nonsurgical patients. The situation in postoperative patients may be aggravated due to the stress response (13). Pharmacological combinations are often used in combination with benzodiazepines (62). Dosages are generally larger for surgical patients than for those in detoxification units (13).

Comparison of the efficiency of three different current combination regimes (benzodiazepine/haloperidol, benzodiazepine/clonidine, clormethiazol/haloperidol) for the treatment of AWS in patients with multiple injuries showed no difference in controlling the AWS or the duration of intensive-care-unit stay (63). Owing to the typical side effects of the different agents, one may find different rates of complications. The bronchial hypersecretion together with ventilatory depression of chlormethiazole leads to an increased rate of pulmonary complications. The sympathicolytic effect of the α_2-agonist clonidine leads to a higher rate of cardiac complications, mainly bradyarrhythmias (13,63).

To treat surgical intensive-care patients for AWS it has been recommended to start with a benzodiazepine and then add clonidine or haloperidol. The patient should be monitored closely by means of the Revised Clinical Institute Withdrawal Assessment for Alcohol Scale (CIWA-Ar) (67), and by titration of the medication a score of <10 should be maintained. The electrolytes (potassium and magnesium!) should be controlled closely and substituted, if necessary (13). Thiamine should be given.

VIII. PHARMACOPROPHYLAXIS

What kind of strategies exist to prevent postoperative withdrawal and its complications? The need for an alcoholic to withdraw from alcohol prior to operation has been under question. Most surgeons do not ask their patients to abstain from drinking before undergoing surgery. Instead prophylaxis is offered to the patient with alcohol misuse to reduce morbidity. Which pharmacological intervention is used has a minor impact. Even alcohol given perioperatively to the patient was able to reduce morbidity (13,62,64,65).

In vitro studies suggest that small doses of alcohol may be immunoprotective (66). The dosage of alcohol used as a prophylactic should not exceed 0.5 g/day, because higher doses may be immunodepressant. Alcohol in the treatment of withdrawal is obsolete (13).

The pharmacoprophylactic agents used to prevent alcohol withdrawal do not differ from the agents used to treat AWS. The dosage is usually lower but has to be adjusted to the patient's needs (13). It is important to recognize patients at risk to initiate prophylaxis. Among 121 patients with upper-digestive-tract cancer and preoperative chronic alcohol misuse 70 patients were diagnosed as alcohol dependent (8). One-third of 70 patients at risk were not diagnosed preoperatively and developed AWS requiring prolonged intensive-care-unit stay (mean

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difference 14 days) together with a significantly increased incidence of severe complications compared with patients who received prophylactic therapy. However, one-quarter of the patients with prophylaxis still developed AWS, although significantly milder than in those chronic alcoholics who had developed unforeseen AWS. The severity of the AWS was measured with the CIWA-Ar (67). The length of the intensive-care-unit stay was comparable in these patients with dependence and prophylaxis and patients with alcohol misuse without dependence (8).

IX. ABSTINENCE

Another possible form of intervention is preoperative abstinence (68). One month of disulfiram-controlled preoperative abstinence reduces postoperative morbidity in alcohol misusers undergoing colorectal surgery. Correspondingly, the need for nursing care was lower compared to the high complication rate in the control patients who continued to drink. Although reduced, the postoperative morbidity in the intervention group was still higher (31%) than in most studies in unselected colorectal patients, though a wide range has been reported (68).

The mechanism of the improved outcome after this intervention is probably reversibility of the ethanol-induced organ dysfunction as a result of abstinence. In nonsurgical patients dysfunctions are often reversible after withdrawal from alcohol within weeks or months (39,40,68). The time for organ functions to recover has not been evaluated in full detail. The alcohol-induced changes of organ functions are not uniformly reversible and may be irreversible.

The DSM-III-R, defines full remission after 6 months of abstinence and the DSM-IV after 12 months (21,69,70), but this has not been evaluated in the perioperative context.

Nondrinkers are generally found to have a general mortality between that of the moderate and high consumers (71–79). This may be due to problems with selection bias related to health in these studies. Sick people may not drink because of their illness, leading to an apparent increased risk of mortality among nondrinkers (80).

According to the above-mentioned criteria, former alcoholics are nondrinkers. Our own data suggested that former alcoholics, abstaining for more than 6 months (median 5 years, range 6 months–21 years), undergoing cancer surgery of the upper digestive tract do not have a reduced morbidity, probably because of confounding variables, such as heavy smoking (81). All 25 abstainers in this cohort of 400 patients with cancer of the upper digestive tract were smokers. Owing to the increased preoperative pulmonary morbidity, the complication rate was comparable to the complication rate of alcoholics and increased compared

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to nondrinkers and social drinkers. This underlines the importance of assessing comorbidity.

X. DIAGNOSIS

Before any intervention a diagnosis is needed.

In assessing the alcoholic patient, history and physical examination are still basic tools. The use of the short and accurate CAGE questionnaire (four items: *C*ut down, *A*nnoyance by criticism, *F*eeling guilty, *E*yeopener) was found to be valuable (82). Accordance with the DSM criteria was found to be acceptable.

Laboratory markers are additional valuable tools.

Less than one-fifth of the patients were suspected as being alcohol dependent at the first contact with the consultant (83). In a study of cancer patients during the staging procedures' several consultations, the additional information gained by laboratory markers (carbohydrate—deficient transferrin, γ-glutamyl-transferase) and by the CAGE questionnaire gradually increased the detection rate up to a reasonable 91%.

The diagnostic quality of a biochemical marker can be assessed by the receiver-operated characteristics (ROC). ROC curves are formed by putting sensitivity and specificity over a range of cutoffs. The area under the curve can be used as marker for the quality of a biochemical marker. Carbohydrate-efficient transferrin (CDT) is performing much better than conventional laboratory markers as mean corpuscular volume (MCV) and γ-glutamyl-transferase (γ-GT) (84–87). With regard to the decrease in sensitivity of CDT after surgery, volume shifting may contribute to a more rapid decrease in detectable CDT when compared to the CDT course of nonsurgical patients (85,86). CDT can also be used to identify patients at risk of developing posttraumatic complications (88).

The use of short-term consumption markers such as a urinary ratio of 5-hydroxytryptophol to 5-hydroxyindole-3-acetic acid in the range of up to 24 hr after the last consumption can add important information about previous drinking (89). Drinking the day before surgery is usually not detectable the next morning by determination of the blood alcohol level, but is associated with an increased rate of complications (89).

Further research will show the relevance of these and other markers to assess the individual perioperative risk.

When discussing possible elevated values it may improve patient's compliance to accept prophylactic procedures.

There is still no alcohol-dependence marker; all known markers can only detect abuse. Although associated with an increased perioperative risk, alcohol abuse does not require specific withdrawal prophylaxis.

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XI. CONCLUSION

There is repeated emphasis on performing a thorough preanesthesia assessment in patients with suspected chronic alcohol use. These patients are difficult to diagnose and to treat in surgical settings if complications arise (13–15). More evidence is needed to develop multimodal strategies to deal with this group of alcohol-misusing patient at high risk, in diagnosis, risk stratification, prophylaxis, and therapy.

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14

Alcohol and the Energy Balance

Is Alcohol a Risk Factor for Obesity?

Paolo M. Suter
University Hospital, Zurich, Switzerland

I. INTRODUCTION

Cardiovascular diseases represent the most important cause of mortality and morbidity in Western societies (1,2). The major cardiovascular risk factors are smoking, hypercholesterolemia, hypertension, glucose intolerance, and insulin resistance (3–5). These primary risk factors are affected by many different modifiable and nonmodifiable factors; however, overweight and obesity represent the most important single modulator of basically all primary risk factors. Moderate alcohol consumption has a potential protective effect on coronary artery disease risk (6,7). However, alcohol has a comparatively high caloric value (7.1 kcal/g) and represents an important source of energy (8) and may thus enhance energy intake and affect body weight. At present it is still controversial whether alcohol calories do count and whether alcohol consumption represents a risk factor for weight gain and obesity. Many different risk factors for obesity and weight gain have been identified, and in view of the pandemic of obesity the control of any risk factor for weight gain would be of great public health importance in the long term. In this chapter the potential importance of alcohol as a risk factor for obesity is addressed. Initially the epidemiological data are summarized; then the metabolic effects of alcohol on the energy balance equation are discussed, and finally, the question ''How much do alcohol calories count?'' is addressed.

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II. THE RELATIONSHIP BETWEEN ALCOHOL INTAKE AND BODY WEIGHT IN EPIDEMIOLOGICAL STUDIES

The prevalence of obesity is increasing worldwide (9,10). At present more than one-third of the U.S. adult population is overweight [i.e., body mass index (BMI) ≥ 27.3 kg/m^2 and 27.8 kg/m^2 for women and men, respectively] (11). The prevalence of obesity varies according to the geographic area, sex, age, socioeconomic factors, patterns of physical activity, and metabolic factors. Whether other lifestyle factors such as alcohol intake contribute to the variability of obesity is not known.

Many studies tried to address the effect of alcohol on body weight; however, most, if not all, studies reported opportunistic data; i.e., the primary issue of these studies was not elucidation of the relationship between alcohol intake and body weight, but other issues such as cardiovascular disease risk or cancer risk. This factor is important to remember, since the initial design and methodology of a study is of crucial importance to assess alcohol intake correctly and to use adequate instruments for identification of certain relationships.

Alcohol provides 6–10% of the total caloric intake of U.S. adults; however, in heavy alcohol consumers this may increase up to 50% (8,12). In the U.S. alcoholic beverages represent the third most important source of energy after white bread and sweets (8); in England beer represents the fifth most important source of energy after white flour, whole milk, potatoes, and sugar (13). Despite this rather high amount of energy supplied with the ingestion of alcoholic beverages the efficiency of the alcohol calories as a usable source of energy is not known. Although Atwater and Benedict suggested in their classic studies (14) that alcohol is comparable to other energy sources such as carbohydrate and fat, it is still controversial whether alcohol energy is indeed equivalent to other energy sources and to what degree alcohol is a usable source of energy. Unfortunately, epidemiological studies cannot provide an answer to the latter controversy.

As summarized in Table 1, the epidemiological studies regarding the relationship between body weight and alcohol intake are inconsistent and controversial. The rather controversial and conflicting data look confusing; however, it is conceivable that all studies do indeed represent a real and accurate relationship, but only for the corresponding population in which the data have been collected. Many aspects of alcohol metabolism do show a wide variation from one individual to another and also from one population to another. This variability of the effect of alcohol on body weight is due to factors such as the absolute amount of alcohol consumed, the frequency of consumption, drinking pattern especially regarding concomitant food intake and energy substrate composition of the food eaten, the body weight status, the family history of overweight, and probably also the genotype of the alcohol-metabolizing enzymes (15,16). The wide variability of the alcohol effects is well illustrated in a recent meta-analysis about the nadir of alcohol consumption with the lowest mortality risk (17). In the study

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Table 1 The Relationship Between Body Weight and Alcohol Intake in Epidemiological Studies

Positive	No relationship	Negative
Rose et al. (53) (men only)	Rose et al. (53) (women only)	Colditz et al. (85)
Arkwright et al. (103)	Colditz et al. (85)	Camargo et al. (104)
Arkwright et al. (105)	Gruchow et al. (106)	Criqui et al. (107)
Barboriak et al. (108)	Higgins et al. (109)	Cooke et al. (110)
Cooke et al. (110)	Jones et al. (111)	Donahue et al. (112)
Colditz et al. (85)	Klatsky et al. (113)	Fisher et al. (57)
Dyer et al. (114)	Shah et al. (115)	Gordon et al. (116)
Ferro-Luzzi et al. (117)	Stampfer et al. (118)	Gruchow et al. (119)
Garg et al. (120)	Schatzkin et al. (121)	Hillers et al. (54)
Gyntelberg et al. (122)	Willett et al. (123)	Kivelä et al. (124)
Jacobsen et al. (125)	Jacobsen et al. (125)	Jones et al. (111)
Klatsky et al. (113)		Klatsky et al. (113)
Kromhout et al. (126)		Kozararevic et al. (127)
Kivelä et al. (124)		MacMahon et al. (128)
Kozarevic et al. (129)		Milon et al. (130)
Lang et al. (131)		Schatzkin et al. (121)
Rissanen et al. (132)		Simko et al. (133)
Shaper et al. (134)		Weissfeld et al. (135)
Seppä et al. (136)		
Savdie et al. (137)		
Suter et al. (100)		
Shephard et al. (138)		
Trevisan et al. (139)		
Wannamethee et al. (140)		
Weatherall et al. (141)		
Meyer et al. (142)		

Source: Adapted from Ref. 102.

by White the amount of alcohol that was associated with the lowest mortality was 7.7 drinks/week for U.S. men (95% CI 6.4–9.1) and 12.9 for British men (95% CI 10.8–15.1) (17). These wide ranges of alcohol intake may have very different metabolic consequences also on body weight. It can be hypothesized that an equally heterogeneous picture will be found for the relationship between alcohol and body weight.

Most of the controversy in the epidemiological studies about alcohol and body weight can be explained as being due to the presence of several confounding factors (see Table 2). It is important to note that some of these confounders cannot be controlled for in epidemiological studies (such as pattern of drinking and eating, as well as individual genetic factors).

One of the foremost difficulties is the correct assessment of alcohol intake in epidemiological studies (18–20). As a function of the instrument and the mea-

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Table 2 Important Confounders and Their Effect on the Relationship Between Alcohol Intake and Body Weight in Epidemiological Studies

Potential confounder	Effect
Absolute amount of alcohol consumed	Degree of MEOS induction
	Eating pattern
	Food composition
Frequency of drinking	Correlation with the absolute amount of alcohol consumed
	Effect on the eating pattern
Liver function	Degree of induction
	Degree of liver damage (cirrhosis)
Body weight status	The alcohol effects seem to be more pronounced in obese subjects
Concomitant food intake	Associated energy intake and substrate composition
	Potential for substrate interactions
	Modulation of blood alcohol level
Smoking	Stimulation of energy expenditure (EE)
	Effect on food intake due to effects on appetite/mood
	Interaction with alcohol regarding EE
Pattern of physical activity	Alcohol may modulate the non–exercise-associated thermogenesis (NEAT)
Beverage type	Drinking and eating pattern varies according to beverage type
	Associated life-style
Ethnicity	Genotype of alcohol-metabolizing enzymes
Age	Age-related changes in alcohol metabolism
	Altered body composition and volume of distribution
	Altered life-style including physical activity and eating
Socioeconomic status	
Gender	
Genetic background	Genotype of the alcohol-metabolizing enzymes
	Family history of obesity

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surements units used the accuracy of the assessment may vary widely. One important modifier of the accuracy of alcohol intake seems to be the absolute amount of alcohol consumed: heavy and light consumers may have reasons to over- and/or underreport their alcohol intake whereas socially acceptable (or even recommended) moderate alcohol intake is generally more adequately reported. Social reasons as well as gender-specific factors may lead to a higher prevalence of intentional wrong reporting in women than in men, thus explaining in part some of the discrepant results according to gender (21).

The absolute amount of alcohol as well as drinking frequency is probably the most important modulator of the relationship of interest (Fig. 1). The absolute amount and drinking frequency represent the major modulators of the drinking pattern regarding the amount and composition of the ingested food, i.e., whether alcohol is added to the usual food sources or is substituted for normal food energy (Fig. 2). Most light to moderate alcohol consumers add the alcohol to their usual energy intake, whereas the substitution of usual food sources by alcohol is the

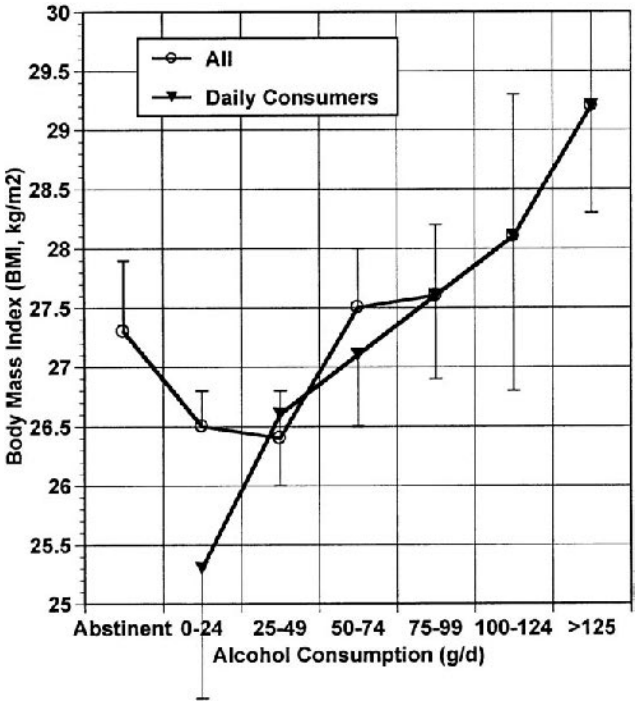

Figure 1 Relationship between alcohol intake and body weight in a group of hotel and restaurant keepers (From Ref. 142).

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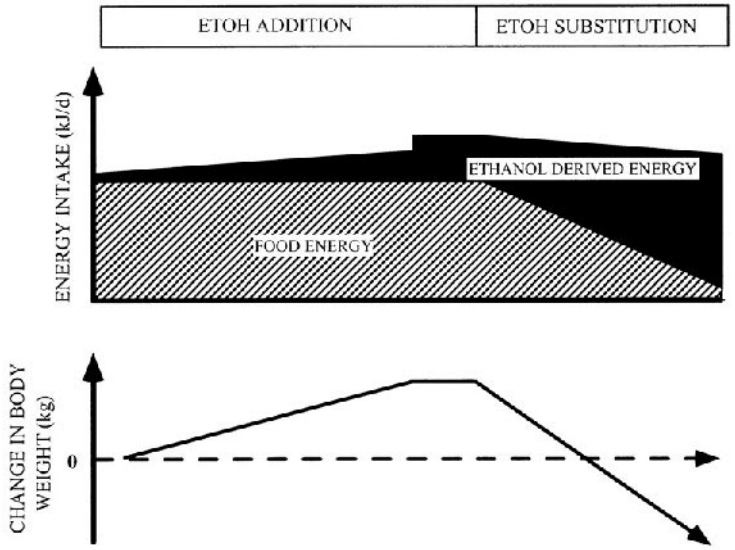

Figure 2 The effect of ethanol on body weight dynamics (From Ref. 102).

typical drinking pattern of the heavy alcohol consumer leading to primary malnutrition (22). The drinking/eating pattern, i.e., whether alcohol is added or substituted, is difficult to assess and usually not reported in epidemiological studies. As will be discussed later, addition or substitution of alcohol for the usual food intake would have very different effects on body weight in the long term. As will be discussed later, the substrate composition may further modify the relationship of interest. Evidence from interventional studies suggests that the alcohol effects on body weight may vary according to the body weight. The effect of alcohol on body weight may be more pronounced in obese subjects and in subjects eating a high-fat diet.

The addition or substitution would lead to different blood alcohol levels with different metabolic consequences including the degree of induction of the microsomal ethanol oxidizing system (MEOS) and thus the degree of energy wastage (see below).

Whether menopausal status affects the relationship between body weight and alcohol is not known. Other factors such as smoking, physical activity, psychosocial factors, caffeine consumption, and medication intake may further affect the association of interest (Table 2).

Epidemiological studies show that alcohol represents an important energy source in the daily diet; however, the effect of alcohol on body weight is not

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consistent and shows a similar variability. Despite many epidemiological studies about the relationship between alcohol and body weight, no conclusive statement can be made. It is conceivable that all reported data are correct; however, they are applicable only to the population in which the data have been collected. Interventional studies may help to clarify some of the controversy.

III. INTERVENTION STUDIES

Only a few interventional studies addressed the issue of the effect of alcohol on body weight regulation as the primary aim. The results from these interventional studies are not conclusive since some studies reported no effect (23–29), others a positive effect (30–33) as well as a negative effect (30–32,34). Some of the controversy can be explained by methodological issues such as a too short study duration, bias due to confounding factors such as physical activity, energy substrate composition of the diet, usual alcohol intake, genetic factors (including positive family history of obesity), and body weight status at the time of the intervention.

Body weight may be an important modifier of the relationship. An increased body weight is associated with a decreased lipid-oxidizing capacity, which may be a cause or a consequence of the increased body weight (35). Alcohol itself elicits a suppression of lipid oxidation and it is conceivable that the suppressive effect of alcohol on body weight is more pronounced in subjects with a higher body weight. We reported recently that the effect of alcohol on body weight is found only in subjects with a positive family history of obesity (36), a group of subjects who may show, besides others, a low capacity for fat oxidation. In a small interventional study by Crouse and Grundy only in individuals with a heavier body weight status did alcohol lead to body weight gain (30).

In a study by Clevidence et al. (37) a less efficient use of alcohol energy depending on body weight was observed. For the maintenance of body weight with isoenergetic diets with or without alcohol, leaner women (mean BMI 22.6 $\pm$ 0.8 kg/m^2) had to ingest approximately 300 kcal more than the heavier subjects (mean BMI 25.2 $\pm$ 1.0 kg/m^2). These data support the observation that all subjects do not use energy from alcohol with equal efficiency. This modulatory effect of body weight on the effects of alcohol is paralleled by the observation that the risk of alcohol-induced hepatic steatosis is strongly modulated by the absolute body weight (38), i.e., fat intake. This study was done in free-living subjects and it is very difficult to control for other factors as listed in Table 1. It is interesting to note that the subjects ingested about 30 g/day of alcohol, corresponding to 840 kJ/day and during alcohol intake they had to ingest an additional 800 kJ/day for weight maintenance from nonalcoholic energy sources (37). This would support the concept of energy wastage. Studies regarding energy metabolism in

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free-living subjects are subject to many confounders and thus the results have to be interpreted with great caution. Several metabolic studies with long-term measurements of the effect of alcohol on energy metabolism do not support the concept of the proposed energy wastage mechanisms (39–41). As will be discussed later, it is conceivable that energy wastage is observed in subjects with an induction of the MEOS.

Cordain et al. (29) reported that the addition of two glasses of wine per day for 6 weeks does not affect the body weight or any parameter of energy metabolism in young healthy men. In view of the known metabolic effects of alcohol on fat oxidation rates (39), it is surprising that the addition of about 35 g of alcohol (i.e., two glasses of wine) to the usual daily energy intake is free of any effect on body weight. Although this study is well designed and controlled for, it is very difficult to control for some variables of daily life such as the NEAT (42). Nevertheless the results of the study by Cordain et al. are certainly valid for the studied population; however, these results may not be generalized (43). All subjects in this study were healthy and had a normal body weight (43) and the participation was volunteered. As already mentioned, body weight status may be an important determinant of the alcohol effects. Furthermore, the 6-week duration of the intervention may be too short. Nevertheless it seems that alcohol calories did not count in this study population; however, as will be discussed later, this amount of alcohol may count in another population, especially when the alcohol is ingested for periods longer than 6 weeks in combination with a high-fat diet.

Excessive alcohol consumers will lose body weight due to primary as well secondary malnutrition (Fig. 2). Upon cessation of excessive alcohol intake and improvement of the mechanisms leading to secondary malnutrition as well as the increased food intake, body weight will increase during a detoxification program. This weight gain during detoxification is often used as an argument that alcohol energy does not count. This is a wrong conclusion and misinterpretation of a basic physiological and metabolic phenomenon: the heavy alcohol consumer has a primary form of malnutrition (i.e., low intake of food) and in the absence of the toxic effects of alcohol, food intake increases and body weight gain occurs.

Although interventional studies are not conclusive, they show that some individuals may be prone to weight gain upon alcohol intake. The metabolic effects of alcohol could explain some of the observed alcohol effects in epidemiological and interventional studies.

IV. METABOLIC EFFECTS OF ALCOHOL

Alcohol has three major characteristics: it is a source of energy, it is a psychoactive drug, and it is a toxin. Depending on the dose and the frequency of consumption, one or the other of these characteristics may prevail. In the present context

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the importance of alcohol as a source of energy as well as a toxin is of major interest.

Compared to other energy sources (i.e., fat and carbohydrates) the energy content of alcohol is rather high (7.1 kcal/g or 29 kJ/g). The energy derived from alcohol has one very special feature that is very different from the other energy sources: alcohol energy cannot be stored in the body. Owing to the lack of a direct storage possibility, an accumulation of alcohol in the body must be avoided because of the high toxic potential. Accordingly, alcohol has an absolute priority in metabolism and thus has a very high potential to affect many different metabolic pathways including the pathways of energy metabolism.

Discussion of the basic requirements for body weight stability may help to explain the alcohol effects on energy metabolism and body weight regulation. For weight stability two requirements must be met: first, the energy balance and, second, the substrate balance must be maintained (35,44,45). As compared to the first requirement the second requirement is much less well known, although it may be of even greater importance in the long term. The maintenance of substrate balance implies that the oxidation rate of each single substrate (i.e., fat, carbohydrates, and to a smaller degree also protein) has to correspond to the amount that is ingested with food. It is evident that the energy balance becomes positive when the oxidation rate of a substrate is lower than the amount that is ingested. In a metabolically healthy person the substrate balance for carbohydrates and protein is rather easily maintained and any excess of these substrates is associated with an increased oxidation rate of the corresponding substrate thus assuring the maintenance of an equilibrated substrate balance. Fat calories behave differently: during excessive fat intake fat oxidation is not stimulated thus leading to a positive fat balance. The positive fat balance is the most important factor in the development of overweight and obesity. The lipid oxidation rate is mainly influenced by two factors: the absolute fat mass and the level of physical activity. Fat oxidation is stimulated by aerobic exercise and by an increased fat mass (35,44,46). Thus in an individual body weight will increase as a response to a longer period of a positive fat balance until at a higher level of body weight a new steady-state condition of fat oxidation is achieved (44). The higher fat mass at the higher body weight level is associated with a higher fat oxidation and thus weight stability for a given energy (fat and carbohydrate) intake. A positive fat balance is a much easier way for the induction of weight gain than a positive carbohydrate balance; however, the ratio of fat to carbohydrates is equally important. Carbohydrates reduce the oxidation of fat and alcohol shows a similar effect (see below).

V. ALCOHOL AND THE ENERGY BALANCE

The energy balance (44) is defined as follows:

Energy balance = energy intake − energy expenditure

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Alcohol may affect both components of this equation. Owing to its high energy content alcohol enhances the intake of energy. Alcoholic beverages usually do not have a high nutrient density; therefore, alcohol calories are described as ''empty calories'' (47). In agreement with the ingestion of other energy substrates above the energy requirements it is conceivable that the ingestion of alcohol energy above the requirements would lead in the long run to a positive energy balance due to the higher energy intake.

Regulation of the ingestion of food is very complex and still largely unknown (48,49). In sharp contrast to other energy sources, alcohol ingestion is not subject to any specific regulatory mechanisms; accordingly, alcohol energy is also denoted as ''unregulated calories (energy)'' (50,51). In view of today's life-style alcohol contributes considerably to the daily energy intake. Windham et al. estimated that alcohol contributes about 160 kcal/day (corresponding to about 20–25 g alcohol or 1 drink) in subjects of drinking age (52).

The common drinking pattern of the moderate alcohol consumer is addition of alcoholic beverages to the usual food intake (53). Addition of alcohol energy to the usual food leads to the development of a positive energy balance. The substitution of usual energy sources by alcohol is generally seen in heavy alcohol consumers and only rarely seen in a few ''sophisticated'' moderate consumers. This drinking pattern of the heavy alcohol consumers represents the major cause for the primary malnutrition. Owing to these drinking patterns the association between alcohol intake and body weight is biphasic (Fig. 2): at low to moderate levels of consumption alcohol enhances the development of weight gain whereas at higher levels, owing to direct and indirect alcohol effects, the opposite is found. The lower body weight of heavy alcohol consumers is due to primary as well as secondary malnutrition. Another factor confounding the epidemiological relationship between alcohol and body weight is the rather high prevalence of meal skipping with increasing alcohol intake (54,55). The level of alcohol intake at which the frequency of meal skipping occurs is not known and may not only be found in heavy alcohol consumers.

Regulation of appetite and energy intake is of central importance in the regulation of body weight. Any factor interfering with regulation of the perception of hunger, appetite, and intrameal and intermeal satiety may affect food intake in the short and long term and thus body weight status. The different effects of the single energy substrates on appetite regulation are not yet fully elucidated. Alcohol has a high potential to interfere with the very labile regulation of food intake. Evaluation of alcohol's effects on long-term food intake and appetite regulation is very difficult; however, with the help of short-term studies (such as preload studies) important information may be gathered. Several epidemiological studies described a positive relationship between alcohol intake and fat intake. In a cross-sectional epidemiological study we described a positive significant relationship between self-judged fat intake and the level of alcohol intake (56).

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This is in agreement with studies reporting an inverse association between alcohol intake and carbohydrate intake (54,57). Other studies reported no or only a minor effect of alcohol on fat and/or carbohydrate substrate composition or overall energy intake (53). In view of the high energy content of alcohol a small increase in fat intake will lead to a considerable additional intake of energy and, if persistent, will contribute to weight gain. The appetite-enhancing effects of alcohol have been empirically known for many years; however, only a few well-controlled experimental studies addressed this important aspect. The appetite-modulating effects of alcohol have been studied in a recent study by Westerterp-Plantenga and Verwegen (58). In this study the effect of an alcoholic preload (1 MJ) as beer or wine 30 min before lunch on consecutive food intake was evaluated in 52 subjects and compared to the effect of isocaloric preloads of protein, fat, or carbohydrates. The alcoholic preload led to an increase in energy intake in the range of 20% over the nonalcoholic control days. The number of grams of food eaten per minute was also significantly higher on the alcohol days; in addition, the duration of the meals was increased and satiation occurred at a later time (58).

These data support the concept that the dietary fat-alcohol combination favors overfeeding and that this combination changes the perception of hunger, satiety, and/or fullness (50,51,58). In view of the effect of alcohol on food intake and appetite regulation, it is very difficult to control these unfavorable effects in daily life and in an environment with hardly little food scarcity.

Although alcohol may contribute considerably to the overall energy intake, it is still controversial to what extend the alcohol energy is also a usable source of energy, i.e., to what extent the alcohol energy can be used for ATP production. Nearly 100 years ago Atwater (14) suggested that the alcohol calories do represent an energy source equivalent to carbohydrates or fat. The metabolism of alcohol in the alcohol dehydrogenase (ADH) pathway leads to the production of 16 molecules of ATP per mol of alcohol (59). In the latter pathway, which is operating in the moderate consumer, the only energy-using metabolic step is activation of acetate to acetyl-CoA where 2 ATP molecules are used. On the contrary, in the metabolism of alcohol in the microsomal ethanol-oxidizing system (MEOS) less than 10 molecules of ATP per mol of alcohol are produced (59). Theoretically a higher ATP production would be expected, which is in agreement with the concept of ''energy wastage'' formulated by Pirola and Lieber (23,60–62). Oxidation of alcohol in the MEOS system requires $NADPH^+$, whereas oxidation of alcohol in the ADH pathway produces $NADH^+$, which can be used for ATP production. Oxidation of alcohol in the MEOS leads to an increased thermogenesis, which may be caused by an alcohol-induced functional impairment of the mitochondria (23,60–62) or by catecholamine-mediated effects (63). Acetaldehyde may be in part responsible for enhancement of some of the catecholamine effects. In the present context it is conceivable that due to the genetic variability

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of alcohol metabolism and the degradation of acetaldehyde, the heterogeneous response pattern regarding body weight changes upon ingestion of alcohol may be mediated by these phenomena. Others suggested that the reoxidation of acetaldehyde may cause energy wastage (64,65); however, this pathway is in vivo barely functional.

Despite the biochemical and experimental evidence, the concept of ''energy wastage'' is still controversial (see below). It is a fact that depending on the state of MEOS induction a considerable amount of the ''alcohol energy'' may be used up; nevertheless, a rather large fraction of the alcohol energy is available for ATP production. The major determinant of potential energy wastage is the degree of MEOS induction.

Independently from the pathway of alcohol degradation, acetate represents the major metabolite and the carbons of alcohol are shuttled to the peripheral organs in the form of acetate. With the help of stoichiometric calculations the calculated thermic response of the degradation of alcohol to acetate based upon ATP utilization is 12% and 27% of the energy content of the ingested dose of alcohol for the ADH and MEOS pathway, respectively (66). The metabolic fate of acetate is severalfold: first, acetate can be oxidized completely to CO_2 and H_2O or acetate can be used in de novo lipogenesis. Stoichiometry reveals a thermic response of 12% and 23% for the two former metabolic options (66). De novo lipogenesis from alcohol would accordingly be accompanied by an overall thermic response of 35% in the ADH pathway and 50% in the MEOS pathway. As compared to the usual energy sources (fat and carbohydrates), the thermic response of alcohol is rather high and thus from the theoretical point of view, alcohol energy can be regarded as a less usable form of energy. This conclusion is, however, only partially correct, since the metabolism of alcohol leads to other metabolic interactions thus interfering with the energy balance.

Ingestion of alcohol leads to an increase in energy expenditure as a function of the principal metabolic pathway of alcohol degradation. Using 24-hr indirect calorimetry measurements in a calorimetric chamber we evaluated the effect of alcohol on energy metabolism in healthy young nonalcoholic men (mean $\pm$ SD age 24 $\pm$ 2 years, weekly alcohol consumption 48 $\pm$ 44 ml alcohol). The subjects were studied in two experimental sessions of 2 days. The first day of each session was a control day, where normal food was ingested according to the energy requirements of the individuals. The second day of each session corresponded to the alcohol day where 25% of the energy requirements of the study subjects was given as alcohol. In one session the alcohol was added to the usual food intake, and in the other session carbohydrates and fat were isocalorically substituted by alcohol. Accordingly, the subjects received on the day of the alcohol addition 125% of their energy requirements and on the day of alcohol substitution 100%. The daily alcohol intake was 96 $\pm$ 4 g/day and was ingested in three smaller doses over the day together with the main meals (39).

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Independently of whether alcohol was added or substituted, energy expenditure increased by 4 ± 1% and 7 ± 1% ($p > 0.001$ and $p < 0.025$), respectively (39). This increase in energy expenditure corresponds to about 20–25% of the energy content of the ingested alcohol (66). The same study population was evaluated by indirect calorimetry in a ventilated hood system after the ingestion of 31.9 ± 0.6 g alcohol without concomitant food (this dose corresponds to one-third the dose of the chamber study). In this setting energy expenditure increased by 7.4 ± 0.6% over baseline values corresponding to an alcohol-induced thermogenesis of 17.1 ± 2.2% (66). The measured values of alcohol-induced thermogenesis are in good agreement with the stoichiometrically calculated values (see above). Our measured and calculated data have been confirmed by other groups using similar long-term indirect calorimetry measurements (41).

Compared to other nutrients, the thermogenesis of alcohol is comparatively high. The thermic response of a mixed meal is in the range of 12%, carbohydrates around 8%, and fat approximately 3%. Compared to the usual food sources, a larger fraction of the energy content of alcohol is ''wasted''; nevertheless, a considerable fraction of the alcohol energy remains available as a usable source of energy.

Several studies were not able to measure a thermic effect of alcohol (14,24,67,68), and the reported alcohol-induced thermogenesis was very different from the one we measured in our studies (for review see ref. 69). In one study by Perkins et al. (70) ingestion of the same amount of alcohol as in our studies (i.e., 0.5 g/kg body weight) led to a thermogenic response of only 1% of the alcohol dose given. These discrepancies can be explained largely by methodological issues such as administration of a too small dose of alcohol, only short-term and intermittent indirect calorimetry measurements, administration of alcohol together with food, evaluation of heterogeneous populations regarding their usual alcohol intake (i.e., inclusion of heavy alcohol consumers who had an induction of the MEOS). In the study by Perkins et al. (70) a respiratory mask on the subject's face was used for air collection, which is not a very reliable methodology.

Aging is associated with many different functional changes including alterations in alcohol metabolism (71,72). Despite these changes alcohol-induced thermogenesis seems not to be affected by age alone as assessed with indirect calorimetry (73).

From the available evidence it can be said that the thermic effect of alcohol in moderate healthy alcohol consumers is between 15% and 25% of the energy content of the consumed alcohol. There are no reliable data about the thermic effect of food in heavy alcoholics. In view of the present knowledge, the thermic effect of alcohol is much higher (eventually up to 50% or even more) in heavy alcohol consumers. In the latter population group the concept of ''energy wastage'' may be applicable.

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VI. EFFECT OF ALCOHOL ON SUBSTRATE BALANCE

Owing to this absolute preference in the metabolism and the lack of any storage of alcohol in the body, the substrate balance for alcohol is always maintained. The clinical features of alcohol-induced liver steatosis are well known. The pathogenesis of steatosis is multiple and one prominent pathogenetic feature is suppressed lipid oxidation at the level of the liver as well the periphery with an increased flux of lipids to the liver (74). The clinical entity of liver steatosis suggests that a similar phenomenon may occur at the whole-body level.

What is the effect of alcohol on whole-body substrate oxidation rates? We addressed this question in a indirect calorimetry study over several 24-hr periods (39). The study design was outlined earlier. Briefly, 25% of the energy requirements of healthy nonalcoholic men were either added as alcohol to the usual food intake or isocalorically substituted for fat and carbohydrates. The subjects were then monitored for 24 hr in an indirect calorimetry chamber and the substrate oxidation rates were calculated using indirect calorimetry equations. Neither the addition nor the substitution of alcohol affected the carbohydrate oxidation rates. Protein oxidation rates were not affected on the day of alcohol addition (compared to the control day without alcohol). On the day of substitution protein oxidation rates were significantly elevated during the night. This is in agreement with the increased protein requirements in alcohol consumers (22).

Independently of whether the alcohol was added or substituted, lipid oxidation was suppressed. Addition of alcohol led to a suppression of lipid oxidation by 49.4 $\pm$ 6.7 g fat (corresponding to 36 $\pm$ 3% of the control day, $p < 0.001$) and 44.1 $\pm$ 9.3 g on the substitution day (corresponding to 31 $\pm$ 7% of the control day, $p < 0.0025$). Suppression of the oxidation rate was seen only during the period of the day when alcohol was metabolized actively, i.e., as long as alcohol was found in the blood. Other studies using a similar design although shorter measurement periods revealed the same effects (41). In the latter study alcohol addition led to a suppression of fat oxidation corresponding to 74 $\pm$ 54% of the energy content of alcohol. The study by Murgatroyd et al. (41) also used whole-body indirect calorimetry; nevertheless, the extremely large standard deviation is surprising. In view of the adequate methodology as well the well-known laboratory in which the study was done, the results must be regarded as accurate. Interpretation of the large variability of the response to alcohol intake would further support the concept of the heterogeneity of the responses upon alcohol ingestion.

Our data, as well as data from most other studies, apply to young healthy nonalcoholic subjects. It is not possible to make a conclusion about the effect of alcohol on thermogenesis and substrate oxidation rates in heavy alcohol consumers. It is well known that liver cirrhosis has a large impact on energy metabolism

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and substrate oxidation rates and energy metabolism (75,76) and more energy (including eventually alcohol energy) may be dissipated.

Suppression of lipid oxidation is due to the increased production of acetate during the metabolism of alcohol and the utilization of acetate in the periphery as an important source of energy (77,78), however at the expense of other energy sources mainly lipids. It has been suggested that the measured suppression of lipid oxidation by ethanol could be due to an increased de novo lipogenesis without a change in fat oxidation thus also leading to a positive fat balance and mimicking a suppression of lipid oxidation (79). Elegant studies by Siler et al. confirmed that a moderate load of alcohol hardly affects de novo lipogenesis (80). In this study stable-isotope mass spectrometric methods in combination with indirect calorimetry were used to study the metabolic fate of a moderate alcohol load (24 g). Using these complex methodologies the researchers showed elegantly that alcohol did not activate the pathways of hepatic de novo lipogenesis, but that the largest fraction of the carbons from alcohol were shuttled to the peripheral tissues with the help of acetate (70–80% of the load). In the peripheral tissues acetate leads to an alteration of the fuel selection with an inhibition of lipolysis. Siler at al. (80) calculated that less than 5% (i.e., about 1 g) of the given moderate alcohol load of 24 g was used for de novo lipogenesis.

Atwater and Benedict concluded in 1902 from their measurements of the effect of alcohol on substrate oxidation rates that the effects of alcohol on fat oxidation ''are hardly large enough to be of consequence.'' This may be correct in the short term or in sporadic alcohol consumers. If somebody consumes alcohol repeatedly over months and years, especially in combination with a high-fat diet, small metabolic changes may become important. Nevertheless it is possible that the alcohol-induced effects on energy expenditure and fat oxidation may be different in chronic moderate alcohol consumers due to alterations in the metabolic pathway of alcohol degradation.

Evidence suggests that alcohol leads to suppression of lipid oxidation by about one-third during the period when alcohol is metabolized. It is conceivable that in moderate nondaily consumers weight gain due to the described metabolic effects will occur. At present the level of daily alcohol intake that leads to an induction of the MEOS is not known. Preliminary data from H. K. Seitz et al. (oral communication, Titisee, Germany, December 1999) suggest that a *daily* intake of 40 g may suffice for certain degree of MEOS induction based upon the chlorzoxazone assays. It is possible that if these observations of MEOS induction at this rather low level of alcohol intake prove to be correct, some of the weight-gain-enhancing effects of alcohol may be compensated for even at moderate—but daily—levels of consumption.

Alcohol influences the basic requirements for body weight stability, i.e., energy balance and substrate balance, and in view of the present evidence, moder-

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ate alcohol consumption has to be regarded as a risk factor for weight gain and in the long term also overweight and obesity.

VII. ALCOHOL AND FAT DISTRIBUTION

During the last 40 years evidence has accumulated that not only absolute body weight and/or fat mass, but also body fat distribution pattern represents an important modifier of disease risk (81–83). With accumulation of fat in the abdominal area, which corresponds to so-called android obesity, the risk for several adverse health outcomes is increased independently from the absolute body weight. Abdominal obesity is associated with a higher overall cardiovascular risk, higher blood pressure, a more unfavorable lipid profile, elevated insulin levels, a more pronounced glucose tolerance, and an higher overall morbidity and mortality (83). Alcohol consumption has been identified as a risk factor for abdominal deposition of fat. As mentioned earlier, alcohol suppresses lipid oxidation and the nonoxidized fat is preferentially deposited in the abdominal area. Enhancement of the abdominal fat deposition has been described in several studies (56,84–88). We found a direct relationship between the absolute amount of alcohol consumed and with the alcohol consumption frequency per week (56). Others reported an inverse (87) or no association (86) with alcohol and the fat distribution pattern. Sakurai et al. reported also a positive relationship between alcohol intake and the fat distribution pattern in a group of Japanese self-defense officials (88). The effect of alcohol on the fat distribution pattern is found also without adjustment for energy intake (88). Furthermore, alcohol's effects on central obesity may depend on the type of alcoholic beverage. This may also be caused by the characteristics of the food that is ingested together with the alcoholic beverage (88) and it is interesting to note that the beverage-specific effect—for instance of shochu in the Japanese study (88)—disappears after adjustment for total alcohol. We have made a similar observation in an ongoing cross-sectional study, where beer alcohol (and not wine alcohol) was associated with an increased abdominal fat mass (Schindler and Suter, unpublished data) and the relationship disappeared with the inclusion of total alcohol intake in the model. This suggests that some concomitant factors [such as certain nutrients (mainly fat intake) or pattern of physical activity (89)] may modulate the effects on fat distribution.

Liver transaminase levels correlate with alcohol consumption and in some studies also with body weight (90,91). Recently waist circumference has been identified as a potential predictor of elevated alanine transaminase in Danes (aged 30–50 years) (92). In the present context the latter constellation may be in part caused by the effect of alcohol. An elevated gamma-glutamyl transferase has been identified as an important indicator of an increased abdominal fat mass by other research groups (93).

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The mechanism by which alcohol enhances abdominal fat deposition is not exactly known; however, alcohol-induced endocrine changes may be of central importance. Increased cortisol secretion has been reported to be associated with an altered fat distribution pattern including an increased abdominal deposition of fat (83). Excessive alcohol consumers may show the clinical features of hypercortisolism characterized by a predominantly truncal obesity and muscle wasting (94). This syndrome is designated pseudo-Cushing's syndrome. The clinical entity of the pseudo-Cushing's syndrome is rarely seen in its full-blown clinical entities; however, the increased abdominal fat deposition with increasing alcohol intake may represent an incomplete phenotypic expression of the same metabolic features. Regular alcohol consumption leads to an increased release of glucocorticoids (95) due to alcohol-induced stimulation of the hypothalamic-pituitary-adrenal axis and enhanced secretion of adrenocorticotropic hormone (ACTH) (96). In addition, release of glucocorticoids from the adrenals is directly stimulated by alcohol as well as acetaldehyde (97).

Regular alcohol consumers do show a higher blood pressure and excessive alcohol intake has been identified as one of the major causes of hypertension (98,99). The pathogenesis of alcohol-associated hypertension is not fully known and central nervous system as well as peripheral effects at the level of the smooth muscle cell have been suggested. Recently we formulated the hypothesis that the alcohol-induced increased abdominal fat mass may be an important pathophysiological entity leading to increased blood pressure in alcohol consumers (100). In a cross-sectional study we observed an increase in systolic and diastolic blood pressure as a function of the weekly alcohol consumption. The increase in blood pressure was paralleled by an increase in the abdominal fat mass as assessed by the waist/hip ratio. In a regression model, alcohol was identified as the fourth most important determinant of blood pressure as well as abdominal fat mass (100). The pathophysiological sequence of the alcohol-induced hypertension and its relationship to the abdominal fat mass are summarized in Figure 3.

Besides alcohol consumption, age, excessive energy intake (especially in the form of fat), smoking, psychological stress including depression, physical inactivity, and eventually also yo-yo dieting may all enhance the abdominal deposition of fat. Some of these risk factors are associated with an increased alcohol intake and most of them are modifiable. In view of the importance of abdominal fat mass as a central disease risk modifier, any factor promoting abdominal deposition of fat should be controlled for.

VIII. CONCLUSION AND SUMMARY

In this chapter evidence supporting the concept of moderate alcohol consumption as a risk factor for obesity has been summarized. As discussed, some of the

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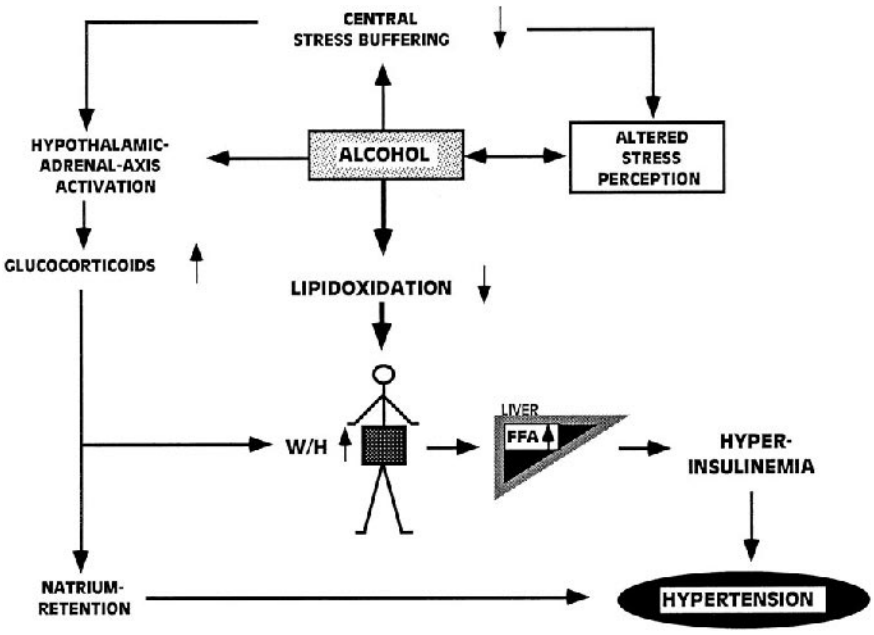

Figure 3 Alcohol and fat distribution pattern (According to Ref. 100). Modulation of the fat distribution pattern by alcohol and the risk of hypertension.

evidence is controversial; however, most of the inconsistencies can be settled and explained. The controversy of this important public health issue is a function of the type of evidence: epidemiological evidence is controversial, whereas experimental metabolic evidence using adequate methodologies is supportive.

In view of present knowledge it would be wrong to ask ''Do alcohol calories count? The more appropriate question would be ''How much do alcohol calories count?'' There is no doubt that alcohol calories count; however, they count very differently from one individual to the other. To explain the effect of alcohol on cardiovascular risk the question ''Is it the drinker or the drink?'' has been addressed recently (101). In the present context the same question should be addressed. The effects of alcohol on body weight depend on the drinker as well the drink: The alcohol calories count more in an overweight person, with a high-fat diet, low levels of physical activity and a positive family history of obesity as well as the alcoholic drink (type, frequency, and amount of alcohol consumed). The alcohol consumer with a specific preference for a certain drink (wine, beer, liquor, or a combination thereof) shows certain—even though not yet known—traits that may modify the alcohol effects. Accordingly we would

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say that it is *the drink AND the drinker* who determine how much the alcohol calories count. What is moderate for one person may be too much for the other and vice versa, and it seems that the drink as well as the drinker modulates the metabolic effects.

In view of present evidence it can be generalized that in moderate consumers alcohol energy counts considerably as long as no adjustment in substrate intake (especially fat intake) is made. With increasing consumption frequency as well as increasing amounts of alcohol, alcohol calories count less, but they count more with regard to alcohol toxicity such as hepatotoxicity or carcinogenesis (Fig. 2).

Accordingly, no recommendation for a safe consumption level, i.e., a level of intake with no positive body weight effect, can be formulated. It is important to remember that the effect of alcohol on body weight is at least biphasic (see Fig. 2).

From the point of view of body weight regulation a nondaily intake of moderate amounts of alcohol is the safest strategy to follow. Last but not least it should be remembered that body weight control includes adequate physical activity and a prudent diet. If the later two life-style issues are implemented on a daily basis, *one* drink can be enjoyed without a moral dilemma regarding disease risks.

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15

Recent Advances in Fetal Alcohol Syndrome and Alcohol Use in Pregnancy

Ann P. Streissguth
University of Washington School of Medicine, Seattle, Washington

I. INTRODUCTION

Alcohol is a teratogenic drug. Teratogens are drugs or agents that cause birth defects. Intrauterine exposure to teratogens can cause four different types of outcomes: death (including miscarriages, stillborn babies, early mortality), physical malformations (both major and minor), growth deficiency (of prenatal onset), and central nervous system (CNS) effects (ranging from structural brain malformations to functional disturbances (1).

The identification and treatment of the medical problems arising from birth defects (cleft lip and palate for example) are often similar regardless of the cause of the birth defect, and the causes of most birth defects are unknown. However, in the case of alcohol, knowledge that birth defects are caused by prenatal alcohol exposure can certainly lead to effective prevention. In the clinical context, this occurs in terms of the protection of subsequent births; in the public context, it leads to improved public health information and practices. As present fetal-alcohol-affected patients seem to be our ''treatment failures'' in many of our community venues; there is the hope that improved treatment and intervention can result as we arrive at a better understanding of the nature and extent of the brain damage caused by in utero alcohol exposure.

Because alcohol is the most commonly used teratogen in the Western world, large numbers of children are affected by their mothers' alcohol ingestion

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during pregnancy. Prevalence estimates from a population-based study in Seattle, Washington found an incidence of fetal alcohol syndrome (FAS), identified at birth in a cohort of 1975/76 births, to be 3 per 1000 live births. The prevalence of alcohol-related neurodevelopmental disorders (ARND), based on the identification of individual children from research protocols of their neurobehavioral testing during the first 7 years of life, was almost 7 per 1000. Thus, the combined rate of FAS plus ARND was nearly 1 per 100 live births in a primarily middle-class, white, well-educated community (2). Comparable studies have not, to our knowledge, been carried out in Germany, but incidence figures from Roubaix in northern France are comparable for FAS detection in babies (2–4).

Because alcohol is a drug that in large quantities can itself contribute to dysfunctional families, the offspring of alcoholic parents may be raised in compromised environments, removed from their families as a result of neglect and abuse and raised in foster families, or relinquished and raised by adoptive families. A recent study in the Pacific Northwest region of the United States found that 80% of the children diagnosed with FAS or fetal alcohol effects (FAE) were not raised by their biological families (5,6). The overlay of unusual environmental backgrounds in such a large number of children with birth defects and the relatively ''hidden'' nature of their organic brain damage have probably contributed historically to the late date at which alcohol was seriously suspected of being teratogenic and contributed clinically to the failure of communities to identify individuals who are prenatally affected by alcohol.

In 1968 in a French-language paper, Lemoine and colleagues described a large group of children with similar characteristics who were born to alcoholic mothers in Western France (7). In 1957, a French doctoral thesis had described similar anomalies and developmental problems in young children with alcoholic mothers (8). The actual naming of FAS as a clinical entity and its putative relationship to maternal alcohol use occurred independently in 1973 by Jones and Smith in Seattle (9). These authors had previously identified a small group of unrelated children of alcoholic mothers, who had a constellation of similar features including: growth deficiency, certain physical anomalies including a characteristic pattern of minor facial anomalies (see Fig. 1), and some evidence of central nervous system (CNS) dysfunction, ranging from microcephaly through fine and gross motor problems, seizures, or mental retardation (10). It is now well established that prenatal alcohol exposure causes a whole spectrum of disorders.

After the identification of FAS, reports of clinical identification quickly appeared in Germany (11), France (12), Hungary (13), Sweden (14), and other countries. Clarren and Smith reviewed 250 clinical identifications in 1978 (15). Among the European countries, the largest numbers of children (as described in the scientific literature) appear to have been identified in Germany (16,17) and many of these have been followed up over time (18–21). Lemoine and Lemoine

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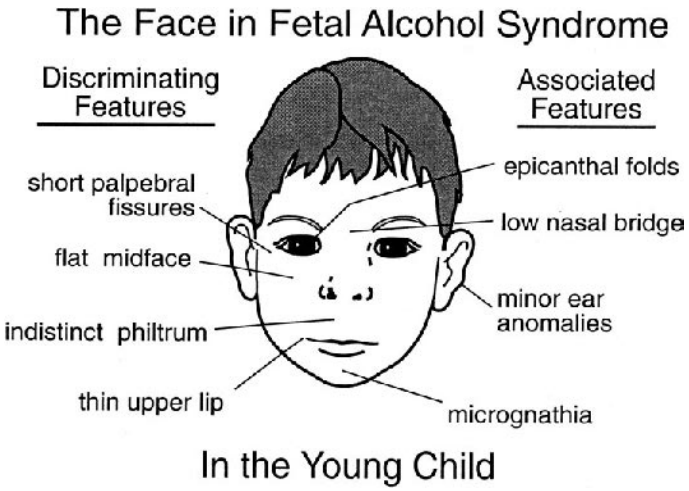

Figure 1 Diagram of FAS facial characteristics in the young child. (From ref. 95, a slide teaching unit on Alcohol and Pregnancy; reprinted by permission.)

in France (22) have reported a seminal 30-year follow-up. Books on children with FAS have appeared in Hungarian (23), Spanish (24), German (25), French (26), and English (27–35), to name a few.

Majewski and colleagues (11) and Vitez and colleagues (36) have described scoring systems for FAS identification based primarily on a variety of physical features. Astley and Clarren (37) have described a scoring system based on facial photographs and another (38) based on facial features, growth deficiency, and presence of CNS dysfunction. Dehaene and colleagues (4) and others have described systems based on overall severity of physical symptoms. The Institute of Medicine (IOM) (32) has suggested a system involving categories of physical and neurobehavioral function that encompass both clinical and research knowledge. All systems require the knowledge that the mother had significant alcohol problems at the time of pregnancy. At this time, the face of FAS continues to be the undisputed hallmark for identification of affected children.

Alcohol was clearly identified as a teratogen by the late 1970s through carefully controlled studies on laboratory animals (39). These animal studies continued in the 1980s, revealing a broad range of types and diversity of brain damage resulting from prenatal alcohol exposure (40,41) and also a broad range of behavioral disorders caused by CNS dysfunction, resulting from prenatal alcohol exposure (42,43). Table 1 shows some of the types of neurobehavioral outcomes reported in studies of laboratory animals and children who were prenatally exposed to alcohol.

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Table 1 Comparable Behavioral Effects Following Prenatal Alcohol Exposure in Humans and Animals

Humans	Animals
Hyperactivity, reactivity	Increased activity exploration and reactivity
Attention deficits, distractibility	Decreased attention
Lack of inhibition	Inhibition deficits
Mental retardation, learning difficulties	Impaired associative learning
Reduced habituation	Impaired habituation
Perseveration	Perseveration
Feeding difficulties	Feeding difficulties
Gait abnormalities	Altered gait
Poor fine and gross motor skills	Poor coordination
Developmental delay (motor, social, language)	Developmental delay
Hearing abnormalities	Altered auditory evoked potentials
Poor state regulation	Poor state regulation

Source: Reprinted by permission of the publisher from ref. 43.

As more children of alcoholic mothers were examined, it became clear that the outcomes among their children were variable: some were much more severely affected than others, and some had the full features of FAS while others had only one or two features. Over the years, the term ''fetal alcohol effects'' has come to be used clinically for children with some but not all of the features of FAS, but born to mothers who abused alcohol during their gestation. This is the context in which the term FAE is used in this chapter. All children identified as having FAE in our work from Seattle were examined by dysmorphologists trained by David W. Smith (44), were found to have some but not all of the features of FAS, and were known to be born to mothers who abused alcohol during pregnancy. They were ascertained in the same manner as those diagnosed with FAS. Recently the IOM (32) suggested that the terms partial FAS, ARND, and alcohol-related birth defects (ARBD) be used to characterize children who were significantly exposed to alcohol in utero and did not have the full FAS. The latter two terms were suggested to apply to those effects (such as those in Table 1) that had been shown in studies of animals and children to be caused by prenatal alcohol exposure (32).

Except for the major birth defects that can be produced by prenatal alcohol exposure (such as congenital heart defects, spina bifida, cleft lip and palate, and so forth) it is now generally recognized that it is the CNS effects of alcohol as a teratogen that are the most debilitating and long lasting across the life span. While the research on laboratory animals has confirmed that prenatal alcohol

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exposure can indeed cause such disorders, it is often difficult to separate out these CNS manifestations in children, especially considering the atypical environmental conditions under which many of the children of alcoholic mothers are raised. Therefore, epidemiological prospective studies are especially important in understanding the CNS deficits caused by prenatal alcohol use in humans, and in separating out the potentially confounding related conditions that could also produce poor developmental outcome. The latter include other substances associated with alcohol use (such as tobacco and drugs of abuse), as well as poor diet, social upheaval, and lower socioeconomic conditions that could also adversely affect the developing child.

Finally, the variability of alcohol use by pregnant women is, of course, enormous. Both dose and timing of exposure are important in the impact of a given teratogen on offspring outcome. For prescription drugs and even smoking, dose is variable across the time of day, but tends to be similar from one day to another. For individual drinkers, however, alcohol dose may not be on a regular daily or even weekly schedule, thus complicating the assessment of exposure within the pregnancy context. The care with which the maternal history is obtained and documented in the medical history is an important aspect of successful identification (34).

In more recent years, the concept of the ''binge'' dose has been studied more carefully. This applies to the massing of drinking rather than the frequency of drinking. Four or five drinks at a sitting is often used as a criterion for ''binge'' drinking, with the understanding that for women, the limit may be lower. In our epidemiological research (45,46) and in the animal literature as well (41), the binge dose is more strongly associated with adverse offspring outcomes than the same amount of alcohol consumed on a regular daily schedule. Thus, seven drinks on Saturday night is a riskier pattern of drinking than one drink a day for 7 days. However, as the Surgeon General of the United States warned in 1981, it is recommended that pregnant women abstain from the use of alcoholic beverages during pregnancy and when they are planning to become pregnant (47). The latter warning is related to the knowledge that an embryo or fetus can sustain damage from alcohol exposure even before the mother recognizes that she is pregnant.

Because animal research has shown that of all the impacts of prenatal alcohol exposure, it is the brain that is the most vulnerable, it is therefore the neurobehavioral manifestations that are the most sensitive outcomes for measuring low-dose or moderate-dose effects. The animal research has led the way in this work. It is now recognized that the characteristic face of FAS (which has been the focus of the clinical identification of affected children) is produced only during a very small window of time in early embryonic development: day 7 in the FAS mouse model (48), day 19–20 in nonprimates in a macaque model (49).

Most recently, a collaborative study from researchers in Berlin, Tokyo, and St. Louis has shown extensive apoptosis (cell death) at 30 times the normal rate

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in nerve cells in brain, from a single high-dose exposure in rats during the period of synaptogenesis (50). In this study, alcohol's action was through the blockade of glutamate transmission and excessive stimulation of GABA transmission. The senior investigator, John Olney, speculated this could occur in humans from one round of heavy drinking during the third trimester of pregnancy.

Mechanisms for ethanol-induced teratogenesis include excessive cell death, reduced cell proliferation, migrational errors in brain development, inhibition of nerve growth factor, and neurotransmitter disruption, among others (40,41). There is no doubt that alcohol is teratogenic (51,52) and that CNS damage can be produced at lower levels of exposure and more variable periods of exposure than the more obvious physical birth defects.

The remainder of this chapter reviews the primary findings from our Seattle longitudinal prospective study on alcohol and pregnancy and how these compare to other studies. It also reviews our research on ''secondary disabilities'' in patients identified with FAS and FAE, the risk and protective factors associated with these secondary disabilities, and the cross-cultural implications of these findings. Finally, our work on preventing FAS/FAE through our targeted intervention program for high-risk mothers who are abusing alcohol and drugs during pregnancy is reviewed.

II. THE SEATTLE LONGITUDINAL PROSPECTIVE ON ALCOHOL AND PREGNANCY, 1974–PRESENT

This population-based study ongoing since 1974 demonstrates broad CNS effects of prenatal alcohol in a primarily low-risk population conceived before general awareness of alcohol's adverse effects on pregnancy outcome and the later lives of those who are exposed.

Two hospitals were selected for study with demographic characteristics representative of the Seattle area. To evaluate alcohol effects in the absence of competing risks, only women enrolled in prenatal care by the fifth month of pregnancy were eligible. The screening interviews from 1529 consecutive consenting women revealed a fairly low-risk life-style in relation to a variety of factors that could relate to offspring health, including diet, drugs, medications, caffeine, alcohol, smoking, family history, and environment. The follow-up birth cohort of approximately 500 infants represented an oversampling of the heavier drinkers and smokers from the original 1529, along with others representing a variety of drinking patterns including abstinence. In this sample, 18% used marijuana, 2% used other street drugs, 12% had not graduated from high school, and 8% were receiving welfare support.

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The primary independent variable, alcohol, was assessed via a quantity-frequency-variability interview with additional questions about higher levels of drinking, intoxications, and problems with drinking. Drinking during two time periods was assessed: during pregnancy (D) and prior to pregnancy or pregnancy recognition (P) [see Streissguth et al. (45,46) for details]. Although 86% of the mothers reported drinking during one or another of these time periods, only 1% reported any problems with alcohol. The majority of mothers were white, married, middle class, and well educated, although a broad range of socioeconomic and racial groups was represented. Heavier-drinking mothers did not differ from the rest of the mothers in terms of nutrition, maternal weight gain, prenatal care, or other pregnancy risk factors such as diabetes, renal disease, thyroid abnormality, or rubella.

Children in the follow-up birth cohort were examined on days 1 and 2 of life, at 8 and 18 months, and at 4, 7, 14, and 21 years (see Fig. 2). Parents were interviewed at each examination; teacher evaluations were obtained at 8 and 11 years. All examinations were conducted blind, without the examiner knowing the subjects' exposure history, living conditions, or previous performance on tests. Follow-up has been excellent, with at least 82% of the original follow-up birth cohort at each assessment. There has been no differential loss of heavily exposed subjects.

Data analyses from the first 4 years of life involved multiple regression analyses of single outcomes against single alcohol predictor variables. Analyses from 7 years onward have incorporated partial least squares (PLS), a method of data analysis that permits simultaneous assessment of the relationship among multiple alcohol predictor scores and multiple outcome scores. PLS is better suited than multiple regression or other alternatives to the complex multifactorial data generated in human behavioral teratology studies such as ours (53). PLS analyses yield latent variables (LVs) for both dose (alcohol LVs) and response (outcome LVs) that demonstrate the salience of prenatal alcohol scores for the outcomes under consideration (45,46,54–56). The alcohol LV, which is computed as a linear combination of all the prenatal dose measures, is very stable over the whole range of outcome ages examined in the present study.

Data on possible confounds were obtained prospectively, prenatally, and at each succeeding examination. More than 150 in number, these variables include maternal nutrition and use of all drugs and medications during pregnancy, sociodemographic and education characteristics of the family, mother/child interactions, major life stresses in the household, childhood accidents, hospitalizations, and illnesses, education experiences of the child, family history of alcoholism, and many others (45,46,57).

All findings reported here have been evaluated in terms of potential confounds. Correlations between the many covariates in the database and the outcome LVs were examined, and then covariates associated with both the alcohol

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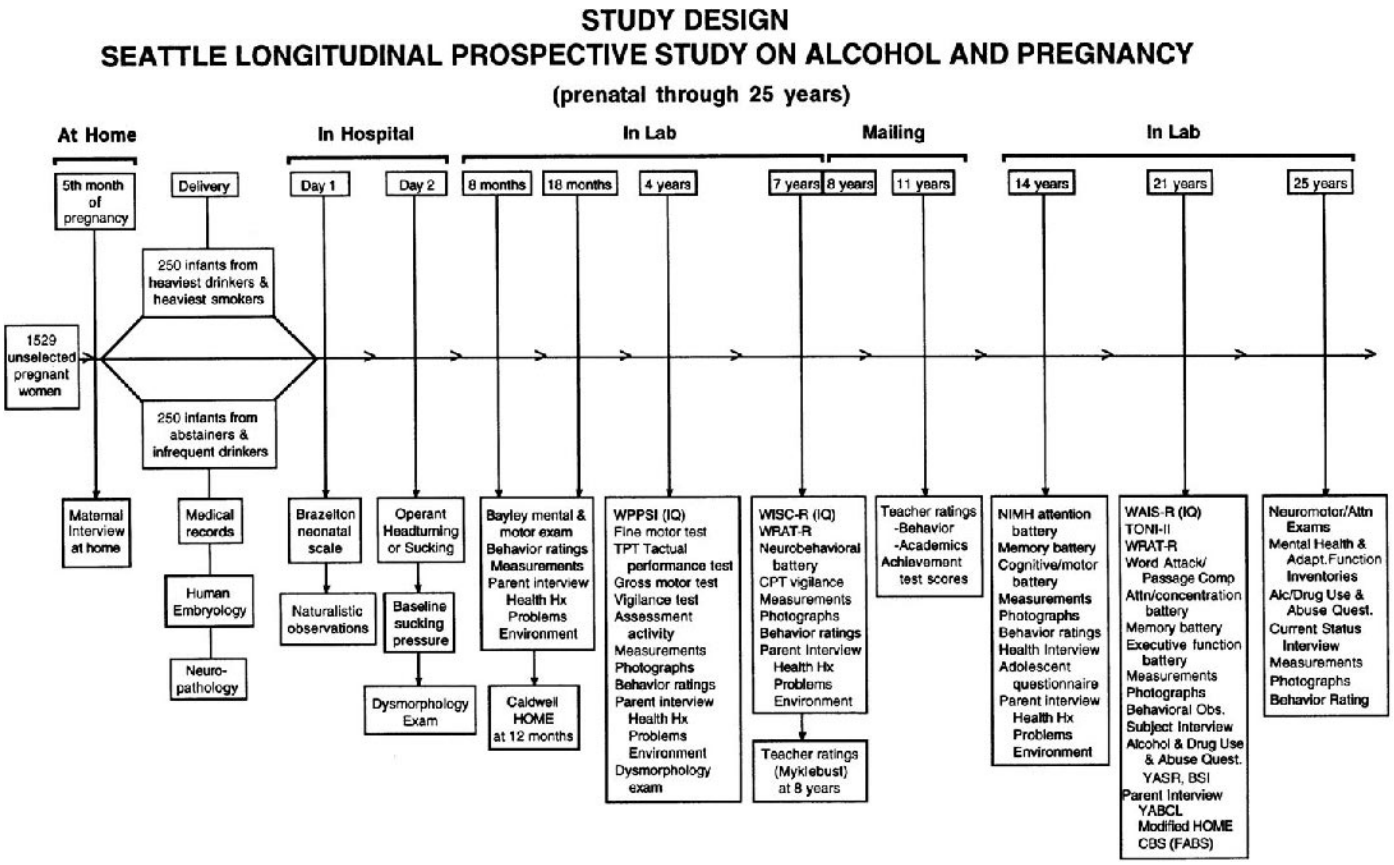

Figure 2 Diagram of the Seattle Longitudinal Study Prospective on Alcohol and Pregnancy showing the age of the cohort at each wave of data collection and the outcomes assessed in blind examinations at the University of Washington School of Medicine.

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LV and outcome LVs were examined in regression analyses to see the extent to which they alter the estimated effects of alcohol dose. Scatterplots and partial residual plots are routinely examined.

Prenatal alcohol exposure, after covariate control, was related to infant outcomes on day 1 of life. These include poor habituation to redundant stimuli and poor response modulation measured on the Brazelton Scale (58); other CNS effects measured with naturalistic observations including increased head turning to the left, tremulousness, hand-to-face movements, time with eyes open, and decreased bodily activity (59); and both hyperresponsive reflexes (incurvation, passive arms reflex) and weak or delayed reflexes (stepping reflex, Moro) (45). On day 2 of life, prenatal alcohol was significantly related to longer latency to suck and lower sucking on a pressure-transducer measure of nonnutritive sucking (60). At 8 months (but not at 18 months) prenatal alcohol was related to subtle decreases in mental and motor development on the Bayley Scales and to increased feeding problems (45,61). Prenatal alcohol was also related to IQ decrements on the WPPSI at 4 years (62) and on the WISC-R (particularly arithmetic and digit span subtests) at 7 years (45,55,63). At 4 years, prenatal alcohol was also related to more ''time in error'' (suggesting slower central processing time), poorer fine motor performance on the Wisconsin Motor Steadiness Battery, and poorer gross motor performance (especially balance) (64).

Attentional deficits, assessed by vigilance tests, were associated with prenatal alcohol exposure at 4, 7, and 14 years. Errors of omission (failing to respond to the target stimulus), errors of commission (responding to the wrong stimulus), and high variability of reaction time were the strongest prenatal-alcohol-related attentional deficits (55,65). Many other neurobehavioral, phonological, and memory tests were also related to prenatal alcohol at 7 years (45,56) and at 14 years (46,66). The 21-year data are still being analyzed.

Academic problems were associated with prenatal alcohol from the first grade on, particularly problems with arithmetic (45,55,66). By the end of the second grade, prenatal alcohol was associated with higher frequency of participation in special programs and classes in school, and with teacher ratings of poor organization, poor attention, poor grammar and word recall, and less tactfulness (63,67). By 11 years of age, prenatal alcohol was associated with lower functioning on standardized school-administered tests of arithmetic and overall achievement and with teacher ratings of distractibility, poor persistence, and restlessness as well as with problems with information processing and reasoning skills (68). By age 14, poor academic performance was reported both by the adolescents themselves and by their parents (69).

Prenatal alcohol exposure was related to examiners' ratings of poor goal directedness, short attention spans, frequent verbal interruptions, excessive talking, general hypertonia, distractibility, poor organization, and a rigid, inflexible approach to problem solving at 7 years (45,56). At 14 years prenatal alcohol was

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related to examiner ratings of high impulsivity and poor organization under stress (69). A surprising new finding from the 14-year examination is that prenatal alcohol exposure is a significant predictor of adolescent alcohol problems and use, a stronger predictor, in fact, than family history of alcohol abuse (57).

The neurobehavioral effects of prenatal alcohol summarized here have been measured in this study from the first day of life through 14 years. New effects were detected at each new age of assessment, as the developing child accrued more testable behaviors (45). The observed alcohol effects on offspring are dose-dependent, generally without a threshold, and are more salient for binge-type maternal alcohol use. Self-reported drinking prior to pregnancy recognition is generally more salient for these outcomes than drinking in midpregnancy, but the two are highly correlated. The results reported are not attributable to such potential confounds as other drug exposures, smoking, or social/demographic factors (45,46). They are also not mediated by low birth weight in this generally low-risk group. In fact, prenatal alcohol effects on height and weight in this group are undetectable after 8 months of age (70). For details of specific analyses, see the original scientific papers from which this overview derives. Streissguth et al. (45) lists all publications prior to 1993. A full listing of published papers from this study is available from the author.

This study, and other prospective studies of prenatal alcohol exposure carried out with other populations and in other countries, demonstrates the short and long-term impacts of prenatal alcohol exposure, particularly on CNS functioning. See, for example, Aronson et al. (71), Coles et al. (72), Day et al. (73), Halmesmäki (74), Jacobson et al. (75,76), Larroque et al. (77,78). These research findings, revealing the breadth of neurobehavioral effects associated with prenatal alcohol even below alcoholic levels, are replicated in almost all instances by experimental animal studies (40,41,43,79). In many animal studies, the behavior can be clearly linked to offspring brain damage.

III. THE STUDY OF PRIMARY AND SECONDARY DISABILITIES IN FAS AND FAE

A second large study, also carried out in Seattle, demonstrates what happens to patients with FAS and FAE in real-life settings. This is a cross-sectional study of patients of various ages who had been diagnosed by dysmorphologists associated with David W. Smith at the University of Washington and who were referred to the Fetal Alcohol Syndrome Follow-up Study between 1973 and 1995 (7,8).

Patients with FAS had (1) a clear history of prenatal alcohol exposure; (2) a characteristic pattern of dysmorphic features including short palpebral fissures,

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midface hypoplasia, smooth and/or long philtrum, and thin upper lip; (3) growth retardation of prenatal onset for height and/or weight; and (4) CNS dysfunction, as manifested by microcephaly, developmental delay, hyperactivity, attention and/or memory deficits, learning difficulties, intellectual deficits, motor problems, neurological signs, and/or seizures. Patients with FAE had a clear history of prenatal alcohol exposure and CNS dysfunction, but did not manifest all of the physical features of FAS.

The goals of this study were twofold: (1) to document the occurrence and range of primary and secondary disabilities that are associated with FAS and FAE; (2) to determine the risk and protective factors associated with these secondary disabilities. A full description of the study is available (5,6).

The 415 patients in the Secondary Disabilities Study were those who were 6 years or older at the time of the study, and for whom a caretaker, or other person familiar with them, was available for a life history interview (LHI). The patients in the secondary disabilities study were 60% white, 25% Native American, 7% black, 6% Hispanic, and 2% Asian and other; 39% of the subjects were between 6 and 11 years old; 39% were 12–20 years old; and 22% were 21–51 years old.

The 473 patients in the Primary Disabilities Study were those who had previously been examined at our unit on the age-appropriate Wechsler IQ tests (WPPSI; WISC-R; WAIS-R), the Wide Range Achievement Test (WRAT-R), and the Vineland Adaptive Behavior Scale (VABS). They ranged in age from 3 to 51 years.

The findings from the 473 patients in the Primary Disabilities Study are as follows: Those with FAS ($n = 178$) had an average IQ of 79, average reading, spelling, and arithmetic standard scores of 78, 75, and 70 respectively, and an average adaptive behavior standard score of 61. Those with FAE ($n = 295$) had an average IQ of 90, average reading, spelling, and arithmetic standard scores of 84, 81, and 76, respectively, and an average adaptive behavior score of 67. (For IQ scores, achievement scores, and adaptive behavior, a score of 100 is normal and a standard derivation is 15.)

The findings from the 415 patients in the Secondary Disabilities Study are as follows:

1. Mental health problems: Over 90% of the patients in each age group were reported to have had problems and nearly 90% had sought help from mental health providers at some time in their lives.
2. Disrupted school experience: 60% of the adolescents and adults had been suspended or expelled from school, or had dropped out of school.
3. Trouble with the law: 60% of the adolescents and adults had been in trouble with authorities, charged, or convicted of a crime.

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4. Confinement: 50% of the adolescents and adults had been in a mental hospital, in an inpatient alcohol/drug treatment program, or had been incarcerated for a crime.
5. Inappropriate sexual behavior: Nearly 50% of the adolescents and adults had been reported to have had repeated problems with one or more of 10 inappropriate sexual behaviors or had been sentenced to a sexual offenders' treatment program.
6. Alcohol/drug problems: Nearly 30% of the adolescents and adults had, or were described as having or having had, alcohol or drug problems or been in treatment for alcohol or drug problems.
7. Dependent living: About 80% of the sample (age 21 and over) were still living ''dependently'' according to study criteria (5).
8. Problems with employment: About 80% of the adults (age 21 and over) had major problems with obtaining and keeping jobs, according to study criteria (5).

Eight ''universal'' protective factors were identified that significantly reduced the odds of an adolescent or an adult with FAS or FAE having any of the six secondary disabilities under investigation (items 1–6 above). In order of their strength, these protective factors are:

1. Living in a stable and nurturant home for over 72% of life.
2. Being diagnosed with FAS or FAE before the age of 6 years.
3. Never having experienced violence against oneself.
4. Staying in each living situation for an average of more than 2.8 years.
5. Experiencing a good quality home (10 or more of 12 ''good'' qualities) from age 8 to 12 years.
6. Being found eligible for Division of Development Disabilities (DDD) services.
7. Having a diagnosis of FAS (rather than FAE).
8. Having basic needs met for at least 13% of life.

In evaluating the backgrounds of these patients with FAS/FAE through the LHI, some alarming statistics are noted. When these are examined in light of their importance as risk or protective factors, the needed interventions become clear.

Early diagnosis of FAS/FAE: While an early diagnosis is a strong universal protective factor for all secondary disabilities, only 11% of these individuals with FAS/FAE were diagnosed by age 6.

Receiving DDD services: While receiving services from the state's DDD is also a strong universal protective factor against secondary disabilities, only 56% of those that applied were found eligible for services and only a subset of these actually received services. Clinical experience suggests that the services

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provided by DDD (such as a case manager, and help with housing, job training, and employment) are useful and necessary services for patients with FAS/FAE.

Freedom from violence and sexual abuse: Violence against individuals with FAS/FAE occurred at an alarming rate: 72% had experienced physical or sexual abuse or domestic violence. Being a victim of violence is related to a fourfold increase in odds of inappropriate sexual behavior.

Help with parenting, family planning, and family support: Thirty females with FAS/FAE had given birth to a child. Of these, 57% no longer had the child in their care; 40% were drinking during pregnancy. The program listed below provides an effective model not only for preventing FAS births, but for intervening with mothers who themselves are fetal alcohol affected (80–83).

IV. BIRTH TO 3 INTERVENTION PROGRAM FOR HIGH-RISK ALCOHOL AND DRUG-ABUSING MOTHERS

The Birth to 3 Program, begun in 1991, is a unique Seattle-based model of paraprofessional advocacy for high-risk women who abused alcohol and/or drugs during pregnancy. In the original demonstration program, funded by a federal grant, five Birth to 3 advocates worked with 65 women from the child's birth to the age of 3, addressing complex social, behavioral, medical, and financial issues. Results of the 36-month exit interview indicated a statistically significant positive impact among clients compared with similarly recruited controls on all five outcome domains studied: alcohol/drug treatment, abstinence from alcohol/drugs, family planning, child well-being, and connection to services (82,83).

A postprogram follow-up was carried out an average of 2 1/2 years after the advocacy program was over to determine whether the successful outcomes were maintained. Data from these interviews and the original exit interview are compared for 47 clients. The proportion of clients abstinent from illicit drugs and not abusing alcohol for a period of 1 year has continued to increase since completion of the program from 38% to 46%; for 2 years, from 16% to 28%. While regular birth control use dropped slightly (76% to 70%), use of more reliable methods (Norplant, Depo Provera, tubal ligation) has stayed virtually the same (47% to 45%) and subsequent births and pregnancies have decreased (births: 27% to 11%; pregnancies: 51% to 36%). Data acquisition is continuing but results to date suggest that the advocacy program set in motion some processes through which ongoing improvement is possible (84).

In 1996, at the end of the federal demonstration project, the program was funded by a local philanthropist to serve a new cohort of 60 women, and the Washington state governor funded a replication program in another city. Since 1997 both sites have been funded by the Washington state legislature. Data from the new cohorts of mothers reveal similar findings to the first study. Additions

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to the program include a special focus on the needs of Native American mothers and children, identification of and special programming for mothers who are themselves fetal alcohol affected (81–83), and the facilitation of diagnostic evaluations for their children. The program is now called the Parent-Child Assistance Program (P-CAP). An important ongoing focus of the program has been on facilitating the modification of community services to be more responsive to the needs of these high-risk mothers and their families to prevent future children being born with fetal alcohol and drug effects. This model program, now replicated in several additional states and provinces of the United States and Canada, shows that it is possible to effectively intervene with high-risk mothers to prevent the birth of additional fetal-alcohol-affected infants and to help mothers stay in recovery.

V. OVERVIEW

Our Seattle prospective study (45,85) shows that there is a pattern of neurobehavioral problems that has been linked to prenatal alcohol exposure in a dose-dependent fashion. These can be measured throughout the life of the child from day 1 through 14 years (and studies into adulthood are continuing). The documented neurobehavioral problems include attentional, memory, fine and gross motor problems, language problems, and academic problems (especially in arithmetic), and problems with speed of information processing. Furthermore these occur against a background of behavioral problems that include impulsivity, poor comprehension, poor frustration tolerance, a rigid problem-solving approach that makes new situations difficult, and unpredictable behavior.

The longitudinal prospective studies, employing careful covariate control and adjustment for competing risks, buttressed with a vast experimental literature, indicate that prenatal alcohol causes neurobehavioral problems that cannot be explained by environmental factors. Alcohol-affected individuals, both with and without the face of FAS, experience the neurobehavioral consequences of prenatal alcohol exposure. The root cause of the atypical behavior is prenatal brain damage from alcohol (86–88).

Our Seattle secondary disabilities study reveals the startling environmental situations in which many children with a diagnosis of FAS and FAE are born and raised (at least for part of their lives). There is no doubt that physical and sexual abuse and frequently changing households represent poor environments for children, and that such experiences are related to increased levels of ''secondary disabilities'' among patients with FAS and FAE. But failing to qualify for services, failing to be detected as needing services, and failing to receive needed treatments and interventions for their birth defects is also a disadvantage. Our secondary disabilities study has identified eight protective factors associated with

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better socioemotional development for patients with FAS and FAE (5). How to implement these conditions is the difficult question, as they often have variable causes. While some represent the direct consequences of the mother's alcohol abuse, others arise from public policy regarding service delivery systems, which inadvertently screen out alcohol-affected children who fail to meet entry requirements to intervention programs they may need.

The recent report of young adults with FAS/FAE studied in Münster (21) finds a much lower rate of socio/emotional problems than our patients in Seattle, despite many apparently similar CNS and learning problems. The degree to which this represents cultural differences in the strength of the social service systems in the two countries or differences in the follow-up procedures between the two studies remains to be investigated. However, a report developed by a large group of Seattle-area parents of patients with FAS/FAE reveals their strong concern about the unavailability of appropriate services for their children, particularly as they reach later adolescence and adulthood (89).

Prenatal alcohol exposure causes a wide range of primary neurobehavioral disabilities that are associated with the primary brain damage occurring in utero. These are measurable as early as the first day of life and on into adulthood. They are life span problems—so interventions must be addressed across the life span. There is no indication yet that early intervention will preclude later interventions, but early intervention could improve general adaptation, promote mental health, and prevent secondary disabilities. Much further research is necessary.

The neurobehavioral effects and maladaptive behaviors associated with prenatal alcohol exposure are not restricted to those individuals with the classic face of FAS—nor to those who meet preexisting criteria for service delivery, such as ''mental retardation,'' ''learning disabilities,'' ''childhood autism,'' ''seizure disorders,'' ''cerebral palsy,'' or even ''low birth weight'' (90,91). Because the risk of secondary disabilities is so high among individuals with FAS/FAE, special interventions will be necessary for schools, social service agencies, mental health and alcohol and drug treatment programs, and juvenile and adult corrections. More research is needed focusing not only on the special needs of children and adults with FAS/FAE, but also on the selection criteria for service delivery. Neurobehavioral criteria, like those in the Fetal Alcohol Behavior Scale (92), may ultimately be more useful than reliance on face or growth dimensions, but further research is needed. Ultimately, it may be brain/behavior studies (such as these presently underway on our unit comprising both MRI brain scans and targeted neuropsychological studies of patients with FAS/FAE) (93,94) that may most successfully identify individual sustaining prenatal alcohol damage.

In conclusion, the scientific literature reviewed here clearly reveals the need for effective prevention efforts. Government warnings about not drinking during pregnancy help women and families make wise choice about their own offspring.

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Programs for high-risk mothers are able to stop alcohol abuse during pregnancy and help another group of women. Together, their strategies safeguard a larger proportion of offspring.

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Quality of Life in Alcohol-Misusing Subjects

Timothy John Peters
King's College, University of London, London, England

John Howard Foster
Middlesex University, Enfield, Middlesex, England

I. INTRODUCTION

Although alcohol misuse clearly, dramatically, and devastatingly interferes with the quality of life (QoL) of the affected individuals, their family, and social networks including workplace colleagues, this topic has been little studied. A recent review by our group revealed only 24 relevant articles indexed under ''alcoholism and quality of life.'' We found that the QoL of alcohol-dependent subjects was very poor compared to general populations and that the factors that contribute to this were psychiatric co-morbidity, the social environment, perceived pain, and disturbed sleep (1). This chapter will briefly describe approaches to the measurement of QoL, including some of the instruments used, some applications to patient assessment and outcome, and new clinical and research questions that have arisen as a result of QoL studies (Table 1).

II. ASSESSMENT OF QoL

Assessment of QoL is usually subdivided into assessment domains. Health may be subdivided into physical, mental, and social domains (2) all of which are of importance in alcohol misusers. The physical domain is subdivided into symptomatology, e.g., pain, nausea, disturbed sleep, and functionality, e.g., ability to

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Table 1 Quality of Life Domains: Two Classifications of Quality of Life in Current Use

A. Physical	
	Psychological
	Social functioning
B. Mental	
	Objective functioning
	Subjective well-being
	Physical
	Objective functioning
	Subjective well-being

Source: Refs. 2, 3.

climb stairs (3). The mental domain similarly subdivides into depression, anxiety, and cognitive functioning. The social domain includes social interactions with family, friends, and work colleagues. Obviously there is some overlap between these categories but the relationship between alcohol dependency and general well-being has components that are implicated in all three domains. It is clear that alcoholics will show impairment in all these areas and probably others as well. However, the area of impairment will vary greatly between patients, at different phases of the natural history of their disease, and, in particular, during relapse and remission.

Assessment of QoL employs a variety of methods ranging from the most objective quantitative instruments administered by health care attendants to a subjective qualitative assessment by the patients themselves. A combination of approaches is clearly relevant in alcoholism: Our own approach has been predominantly quantitative.

In the measurement of QoL one can use either generic or disease-specific instruments. Unfortunately, no fully validated QoL instruments are available for specific use with alcoholics at present. Powell and Crome (4) have found that the range of QoL dimensions mentioned by alcohol-dependent subjects is much greater than that of patients with hip replacements or controls. The research resources required to develop an alcohol-specific questionnaire are expensive and it is doubtful whether such a measure would cover all aspects of QoL in alcohol-dependent subjects. Thus our approach has been to use generic instruments supplementing these with objective measures of alcohol-related symptoms or complications or areas of special interest. The advantage of using a generic QoL instrument is that the QoL of alcoholics can be directly compared to a range of

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other disorders and the instruments are usually fully validated. Table 2 lists some of the generic, condition-specific, and symptom-specific instruments used in QoL studies. Several of these have been applied to patients with alcohol misuse and the results are discussed here.

Table 3 summarizes the results of applying generic, cancer-specific, and depression-specific questionnaires to recently detoxified alcoholics. Scores are abnormal in 80–95% of subjects and levels in alcoholics differ by 2–4-fold from normative values indicating a significantly poorer QoL in these subjects (5). The data with the Rotterdam Symptom Check List (RSCL) are of particular interest. This ''disease specific'' questionnaire was selected because, although this instrument was developed for use with patients with malignant disease, there are striking similarities between the symptomatology of patients with chronic alcohol misuse and extensive malignant disease, though no statistical control was made for affective status. These include lack of appetite, irritability, tiredness, worrying, sore muscles, depressed mood, lack of energy, low back pain, nervousness,

Table 2 Measures Employed to Assess QoL

Generic instruments	
General Health Questionnaire (GHQ12)	
MOS-SF36	
Sickness Impact Profile	
Nottingham Health Profile	
Life Situation Survey	
Euro-QoL (EQ-5D)	
Condition-specific instruments	
Karnovsky Performance Index	
Barthel Index	
SmithKline Beecham QoL Scale	
Rotterdam Symptom Check List	
Symptom-specific instruments	
Sleep	PSQI
Depression	BDI, HADS
Anxiety	HADS
Alcohol dependence	SADQ
Alcohol problems	APQ

Abbreviations: 36 Item Medical Outcome Study Short-Form Health Survey (MOS-SF36), Pittsburg Sleep Quality Index (PSQI), Beck Depression Inventory (BDI), Hospital Anxiety and Depression Scale (HADS), Severity of Alcohol Dependency Questionnaire (SADQ), Alcohol Problem Questionnaire (APQ).

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Table 3 Quality of Life Measures in Dependent Alcoholics

Instrument	Alcoholics (% abnormal)	Normative values (mean/reference range)
LSS	75.5 (80)	>90
GHQ12	6.8 (80)	2.5
NHP	49.7 (82)	47
RSCL (physical) symptoms	39.4 (89)	9.9
RSCL (psychological) symptoms	54.3 (93)	17.0
BDI	19.4 (80)	<9

Abbreviations: Life Situation Survey (LSS), Nottingham Health Profile (NHP), General Health Questionnaire (GHQ12), Rotterdam Symptom Check List (RSCL), Beck Depression Inventory (BDI). For further details see ref. 5.

nausea, desperate feelings about the future, difficulties in sleeping, vomiting, dizziness, decreased sexual interest, itching, anxiety, abdominal pain, diarrhea, heartburn, tingling in hands or feet, and deafness (6). Our data (Table 4) clearly show the severely impaired QoL scores in recently detoxified chronic alcoholics. These scores are significantly worse than those in patients with extensive malignant disease (7). The QoL scores were worse in women than in men, but in both groups improved significantly with continued abstinence.

Further evidence of improvement with continued (3 months) remission and deterioration with relapse is shown in Table 5. In this study the Chubon Life Situation Survey questionnaire was used (8). This instrument uses 20 statements against which the respondent rates his QoL on a six-point (agree very strongly

Table 4 Quality of Life Scores Using the Rotterdam Symptom Checklist

	Domain			
	Psychological (mean SD)		Physical (mean SD)	
General population (201)	17.0	18.1	9.9	18.1
Alcohol-dependent: baseline (60)	54.3	24.8	39.6	21.4
Alcohol-dependent: 3-month remission (22)	34.1	22.2	12.9	12.8
Lung cancer (127)	32.9	26.3	27.5	13.9
Bladder cancer (157)	21.5	21.1	15.9	11.6
Head and neck cancer (274)	25.8	20.2	17.5	11.9

For further details see ref. 7. Number of patients shown in parentheses.

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Table 5 Quality of Life in Dependent Alcoholics, Life Situation Survey Scores in Remission and Relapsing Patients

	Remission (29)		Relapse (50)		
(p)	Baseline	Follow-up	Baseline	Follow-up	Stats
Security	4.0 ± 1.8	4.9 ± 1.7	4.4 ± 1.8	3.5 ± 1.9	<0.01
Self-esteem	3.0 ± 1.5	4.8 ± 1.1	3.1 ± 1.7	2.5 ± 1.4	<0.001
Affection	4.1 ± 1.7	4.3 ± 1.5	3.5 ± 1.6	3.5 ± 1.7	>0.5
Outlook	4.6 ± 1.3	5.2 ± 1.1	4.2 ± 1.8	3.7 ± 1.0	<0.01
Mood/affect	3.8 ± 1.3	4.3 ± 1.4	3.9 ± 1.6	2.8 ± 1.4	<0.001
Sleep	3.1 ± 1.7	4.8 ± 1.6	3.0 ± 1.6	2.9 ± 1.7	<0.001
Total score	74.0 ± 12.5	90.0 ± 15.2	73.0 ± 17.1	62.1 ± 19.3	<0.001

Mean ± SD for (*n*) patients at detox baseline and 3-month follow-up either in remission or relapsed. LSS assesses current situation for 20 QoL indicators using a seven-point scale. Total scores > 100 indicate good QoL. For further details see ref. 7.

to disagree very strongly) scale. The maximum score is 120 with scores of over 100 indicating a good QoL. The baseline scores for the patients were highly significantly reduced for all of the parameters. Deterioration occurred with relapse but a highly significant improvement was seen for most indices with remission (9). This questionnaire is easy to administer and is recommended for routine use in patients with alcohol misuse. It is particularly useful in follow-up studies, treatment-group comparisons, and trials of chemotherapy or other agents. Thus in a recent study comparing patients admitted to a community alcohol treatment unit and offered either a 7-day detox or a 28-day detox and rehabilitation program, no significant differences in outcome were noted using a variety of indices including QoL measures (Table 6) (10). These findings have important implications for local-authority funding organizations, the treatment units themselves, and patient care policies. Objective evaluation of patient outcome should clearly include QoL measures as well as indices including biochemical markers of continued alcohol misuse.

III. IMPAIRED SLEEP

Only one generic QoL measurement has a sleep subscale score, the Nottingham Health Profile (11). Foster et al. (12) found that sleep is predictor of 3-month relapse in a group of 60 socially disadvantaged subjects. Furthermore, sleep latency was found to be the variable that predicted relapse and sleeping badly was associated with a poor 3-month outcome (13). These results have emphasized

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Table 6 Quality of Life Scores in Alcoholics Admitted to a Community Treatment Unit

	Detox (7 day) (n = 30)			Detox + rehab (28 day) (n = 32)		
Measure	Baseline	Follow-Up	Stats	Baseline	Follow-up	Stats
Life Situation Survey	76.1 (13.2)	76.2 (15.9)	0.963	75.1 (10.0)	71.8 (17.0)	0.421
Rotterdam Symptom Checklist	68.7 (12.2)	60.9 (17.9)	0.058	65.5 (18.1)	65.8 (21.9)	0.925
General Health Questionnaire (12 items)	7.2 (3.5)	5.2 (4.9)	0.069	6.8 (3.5)	6.8 (4.2)	0.871
Beck Depression Inventory	19.7 (7.1)	17.7 (9.2)	0.388	20.3 (9.0)	21.2 (12.3)	0.744

Data show mean (SIQ) and were analyzed by two-tailed paired *t*-test. For further details see ref. 10.

the importance of disturbed sleep in chronic alcoholism. Depression either as a consequence of the alcohol misuse or as a comorbid feature appears to correlate well with the impaired sleep, suggesting a causal relationship. The pathogenesis of the impaired sleep is, however, unclear.

A recent study from our group comparing subjective measures of sleep indicates a degree of impairment in patients with nonalcoholic liver disease, suggesting that liver dysfunction may be a contributing factor (14). It is well known that patients with severe liver disease, e.g., hepatic precoma, have seriously disturbed sleep patterns, suggesting that toxic substances accumulating as a result of the liver damage may be causal agents of the sleep disturbance. However, in the patients with nonalcoholic liver disease a correlation between impaired sleep and depression scores was again noted indicating a multifactorial causation for the sleep disturbance. The pineal hormone melatonin has been implicated in the regulation of sleep and there is a report of reduced urinary melatonin excretion in alcoholics (15). However, a small study investigating diurnal melatonin levels in a series of alcoholics admitted to a sleep laboratory showed no clear relationship between objective measures of sleep and melatonin levels and secretion (16).

The strikingly disturbed sleep is well illustrated in Table 7, where the Pittsburgh Sleep Quality Index was measured in a series of alcoholics and controls. All modalities of sleep were clearly impaired. Clinical experience indicates that disturbed sleep may persist for many months even with complete abstinence. This continued impaired sleep is often disturbing to the patients, who may relapse after using alcohol as a nighttime sedative. This is clearly an area for further

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Table 7 Pittsburgh Sleep Quality Index Scores in Controls and Alcoholics

	Controls (49)		Alcoholics (31)		Stats (p)
Total score	3.89	1.0	10.3	4.0	<0.001
Sleep latency	0.61	0.5	1.77	1.5	<0.001
Sleep duration	0.65	0.5	1.67	1.5	<0.0001
Sleep efficiency	0.16	0.5	1.25	1.0	<0.0001
Sleep disturbance	1.00	1.0	1.90	1.5	<0.0001
Sleep medications	0.02	0.0	0.48	0.0	<0.001
Daytime dysfunction	0.80	0.5	1.64	1.0	<0.0001
Sleep quality	0.67	0.5	1.55	1.5	<0.01

Mean and SIQ for patients and controls (n) studied in an outpatient clinic setting. For further details see ref. 14.

clinical research. Studies of the use of antidepressants with or without the use of short term nighttime sedatives, anxiolytic agents, and other sleep modulators would be of particular interest.

To date, QoL measures in alcoholics have used questionnaires developed by medical and paramedical staff with the administration and scoring of the questionnaires performed by similar professionals. Little attention has been paid to the QoL as judged by the patients themselves, although previous studies in other patient groups, e.g., cancer (17) and elderly (18) patients, have suggested that physicians' scores were consistently worse than those of the patients themselves and correlation between the two scores were low. In a recent study this approach has been extended to chronic alcoholics using the well-validated EuroQol 5D Visual Analogue Scale (19). The results, shown in Table 8, are similar to those reported with other patient groups; i.e., the clinician rated the patients' QoL significantly worse than did the patients themselves. These findings question the accuracy of observer-developed QoL measures and indicate the need for more

Table 8 Clinician- and Patient-Rated QoL in Alcoholics

Measure	Alcohol misusers ($n = 52$)	Normative values
EQ-5D VAS (Pt)	55.6 ± 20.5	75–95
EQ-5D VAS (Clin)	40.5 ± 19.8	N/A

Results show mean ± SD on 0–100 Visual Analogue Scale (VAS). There was low correlation between patient (Pt) and clinician (Clin) scores ($r = 0.39$; $p < 0.05$).

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patient-focused measures in which the patients' self-perceptions are given a greater weighting. However, with the well-validated instruments currently available, useful findings, new research avenues, and outcome predictors have been identified. It remains to be seen whether patient self-perception will add further to these results.

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Hepatic, Metabolic, and Nutritional Disorders of Alcoholism

From Pathogenesis to Therapy

Charles S. Lieber
Mount Sinai School of Medicine and Bronx Veterans Affairs Medical Center, Bronx, New York

I. INTRODUCTION

The clinical course and ultimate outcome of alcoholic liver disease is dismal. Indeed, in a prospective survey of 280 subjects with alcoholic liver injury (1) it was found that, within 48 months of follow-up, more than half of those with cirrhosis, and two-thirds of those with cirrhosis plus alcoholic hepatitis, had died. This disastrous outcome is more severe than that of many cancers, yet it is attracting much less concern, both among the public and in the medical profession. This may be due, at least in part, to the general perception that not much can be done about this major public health issue. One purpose of this review is to analyze how concepts about alcoholic liver disease have evolved and how elucidation of the biochemical effects of ethanol allows for a more optimistic outlook in terms of early diagnosis and treatment.

II. PATHOGENESIS

A. Nutritional Factors

1. Respective Role of Alcohol and Nutritional Deficiencies

Besides ethanol's pharmacological action, it has a considerable energy value (7.1 kcal/g). Therefore, a substantial use of alcohol has profound effects on nutritional

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status (2). Such consumption may cause *primary malnutrition* by displacing other nutrients in the diet because of the high-energy content of the alcoholic beverages (Fig. 1) or because of associated socioeconomic and medical disorders. *Secondary malnutrition* may result from either maldigestion or malabsorption of nutrients caused by gastrointestinal complications associated with alcoholism, involving especially the pancreas and the small intestine. Alcohol also promotes nutrient degradation or impaired activation. Such primary and secondary malnutrition can affect virtually all nutrients (vide infra). At the tissue level, alcohol replaces various normal substrates, with the liver being the most seriously affected organ and malnutrition being incriminated as a primary etiological factor of liver dysfunction.

However, even with nutritionally adequate diets, isoenergetic replacement of sucrose or other carbohydrates by ethanol consistently produced a five- to 10-

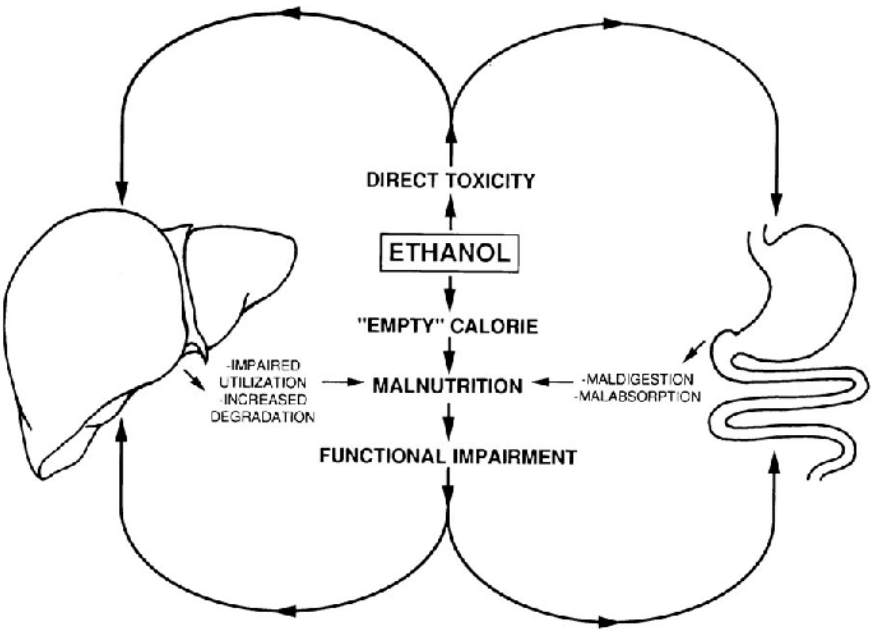

Figure 1 Interaction of direct toxicity of ethanol with malnutrition due to primary or secondary deficiencies. Secondary malnutrition may be caused by either maldigestion and malabsorption, impaired utilization (decreased activation and/or increased inactivation), or increased degradation of nutrients. Both direct toxicity of ethanol and malnutrition (whether primary or secondary) may affect function and structure of liver and gut (From ref. 3.)

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fold increase in hepatic triglycerides (4–6). Furthermore, isoenergetic replacement of carbohydrate by fat instead of ethanol did not produce steatosis (4). Alcohol was also shown to be capable of producing cirrhosis in nonhuman primates, even when given with an adequate diet (7). In addition, the hepatotoxicity of ethanol was established in humans by controlled clinical investigations that showed that even in the absence of dietary deficiencies, alcohol can produce fatty liver and ultrastructural lesions (4,5).

Thus, in humans, ethanol is capable of producing striking changes in the liver even in the presence of an enriched diet; these effects were linked to the metabolism of ethanol (Fig. 2) (vide infra).

2. *Effect of Alcohol on Nutrient Activation*

a. Thiamine and Pyridoxine. Thiamine deficiency in alcoholics causes Wernicke-Korsakoff syndrome and beriberi heart disease, and probably contributes to polyneuropathy.

Neurological, hematological, and dermatological disorders can be caused in part by pyridoxine deficiency. Pyridoxine deficiency, as measured by low plasma pyridoxal-5′-phosphate (PLP), was reported in over 50% of alcoholics without

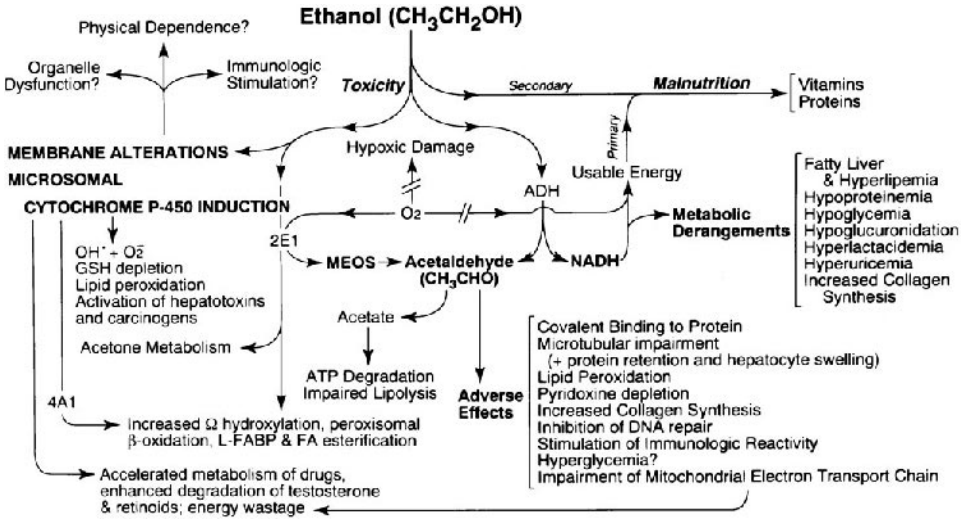

Figure 2 Hepatic, nutritional, and metabolic abnormalities after ethanol abuse. Malnutrition, whether primary or secondary, can be differentiated from metabolic changes or direct toxicity, resulting partly from ADH-mediated redox changes, or effects secondary to microsomal induction, or acetaldehyde production. (From ref. 8.)

hematological findings or abnormal liver function tests (9,10). Inadequate intake may partly explain low PLP, but increased destruction and reduced formation may also contribute. PLP is more rapidly destroyed in erythrocytes in the presence of acetaldehyde, the product of ethanol oxidation, perhaps by displacement of PLP from protein and consequent exposure to phosphatase (9,11). Studies showed that chronic ethanol feeding lowered hepatic content of PLP by decreasing net synthesis from pyridoxine (12–14). The acetaldehyde produced on alcohol oxidation is thought to enhance hydrolysis of PLP by cellular phosphatases (9).

b. Methionine and S-Adenosylmethionine (SAMe). Kinsell et al. (15) found a delay in the clearance of plasma methionine after its systemic administration to patients with liver damage. Similarly, Horowitz et al. (16) reported that the blood clearance of methionine after an oral load of this amino acid was slowed. Since about half the methionine is metabolized by the liver, these observations suggested impaired hepatic metabolism of this amino acid in patients with alcoholic liver disease. Indeed, for most of its functions, methionine must be activated to SAMe, and in cirrhotic livers, Duce et al. (17) reported a decrease in the activity of SAMe synthetase, the enzyme involved, also called methionine adenosyltransferase (Fig. 3).

Various mechanisms of inactivation of SAMe synthetase contribute to the defect (19), one of which is relative hypoxia, with nitric-oxide-mediated inactivation and transcriptional arrest (20). As a consequence, SAMe depletion as well as its decreased availability could be expected and, indeed, long-term ethanol consumption under controlled conditions by nonhuman primates was associated with a significant depletion of hepatic SAMe (21). Potentially, such SAMe depletion may have a number of adverse effects. SAMe is the principal methylating agent in various transmethylation reactions that are important to nucleic acid and protein synthesis. Hirata and Axelrod (22) and Hirata et al. (23) also demonstrated the importance of methylation to cell membrane function with regard to membrane fluidity and the transport of metabolites and transmission of signals across membranes. Thus, depletion of SAMe, by impairing methyltransferase activity, may promote the membrane injury that has been documented in alcohol-induced liver damage (24). Furthermore, SAMe plays a key role in the synthesis of polyamines and provides a source of cysteine for glutathione production (Fig. 3). Thus, the deficiency in methionine activation and in SAMe production resulting from the decrease in activity of the corresponding synthetase results in a number of adverse effects, including inadequate cysteine and GSH production, especially when aggravated by associated folate, B_6, or B_{12} deficiencies (Fig. 3). The consequences of this enzymic defect can be alleviated by providing SAMe, the product of the reaction. SAMe is unstable, but the synthesis of a stable salt allowed for replenishment of SAMe through ingestion of this compound: blood levels of

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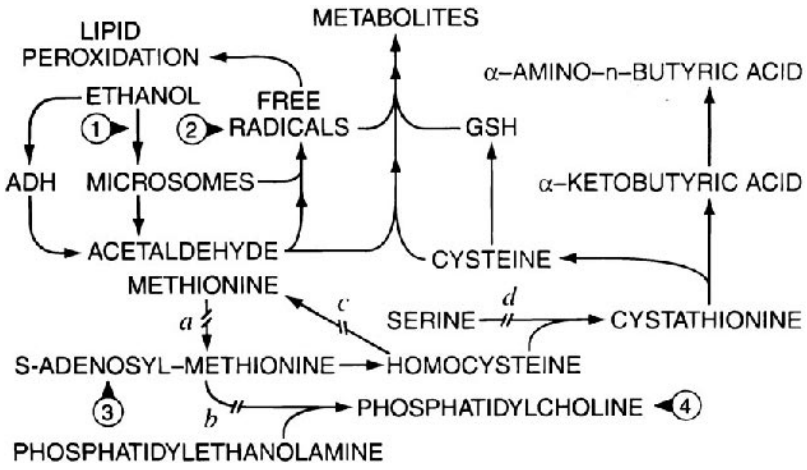

Figure 3 Lipid peroxidation and other consequences of alcoholic liver disease and/or increased free radical generation and acetaldehyde production by ethanol-induced microsomes, with sites of possible therapeutic interventions. Metabolic blocks caused by liver disease (a,b), folate (c), B_{12} (c), or B_6 (d) deficiencies are illustrated, with corresponding depletions in S-adenosylmethionine, phosphatidylcholine, and glutathione (GSH). New therapeutic approaches include (1) down-regulation of microsomal enzyme induction, especially of CYP2E1, (2) decrease of free radicals with antioxidants, (3) replenishment of S-adenosylmethionine and of (4) phosphatidylcholine. (From ref. 18.)

SAMe increased after oral administration in rodents (25) and in humans (26). Although it has been claimed that the liver does not take up SAMe from the bloodstream (27), other results indicated uptake of SAMe by isolated hepatocytes either at pharmacological (28) or at physiological (29,30) extracellular levels. Results in baboons (21) also clearly showed hepatic uptake of exogenous SAMe in vivo.

Clinical trials revealed that SAMe treatment is beneficial in intrahepatic cholestasis (31) including recurrent intrahepatic cholestasis and jaundice caused by androgens or estrogens. Given either orally or parenterally, SAMe improved both the pruritus and the biochemical parameters of cholestasis such as serum bilirubin, alkaline phosphatase, and γ-glutamyl transferase. It was also used successfully in severe cholestasis of pregnancy (32) with few, if any, untoward effects. Furthermore, in a prospective, multicenter, double-blind, placebo-controlled trial (33) performed in 220 inpatients with chronic liver disease (chronic active hepatitis and cirrhosis, including primary biliary cirrhosis), serum markers of cholestasis and subjective symptoms significantly improved after SAMe. Oral administration of 1200 mg/day of SAMe for 6 months also resulted in a signifi-

cant increase of hepatic GSH in patients with alcoholic as well as nonalcoholic liver disease (34).

The most impressive therapeutic success was achieved in a long-term randomized, placebo-controlled, double-blind, multicenter clinical trial of SAMe in patients with alcoholic liver cirrhosis in whom SAMe improved survival or delayed liver transplantation (35). A total of 123 patients with Child class A, B, or C cirrhosis were studied for 2 years. When patients in Child C class were excluded, the overall mortality/liver transplantation was significantly greater in the placebo group (29% vs. 12%, $p = 0.025$), and differences between both groups in the 2-year survival were also significant.

c. Phosphatidylcholine (PC). In the presence of cirrhosis, the activity of phosphatidylethanolamine methyltransferase is depressed (17); this is not simply secondary to the cirrhosis, but may in fact be a primary defect related to alcohol, as suggested by the observation that the enzyme activity is already decreased prior to the development of cirrhosis (36). This enzymatic block has significant pathological effects, which can again be bypassed through administration of the product of that reaction, in this case PC (36) (Fig. 3). This is emerging as a potentially important approach to the treatment of liver disease. Characteristic features of alcoholic liver injury include scarring or fibrosis and striking membrane alterations with associated phospholipid changes (2). In an attempt to offset some of these abnormalities, polyunsaturated lecithin (extracted from soybeans) was fed to baboons given ethanol for up to 8 years (37). Whereas fibrosis or cirrhosis ensued in most of the baboons fed the diet with ethanol alone, no cirrhosis or septal fibrosis developed in the animals fed ethanol with the lecithin. To assess whether PC was the active agent, a more purified extract was fed comprising 94–96% PC. The feeding of this mixture rich in polyunsaturated PCs (PPC), especially dilinoleoylphosphatidylcholine (DLPC), which has a high bioavailability, exerted a remarkable protection against alcohol-induced septal fibrosis and cirrhosis (38).

PPC contains choline, but choline, in amounts present in PPC, had no protective action against the fibrogenic effects of ethanol in the baboon (39). In primates in general, choline plays a lesser role as a dietary nutrient than in rodents, because of lesser choline oxidase activity. In fact, as reviewed elsewhere (40), choline becomes essential for human nutrition only in severely restricted feeding situations.

PPC is rich in linoleic acid, but this fatty acid per se is probably not responsible for the protective effect because the basic diet was supplemented with safflower and corn oil, which are rich in linoleic acid. Thus the polyunsaturated phospholipids themselves appear to be responsible for protection, perhaps because of their high bioavailability and selective incorporation into liver membranes (41).

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In baboons given ethanol that developed significant fibrosis, type I procollagen mRNA content was significantly enhanced (42). The number of stellate cells was also found to strikingly increase in alcohol-fed baboons, contributing to the progression of fibrosis (43). PPC not only corrected the ethanol-induced PC depletion and prevented the associated septal fibrosis and cirrhosis in these baboons (38), but it also reduced the number of activated stellate cells. A similar transformation of the stellate cells was observed in patients with alcoholic liver disease (44). An attenuation of the transformation of stellate cells to myofibroblast-like cells was also observed in vitro (45). In addition, the activity of membrane-bound enzymes was normalized: cytochrome C oxidase, a key enzyme of the mitochondrial electron-transport chain, which requires a normal phospholipid milieu for optimal activity and is severely depressed by chronic ethanol consumption (46), was restored to normal by addition of PC in vitro (47) or by PPC supplementation in vivo (48) with improvement of hepatic mitochondrial respiration, assessed by the respiratory control (determined by the ratio between the ADP-stimulated and nonstimulated O_2 consumption), with glutamate, succinate or palmitoyl-1-carnitine used as substrates.

Another mechanism whereby ethanol may affect phospholipids is via formation of phosphatidylethanol, with possible impact on signal transduction, as shown in isolated rat hepatocytes (49). A third mechanism is increased lipid peroxidation, as reflected by increased F_2-isoprostanes (39), which could explain the associated decrease of arachidonic acid in phospholipids (47). PPC resulted in total protection against oxidative stress, as determined by normalization of 4-hydroxynonenal, F_2-isoprostanes and GSH levels (50). However, beneficial effects of PPC were not limited to a correction of oxidative stress. Indeed, in an experimental model devoid of oxidative stress, namely fibrosis and cirrhosis induced by the injection of heterologous albumin in rats, PPC was nevertheless protective (51). This may reflect, at least in part, the fact that both PPC (52) and DLPC (38) stimulate collagenase production in stellate cells, resulting in a decrease of collagen accumulation.

In patients with hepatitis C, PPC improved the transaminase levels but the effect on liver fibrosis was not assessed (53). However, a clinical trial on alcoholic fibrosis is presently ongoing in the United States.

3. Toxic Interaction of Alcohol with Nutrients

a. Adverse Interaction with Retinol. In addition to the classic aspects of vitamin A deficiency due to either poor dietary intake or severe liver disease, direct effects of alcohol on vitamin A metabolism, and resulting alterations in hepatic vitamin A levels, have been elucidated (54).

- Depletion of Hepatic Vitamin A by Ethanol, Its Mechanism and Pathological Consequences. Alcoholic liver disease is associated with severely de-

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creased hepatic vitamin A levels, even when liver injury is moderate (fatty liver) and when blood values of vitamin A, RBP, and prealbumin are still unaffected (55,56). Malnutrition, when present, can of course contribute to hepatic vitamin A depletion but the patients with low liver vitamin A in the study of Leo and Lieber (56) appeared well nourished, which suggested a more direct effect of alcohol. Under strictly controlled conditions, chronic ethanol consumption was found to decrease hepatic vitamin A in baboons pair-fed a nutritionally adequate liquid diet containing 50% of total energy as either ethanol or isocaloric carbohydrate (57). To avoid the confounding effect of dietary vitamin A, it was virtually eliminated in some experiments. Under those conditions, the depletion rate of vitamin A from endogenous hepatic storage was observed to be 2.5 times faster in ethanol-fed rats than in controls. Two possible mechanisms other than malabsorption can be invoked: increased mobilization of vitamin A from the liver, and enhanced catabolism of vitamin A in the liver or in other organs (57–59). Drugs that induce the cytochromes P-450 in liver microsomes were shown to result in a depletion of hepatic vitamin A (60). A similar effect was observed after administration of ethanol (56,57) and other xenobiotics that are known to interact with liver microsomes, including carcinogens (61). The hepatic depletion was strikingly exacerbated when ethanol and drugs were combined (62), which mimics a common clinical occurrence.

Retinoic acid has been shown to be degraded in microsomes of either hamsters (63) or rats (59,64). In both species, the reported activity was very low compared to the degree of hepatic vitamin A depletion, but two new pathways of retinol metabolism in liver microsomes were described: rat liver microsomes, when fortified with NADPH, converted retinol to polar metabolites, including 4-hydroxyretinol (65). This activity was also demonstrated in a reconstituted monooxygenase system containing purified forms of rat cytochromes P-450 (65), including P-4502B1 (a phenobarbital-inducible isozyme). More recently, it has been shown that other cytochromes (such as P-450 CYP 1A1) also catalyze the conversion of retinal to retinoic acid (66). In addition, a new microsomal NAD^+-dependent retinol dehydrogenase was described (67). The activity of the retinol (67) as well as of the retinal (68) dehydrogenases is inducible by chronic alcohol consumption, thereby contributing to hepatic vitamin A depletion. Finally, metabolism of retinol and retinoic acid was also demonstrated with human liver microsomes and purified cytochrome P-4502C8 (68).

In patients with severe as well as moderate depletion of hepatic vitamin A, multivesicular lysosome-like organelles were detected in increased numbers (69). That a low hepatic vitamin A concentration contributes to these lesions was also verified experimentally in rats (70).

Hepatic vitamin A depletion plays a key role in hepatic fibrosis. Hepatic stellate cells are the principal storage site of vitamin A. The activation of stellate cells into myofibroblast-like cells, which then synthesize collagen, is associated

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with a decrease in vitamin A storage in these cells (71). Retinoic acid, and to a lesser extent retinol, were shown to reduce stellate cell proliferation and collagen production in culture (71–73). Conversely, lack of retinoids could promote fibrosis in these tissues, especially in the liver, consistent with the associated activation of stellate cells (71). Paradoxically, however, vitamin A excess may also promote fibrosis (vide infra).

Increasing evidence for a role of vitamin A in the proliferation and differentiation of a variety of human cells makes it apparent that reduction in vitamin A levels may be significant in the development of tumors. In fact, vitamin A deficiency has been associated with the formation of various types of tumors (74,75). Furthermore, concomitant ethanol consumption and vitamin A deficiency resulted also in an increased severity of squamous metaplasia of the trachea (43,76). This potentiation of vitamin A deficiency by alcohol may predispose the tracheal epithelium to neoplastic transformation. In addition, ethanol-induced vitamin A depletion is associated with decreased detoxification of xenobiotics, including carcinogens such as nitrosodimethylamine (77), thereby playing a role in chemical carcinogenesis (vide supra). Recent data also suggest that functional down-regulation of retinoic acid receptors, by inhibiting biosynthesis of retinoic acid and up-regulating activator protein-1 (c-*jun* and c-*fos*) gene expression, may be important mechanisms for causing malignant transformation by ethanol (78).

In addition to promoting vitamin A depletion, ethanol may interfere more directly with retinoic acid synthesis since both were shown in vitro to serve as substrates for the same enzymes (79). Specifically, one of the mechanisms by which ethanol induces gastrointestinal cancer may be an inhibition of ADH-catalyzed gastrointestinal retinoic acid synthesis, which is needed for epithelial differentiation. Indeed, class I ADH (ADH-I) and class IV ADH (ADH-IV), which function as retinol dehydrogenases in vitro, are abundantly distributed along the gastrointestinal (GI) tract (80).

- Abnormalities Associated with Excess Vitamin A. An excess of vitamin A is known to be hepatotoxic (81,82). The smallest daily supplement of vitamin A reported to be associated with liver cirrhosis is 7500 μg RE (25 000 IU) taken for 6 years (83). These supplements fall well within common therapeutic dosages and amounts used prophylactically with over-the-counter preparations by the population at large.

Potentiation of vitamin A hepatotoxicity by ethanol was first demonstrated in rats fed diets for 2 months with either normal or fivefold-increased vitamin A content, both with and without ethanol (84). Whereas under these conditions ethanol alone produced only modest changes, and vitamin A supplementation at the dose used had no adverse effect, the combination resulted in striking lesions, with giant mitochondria containing ''paracrystalline'' filamentous inclusions and depression of oxygen consumption in state 3 respiration with five different sub-

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strates. The potentiation of vitamin A toxicity by ethanol was also seen in patients treated with 10,000 IU vitamin A per day for sexual dysfunction attributable to excess alcohol consumption (85). In addition to giant mitochondria, filamentous or ''crystalline-like'' inclusions were seen in the liver mitochondria of patients with hypervitaminosis A (86,87).

b. Adverse Interactions of Ethanol with β-Carotene

- Effects of Alcohol on β-Carotene Concentration. Studies in humans revealed that for a given β-carotene intake, there is a correlation between alcohol consumption and plasma β-carotene concentration (88). Thus, whereas in general, alcoholics have low plasma β-carotene levels (88,89), presumably reflecting low intake, alcohol per se might in fact increase blood levels in humans (88). There was also an increase in women with a dose as low as two drinks a day (90). Furthermore, in nonhuman primates studied under strictly controlled conditions (91) and fed ethanol chronically, liver β-carotene was increased, in contrast to vitamin A, which was depleted. Similarly, plasma β-carotene levels were elevated in these ethanol-fed baboons, with a striking delay in the clearance from the blood after a β-carotene load. Furthermore, whereas β-carotene administration increased hepatic vitamin A in control baboons, this effect was much less evident in alcohol-fed animals. The combination of an increase in β-carotene and a relative lack of a corresponding rise in vitamin A suggests a blockage in the conversion of β-carotene to vitamin A by ethanol.

- β-Carotene, Alcohol, Oxidative Stress, and Liver Injury. In the baboon, administration of ethanol together with β-carotene resulted in a more striking hepatic injury than with either compound alone (91), with increased leakage of liver enzymes in the plasma, an inflammatory response in the liver, and, at the ultrastructural level, striking autophagic vacuoles and alterations of the endoplasmic reticulum and the mitochondria (92).

- Extrahepatic Side Effects. Two epidemiological investigations, namely both the ATBC (93) and the CARET (94) studies, revealed that β-carotene supplementation increases the incidence of pulmonary cancer in smokers. Because heavy smokers are commonly heavy drinkers, we raised the possibility that alcohol abuse was contributory (95), since alcohol is known to act as a carcinogen and to exacerbate the carcinogenicity of other xenobiotics, especially those of tobacco smoke (96). Why this should be aggravated by β-carotene is not clear, but β-carotene was found in rat lung to produce a powerful booster effect on phase I carcinogen-bioactivating enzymes, including activators of polycyclic aromatic hydrocarbons (PAHs) (97,98). In addition, since pulmonary cells are exposed to relatively high oxygen pressures, and because β-carotene loses its antioxidant activity and shows an autocatalytic, prooxidant effect at these higher pressures (99), such an interaction is at least plausible and deserves further study, especially

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since recent investigations showed that β-carotene protects against oxidative damage in HT29 cells at low concentrations but rapidly loses this capacity at higher doses (100) and that β-carotene enhances hydrogen-peroxide-induced DNA damage in human hepatocellular HepG2 cells (101). Furthermore, the more recent publications of the ATBC and CARET studies showed that the increased incidence of pulmonary cancer was related to the amount of alcohol consumed by the participants (102–104).

c. Therapeutic Window of Retinoids and Carotenoids. As discussed earlier, detrimental effects result from deficiency as well as from excess of retinoids and carotenoids and, paradoxically, both have similar adverse effects in terms of fibrosis, carcinogenesis, and possibly embryotoxicity. Treatment efforts therefore must carefully respect the resulting narrow therapeutic window, especially in drinkers in whom alcohol narrows this therapeutic window even further by promoting the depletion of retinoids and by potentiating their toxicity.

B. The Alcohol Dehydrogenase (ADH) Pathway and Associated Metabolic Disorders

The hepatocyte contains three main pathways for ethanol metabolism, each located in a different subcellular compartment: (1) the alcohol dehydrogenase (ADH) pathway of the cytosol or the soluble fraction of the cell, (2) the microsomal ethanol oxidizing system located in the endoplasmic reticulum, and (3) catalase located in the peroxisomes (2). Each of these pathways produces specific metabolic and toxic disturbance and all three result in the production of acetaldehyde, a highly toxic metabolite.

1. ADH Isozymes

ADH is the liver's major pathway for ethanol disposition. One *raison d'être* of ADH might be to rid the body of the small amounts of alcohol produced by fermentation in the gut (105). ADH has a broad substrate specificity, which includes dehydrogenation of steroids, oxidation of the intermediary alcohols of the shunt pathway of mevalonate metabolism, and ω-oxidation of fatty acids (106); these processes may act as the ''physiological'' substrates for ADH.

Human liver ADH is a zinc metalloenzyme with five classes of multiple molecular forms, which arise from the association of eight different types of subunits, α, β1, β2, β3, γ1, γ2, π, and χ, into active dimeric molecules.

2. Metabolic Effects of Excessive ADH-Mediated Hepatic NADH Generation

Oxidation of ethanol via the ADH pathway results in the production of acetaldehyde with loss of H, which reduces NAD to NADH. The large amounts of reduc-

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ing equivalents generated overwhelm the hepatocyte's ability to maintain redox homeostasis and a number of metabolic disorders ensue (Fig. 2) (2), including hypoglycemia and hyperlactacidemia. The latter contributes to the acidosis and also reduces the capacity of the kidney to excrete uric acid, leading to secondary hyperuricemia, which is aggravated by the alcohol-induced ketosis and acetate-mediated enhanced ATP breakdown and purine generation (107). Hyperuricemia explains, at least in part, the common clinical observation that excessive consumption of alcoholic beverages commonly aggravates or precipitates gouty attacks. The increased NADH also promotes fatty acid synthesis and opposes lipid oxidation with, as a net result, fat accumulation (108).

3. Hepatic Steatosis and Other Zonal Effects in the Liver

Fatty acids of different origins can accumulate as triglycerides in the liver because of different metabolic disturbances: decreased hepatic release of lipoproteins, increased mobilization of peripheral fat, enhanced hepatic uptake of circulating lipids, enhanced hepatic lipogenesis (vide supra), and, most importantly, decreased fatty acid oxidation, whether as a function of the reduced citric acid cycle activity secondary to the altered redox potential (vide supra) or as a consequence of permanent changes in mitochondrial structure and functions (1,2,109).

C. Microsomal Ethanol Oxidizing System (MEOS)

This pathway has been the subject of extensive research, reviewed in detail elsewhere (110,111). The first indication of an interaction of ethanol with the microsomal fraction of the hepatocyte was provided by the morphological observation that alcohol consumption results in a proliferation of the smooth endoplasmic reticulum (SER) (109). This increase in SER resembles that seen after administration of a wide variety of hepatotoxins (112), therapeutic agents (113), and some food additives (114). Since most of the substances that induce a proliferation of the SER are metabolized, at least in part, by the cytochrome P-450 enzyme system that is located on the SER, the possibility that alcohol may also be metabolized by similar enzymes was raised. Such a system was indeed demonstrated in liver microsomes in vitro and found to be inducible by chronic alcohol feeding in vivo (115) and was named the microsomal ethanol oxidizing system (MEOS) (115,116). It was reconstituted using NADPH–cytochrome P-450 reductase, phospholipids, and either partially purified or highly purified microsomal P-450 from untreated (117) or phenobarbital-treated (118) rats. That chronic ethanol consumption results in the induction of a unique P-450 was also shown by Ohnishi and Lieber (117) using a liver microsomal P-450 fraction isolated from ethanol-treated rats. An ethanol-inducible form of P-450, purified from rabbit liver microsomes (119), catalyzed ethanol oxidation at rates much higher than

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other P-450 isozymes. The purified human protein (now called CYP2E1) was obtained in a catalytically active form, with a high turnover rate for ethanol and other specific substrates (120). Contrasting with hepatic ADH, which is not inducible in primates as well as most other animal species, a fourfold induction of CYP2E1 was found in biopsies of recently drinking subjects, using the Western blot technique with specific antibodies against this CYP2E1 (121), accompanied by a corresponding rise in mRNA (122). Other cytochromes P-450 (1A2, 3A4) are also involved (123).

The induction of CYP2E1 contributes to the metabolic tolerance to ethanol that develops in chronic and heavy drinkers (124). In addition to this tolerance to ethanol, alcoholics tend to display tolerance to various other drugs. Indeed, it has been shown that the rate of drug clearance from the blood is enhanced in alcoholics (125,126). Of course, this could be caused by a variety of factors other than ethanol, such as the congeners and the use of other drugs so commonly associated with alcoholism. Controlled studies showed, however, that administration of pure ethanol with nondeficient diets to either rats or humans (under metabolic ward conditions) resulted in a striking increase in the rate of blood clearance of meprobamate and pentobarbital (127) and propranolol (128). The metabolic tolerance persists several days to weeks after cessation of alcohol abuse, and the duration of recovery varies depending on the drug considered (129). Similarly, increases in the metabolism of antipyrine (130), tolbutamide (125,126,131), warfarin (126), propranolol (132), diazepam (133), and rifamycin (134) were found. Furthermore, the capacity of liver slices from animals fed ethanol to metabolize meprobamate was increased (127), which clearly showed that ethanol consumption affects drug metabolism in the liver itself, independent of changes in drug excretion or distribution or hepatic blood flow.

It is now recognized that CYP2E1, in addition to its ethanol-oxidizing activity, catalyzes fatty acid ω-1 and ω-2 hydroxylations (135–137). Furthermore, acetone is both an inducer and a substrate of CYP2E1 (138–140) (Fig. 4). Excess ketones and fatty acid commonly accompany diabetes and morbid obesity, conditions associated with nonalcoholic steatohepatitis (NASH). Experimentally, obese, overfed rats also exhibit substantially higher microsomal ethanol oxidation, acetaminophen activation, and *p*-nitrophenol hydroxylation (monooxygenase activities catalyzed by CYP2E1) (141). These diabetic rats are experimental models relevant to NASH, and indeed, the hepatopathology of NASH appears to be due, at least in part, to excess CYP2E1 induction (142).

Clinically, a most important feature of CYP2E1 is not only ethanol oxidation, but also its extraordinary capacity to convert many xenobiotics to highly toxic metabolites, thereby explaining the increased vulnerability of the alcoholic to *industrial solvents* (e.g., bromobenzene and vinylidene chloride), *anesthetic agents* [e.g., enflurane (143) and methoxyflurane], commonly used *medications* (e.g., isoniazid, phenylbutazone), *illicit drugs* (e.g., cocaine) and over-the-counter

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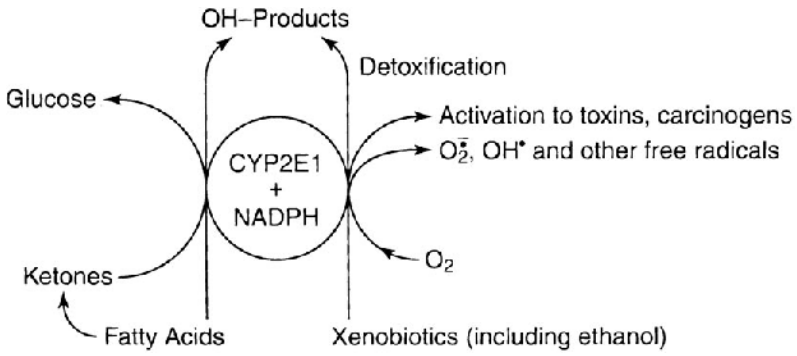

Figure 4 Physiological and toxic roles of CYP2E1, the main cytochrome P-450 of the microsomal ethanol-oxidizing system (MEOS). Many endogenous and xenobiotic compounds are substrates for CYP2E1 and their excess induces its activity through various mechanisms, resulting in an array of beneficial as well as harmful effects (From ref. 111.)

analgesics (e.g., acetaminophen) (144), all of which are substrates for, and/or inducers of, CYP2E1. The effects of acetaminophen, ethanol, and fasting are synergistic (145), because all three deplete the level of reduced glutathione, a scavenger of toxic free radicals.

CYP2E1 also generates several species of active oxygen (Figs. 2, 4), which, in concert with a decrease in the level of GSH, promote injury by inactivation of enzymes and peroxidation of lipids. In patients with cirrhosis, hepatic depletion of α-tocopherol (146), a major antioxidant, potentiates this effect. GSH offers one of the mechanisms for the scavenging of toxic free radicals. Replenishment of GSH can be achieved by administration of precursors of cysteine (one of the amino acids of this tripeptide) such as acetylcysteine or SAMe (21,111).

III. THERAPEUTIC IMPLICATIONS

A. Nutritional Therapy in Alcoholism

Individuals consuming over 30% of total calories as alcohol have a high probability of ingesting less than the recommended daily amounts of carbohydrate, protein, fat, vitamins A, C, and B (especially thiamine), and minerals such as calcium and iron (vide supra). It is sensible to recommend a complete diet comparable to that of nonalcoholics to forestall deficiency syndromes, although this does not suffice to prevent some organ damage due to the direct toxicity of alcohol, including alcoholic liver disease.

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Damage due to lack of thiamine is serious but treatable with a great margin of safety; therefore, thiamine deficiency should be presumed and, if not definitely disproved, parenteral therapy with 50 mg of thiamine per day should be given until similar doses can be taken by mouth. Riboflavin and pyridoxine should be routinely administered at the dosages usually contained in standard multivitamin preparations. Adequate folic acid replacement can be accomplished with the usual diet. Additional replacement is optional unless deficiency is severe. Vitamin A replacement should be given only for well-documented deficiency, and to patients whose abstinence from alcohol is assured (vide infra).

Zinc replacement is indicated only for night blindness unresponsive to vitamin A replacement. Magnesium replacement is recommended for symptomatic patients with low serum magnesium. Iron deficiency that has been clearly diagnosed may be corrected orally.

The nutritional management of acute and chronic liver disease due to alcoholism should include feeding programs to achieve protein replenishment without promoting hepatic encephalopathy, as reviewed elsewhere (2).

A pathogenic concept is emerging that is particularly useful therapeutically. Whereas it continues to be important to replenish nutritional deficiencies, when present, it is crucial to recognize that because of the alcohol-induced disease process, some of the nutritional requirements change. This is exemplified by methionine, which normally is one of the essential amino acids for humans, but needs to be activated to SAMe, a process impaired by the disease (vide supra). Thus, SAMe rather than methionine is the compound that must be supplemented in the presence of significant liver disease (111). Similarly, serious consequences of the decreased phosphatidylethanolamine methyltransferase activity associated with alcoholic liver disease (Fig. 3) can be offset by the administration of PPC (vide infra).

B. Redox Shift, Oxidative Stress, Toxicity of Acetaldehyde, and Antioxidant Therapy

The redox shift and oxidative stress related to the metabolism of ethanol (Fig. 2) have been shown to play a major role in the pathogenesis of alcoholic liver disease (vide supra) and hence in treatment. The ADH-mediated, ethanol-induced redox changes may play a role in fibrogenesis. This was suggested by the observation that methylene blue, a scavenger of reducing equivalents, totally inhibits the stimulatory effect of acetaldehyde on $\alpha 1$ (I) procollagen and fibronectin gene expression in stellate cells (147). Furthermore, a major source of oxygen-free radicals is the microsomal ethanol-oxidizing system (MEOS). The induction of MEOS is associated with proliferation of the endoplasmic reticulum (vide supra), which is accompanied by increased oxidation of NADPH, with resulting H_2O_2 generation (148). There is also increased superoxide radical production (149). In

addition, the CYP2E1 induction contributes to the well-known lipid peroxidation associated with alcoholic liver injury (Fig. 3). Lipid peroxidation correlated with the amount of CYP2E1 in liver microsomal preparations, and it was inhibited by antibodies against CYP2E1 in control and ethanol-fed rats (150,151). Indeed, CYP2E1 is rather ''leaky'' and its operation results in a significant release of free radicals, including 1-hydroxyethyl-free radical intermediates (152,153), confirmed by detecting the hydroxyethyl radicals in vivo (154). The presence of CYP2E1 and its up-regulation by ethanol was also shown in Kupffer cells (155).

The product of ethanol oxidation, namely acetaldehyde, promotes glutathione (GSH) depletion (vide supra), resulting in free-radical-mediated toxicity, and lipid peroxidation. Indeed, in isolated perfused livers acetaldehyde was shown to cause lipid peroxidation (156,157). One of the three amino acids of the tripeptide GSH is cysteine. Binding of acetaldehyde with cysteine and/or GSH may contribute to a depression of liver GSH (158) (Fig. 3). Rats fed ethanol chronically have significantly increased rates of GSH turnover (159). Acute ethanol administration inhibits GSH synthesis and produces an increased loss from the liver (160). GSH is selectively depleted in the mitochondria (161) and may contribute to the striking alcohol-induced alterations of that organelle since GSH offers one of the mechanisms for the scavenging of free radicals (Fig. 3).

Since the up-regulation of CYP2E1 activity by ethanol results in increased generation of reactive radicals and toxic metabolites, it has been suggested that CYP2E1 inhibitors may eventually provide useful tools for prevention and treatment of the hepatotoxicity associated with heavy drinking. Several compounds were shown to exert some inhibiting effects on CYP2E1 (110) including chlormethiazole (162–164). Experimentally, a decrease in the inducibility of CYP2E1 was found to be associated with a reduction in associated liver injury (165,166), but when the liver pathology was semiquantitated, it was only partially ameliorated by CYP2E1 inhibitors (167,168). More effective inhibitors are being developed (169) but there is still a need to obtain CYP2E1 inhibitors that are not only effective but also innocuous enough for prolonged use in humans. Interestingly, PPC feeding down-regulated the CYP2E1 induction by ethanol (170). Unlike some of the experimental CYP2E1 inhibitors referred to above, PPC is innocuous; it is currently being tested in humans.

Bile acids may also play an important role. That ethanol impairs bile acid excretion was already shown by Lefevre et al. (171) and Vendemiale et al. (172). More recently, Vendemiale et al. (173) found that tauro-ursodeoxycholate administration protects proteins and lipids from ethanol-induced oxidative damage without influencing the GSH content and compartmentalization. Furthermore, in a placebo-controlled, crossover study, administration of ursodeoxycholic acid for 4 weeks reduced both bilirubin and liver enzyme levels in patients with alcoholic cirrhosis who were actively drinking (174). Bile acids such as taurine were found

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to have protective properties against ethanol-induced hepatic steatosis and lipid peroxidation during chronic ethanol consumption in rats (175), possibly again because of inhibition of the activity of CYP2E1, assessed indirectly by hepatic 4-nitrophenol hydroxylation. Similarly, ursodeoxycholate protected against ethanol-induced liver injury (176) by improving ATP synthesis and preserving liver mitochondrial morphology; it also attenuated alcoholic fatty liver in rats (177). Finally, Neuman et al. (178) had also shown favorable effects of tauro-ursodeoxycholic and ursodeoxycholic acid on ethanol-induced cell injuries using a human Hep G2 cell line.

In patients with cirrhosis, diminished hepatic vitamin E levels have been observed (146), as confirmed by von Herbay et al. (179). However, in alcoholics without cirrhosis, hepatic vitamin E levels were generally within the normal range (146) raising the question of the usefulness of supplementation in that group of subjects. Moreover, even in baboons with normal vitamin E liver levels, alcohol administration resulted in the production of septal fibrosis in most, and full-blown cirrhosis in some (38,50). Thus, while vitamin E deficiency increases the hepatic vulnerability to ethanol, even in the presence of normal α-tocopherol, ethanol is hepatotoxic and causes fibrosis. Effectiveness of vitamin E supplementation in the prevention of alcoholic liver injury is now being evaluated in humans.

Another antioxidant that has shown promising results in experimental animals in silymarin. The results of an ongoing double-blind, placebo-controlled trial are presently awaited (see chapter by H. Seitz, this volume).

Iron overload may play a contributory role, as chronic alcohol consumption results in an increased iron uptake by hepatocytes (180) and as iron exposure accentuates the changes of lipid peroxidation and in the GSH status of the liver cell induced by acute ethanol intoxication (181). Hence iron chelation is being explored therapeutically.

C. Prevention and Treatment of Alcoholic Fatty Liver

A key pathogenic observation was the discovery that alcohol interferes with the oxidation of fatty acids (108,182). As a result, alcohol promotes the deposition of dietary fat in the liver, both in rats (183) and in humans (184). Consequently, decreasing the amount of dietary fat resulted in a reduction in the severity of the alcoholic fatty liver (185). Furthermore, replacement of the dietary long-chain triglycerides with medium-chain triglycerides (MCT) also significantly reduced the alcohol-induced steatosis (186), most likely because medium-chain fatty acids have a much greater propensity to undergo oxidation than the long-chain ones (187). This protective effect of MCT has been more recently confirmed (188) and may be of therapeutic interest, particularly for relatively short-term interventions in patients recovering from alcohol-induced liver injury.

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Replenishment of phosphatidylcholine restored the capacity of the mitochondria for fatty acid oxidation, with a corresponding reduction of hepatic fat accumulation (48), which supports the therapeutic use of PPC (vide supra).

D. Treatment of Inflammation

Effective therapy should limit the catabolic processes underlying alcoholic hepatitis (AH) and promote anabolism. The inflammatory nature of AH and the possibility that immunological factors influence its perpetuation suggested that therapy with corticosteroids might reduce short-term mortality (189,190). Furthermore, in experimental models of liver failure, corticosteroids led to decreased ammonia levels through improved liver function (191). Corticosteroids might, therefore, indirectly decrease hepatic encephalopathy. A meta-analysis of the randomized trials concluded that corticosteroids do indeed reduce short-term (2-month) mortality in patients with severe AH, if patients with acute GI bleeding are excluded (192). This conclusion was confirmed in a subsequent trial (193).

Good candidates for corticosteroid therapy are patients with severe disease with either spontaneous hepatic encephalopathy or high bilirubin and prothrombine time (PT), with a Maddrey Index of 4.6 (PT-control) + serum bilirubin $>$ 32. It is strongly recommend that a liver biopsy be performed to confirm the diagnosis before initiation of treatment, because a small, but significant, proportion (12%) of patients who fulfill the clinical and laboratory criteria for severe AH lack the appropriate histological findings (194).

Prednisolone, rather than prednisone, is recommend because hepatic metabolism of prednisone to its biologically active form may be incomplete in patients with liver disease (189). The initial dosage is 40 mg/day of prednisolone (orally or intravenously) or 32 mg/day of methylprednisolone for 28 days, then a 2-week taper. Therapy is generally well tolerated.

Other approaches pertain to antiendotoxin therapy (including antibiotic treatment) as well as inhibition of Kupffer cell activation with associated decreased production of tumor necrosis factor (TNF)-α and other cytokines. None of the latter approaches has yet been applied clinically. Of interest again are 2 CYP2E1 inhibitors. Indeed, a potent stimulant of Kupffer cell activation is the gut-derived endotoxin lipopolysaccharide (195–197), but an additional possible stimulator of Kupffer cells also is CYP2E1, shown to be increased after chronic alcohol consumption not only in hepatocytes (vide supra) but also in Kupffer cells (155). In both acute and chronic liver diseases, Kupffer cells become activated to produce cytokines and reactive oxygen radicals (198–203), known mediators of hepatocellular injury (see chapter by Oneta, this volume).

Derangements of immune systems are also present in alcoholic liver disease (204), but there is still some debate whether they represent a consequence or a

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cause of the liver injury. Indeed, acetaldehyde is a very reactive compound that forms adducts with a variety of proteins. These, in turn, represent neoantigens and elicit an antibody response that may reflect, as well as contribute to, the pathogenesis of alcoholic liver injury.

E. Prevention and Treatment of Fibrosis

Individual susceptibility to the development of cirrhosis varies. Therefore, recognition of those individuals prone to progress to cirrhosis at an early, and still reversible, stage is of practical importance for prevention and treatment. In that regard, recognition of perivenular fibrosis on liver biopsy is valuable (vide supra). This lesion had been described in association with full-blown alcoholic hepatitis (205), but it can occur already at the fatty liver stage in the absence of hepatitis (206). Experimental data suggested that perivenular fibrosis is a common and early warning sign of impending cirrhosis if drinking continues (206), and similar conclusions were derived from clinical studies in alcoholics (207) and, interestingly, in diabetics (208), consistent with the pathogenic role of CYP2E1 in NASH (vide supra).

The accumulation of hepatic collagen during the development of cirrhosis could theoretically result from increased synthesis, decreased degradation, or both. The role of increased collagen synthesis was suggested by enhanced activity of hepatic peptidylproline hydroxylase in rats and primates and increased incorporation of proline ^{14}C into hepatic collagen in rat liver slices (209). Increased hepatic peptidylproline hydroxylase activity was also found in patients with alcoholic cirrhosis (210) and hepatitis (211). In baboons given ethanol who developed significant fibrosis, the hepatic type I procollagen mRNA content was significantly higher (per liver RNA) as determined by hybridization analysis (42). Concerning the respective role of hepatocytes and stellate cells, a consensus has been reached that stellate cells, which are ''activated'' after chronic alcohol consumption (43,44), appear to play the major role in the production of collagen in the liver. Normal stellate cells, when isolated and cultured on plastic, undergo spontaneous transformation into transitional-like cells, thereby mimicking in vitro the condition that prevails in vivo after chronic alcohol consumption (212). These cells in culture produce collagen (212). When acetaldehyde, the active metabolite of ethanol, is added to these cells, they respond with a further increase in collagen accumulation (212) and synthesis, with increased mRNA for collagen (147) because collagen synthesis by liver stellate cells is released from feedback inhibition by the carboxy terminal propeptide of procollagen by adduct formation of acetaldehyde with the latter (213).

Collagen accumulation not only reflects enhanced synthesis, but it also results from an imbalance between collagen degradation and collagen production.

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Thus, cirrhosis might, in part, represent relative failure of collagen degradation to keep pace with synthesis. Interestingly, PPC may affect this balance. Indeed, addition of PPC to transformed stellate cells was found to prevent the acetaldehyde-mediated increase in collagen accumulation, possibly by stimulation of collagenase activity (52) as well as by reduction of collagen production through a decrease in stellate cell activation demonstrated for the active component of PPC, namely DLPC (214).

Finally, the aldehydic products of lipid peroxidation (215,216) may also play a role in fibrogenesis. As discussed earlier, PPC and DLPC exert remarkable antioxidant effects, both in vitro and in vivo. Thus, PPC may attenuate the development of cirrhosis after chronic alcohol administration in baboons (38) and CCl_4 in rats (51) through, at least in part, its antioxidative effects (50). However, it should be pointed out that PPC is active as an antifibrotic agent even in the absence of oxidative stress, as demonstrated with the model of fibrosis and cirrhosis caused by heterologous albumin in the rat (51). In that case, the other antifibrotic properties of PPC (vide supra) are obviously involved.

Colchicine attenuates the inflammatory response associated with alcohol injury to the liver and may also decrease collagen deposition. Kershenobich et al. (217) showed a significant reduction in mortality and reversal of fibrosis on serial biopsies but there was a difference in the severity of the liver disease in the treated versus the placebo group, and there was a high dropout rate (218). Therefore, confirmatory studies are desirable and thus ongoing.

Viral hepatitis B or C commonly accompanies chronic hepatitis in alcoholics. The association is particularly striking for hepatitis C. Indeed, in these patients, even in the absence of risk factors for the hepatitis C virus (219), portal or lobular inflammation (or both) are strongly associated with antibody to this virus, suggesting that alcohol may favor acquisition, replication, or persistence of the virus. There may be potentiation of associated liver disorders, including fibrosis, but no satisfactory specific antiviral therapy is currently available, since α-interferon is relatively contraindicated in alcoholics, unless they stop drinking.

F. Transplantation

Orthotopic liver transplantation is an appropriate option for patients with alcoholic cirrhosis in whom liver failure progresses and life-threatening complications develop despite abstinence from alcohol (220). Most patients are compliant with therapy and resume a satisfactory degree of social and occupational function (221,222). Between 10% and 20% of patients resume drinking; however, recidivism is frequently difficult to confirm (223,224). There is no satisfactory solution to this problem as yet but the obvious answer would be a better control of relapse of drinking (vide supra).

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Gut-Derived Bacterial Toxins (Endotoxin) and Alcoholic Liver Disease

J. Christian Bode
Robert-Bosch Hospital, Stuttgart, Germany

Alexandr Parlesak and Christiane Bode
Hohenheim University, Stuttgart, Germany

I. INTRODUCTION

Excessive use of alcoholic beverages remains the most important cause of cirrhosis in most Western countries. Nearly all alcohol abusers will develop fatty liver. A highly significant positive correlation has also been demonstrated between both the magnitude and duration of alcohol consumption and the development of alcoholic cirrhosis. However, among alcohol abusers, only about 20–40% develop a progressive type of alcoholic liver disease, such as alcoholic hepatitis and fibrosis, which may eventually lead to cirrhosis (1,2). This can partly be explained by differences in lifetime alcohol consumption, but in addition interindividual differences in the susceptibility to the direct and indirect injurious effects of alcohol are assumed to be important determinants for the development of progressive alcoholic liver disease.

Despite extensive basic and clinical research for about five decades the mechanisms that lead to a progressive type of alcoholic liver disease have still not completely been clarified. Mechanisms that have been proposed for the complex pathogenesis of alcoholic hepatitis and fibrosis include manifold metabolic alterations, direct cellular toxicity of acetaldehyde, promotion of oxidative stress, immunological mechanisms, regional hypoxia, and various nutritional factors (3–5). More recent experimental and clinical data suggest that a hepatic necroin-

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flammatory cascade, which is initiated by gut-derived endotoxins (lipopolysaccharides, LPS), triggers progressive alcoholic liver injury.

Evidence that intestinal bacteria might contribute to acute and chronic experimental liver damage was already published half a century ago. Several authors found that long-term administration of nonabsorbable antibiotics prevented liver damage and the development of liver fibrosis in rats receiving a choline-deficient diet (6,7). The assumption that LPS, the main toxin of gram-negative bacteria, plays a key role for the development of dietary cirrhosis in rats was confirmed in an elegant study, in which it was shown that the protective effect of oral neomycin administration against the development of dietary cirrhosis in rats is completely abolished by adding LPS to the choline-deficient diet (8). Since then LPS-mediated injury has been implicated in the pathogenesis of several types of experimental liver disease and has causally been related to complications and extrahepatic manifestations of acute and chronic liver disease in humans. The rapidly increasing knowledge of functional disturbances and tissue damage in the liver caused by LPS has been summarized in extensive reviews (9,10). Nearly two decades ago our group proposed for the first time that alcohol-induced changes in the gut, and their consequences, especially endotoxemia, may play an important role in the pathophysiology of alcoholic liver disease in humans (11). The evidence supporting the validity of this hypothesis, which has accumulated from numerous experimental and clinical studies, will be briefly summarized in this chapter.

II. ALCOHOL-INDUCED MUCOSAL INJURY IN THE UPPER GASTROINTESTINAL TRACT

Consumption of large doses of alcoholic beverages has been shown to be an important cause of hemorrhage-erosive gastric lesions both in animal experiments and in humans (11,12). Although low or moderate alcohol doses do not cause such damage in healthy subjects, even a single episode of heavy drinking can induce mucosal inflammation and hemorrhage lesions (13). Mechanisms that are assumed to contribute to the development of the mucosal injury caused by alcohol are summarized in Table 1.

Similar mucosal injury with erythema, subepithelial bleeding, and erosions occurs in the duodenum following excessive alcohol consumption (14). Even in healthy people, a single episode of heavy drinking can result in duodenal erosions and bleeding. Animal studies have indicated that several mechanisms contribute to the development of these mucosal injuries. First, alcohol can directly disturb the integrity of the mucosal epithelium. Second, alcohol induces the release of noxious signaling molecules, such as proinflammatory cytokines, leukotrienes, and histamine. The latter leads to an accumulation of polymorphonuclear leuko-

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Table 1 Factors Involved in the Pathophysiology of Alcohol-Induced Mucosal Injury in the Stomach

Increase in the permeability of the membranes of the epithelial cells
Disturbances of the ''mucosal barrier,'' backdiffusion of hydrogen ions
Inhibition of prostaglandin synthesis, especially PGE_2 (loss of the ''cytoprotective'' effect)
Increase in the synthesis of leukotrienes
Damage of the ''tight junctions''
Qualitative and quantitative changes in mucus production
Disturbances of the microcirculation of the mucosa

cytes and disturbances in the mucosal microcirculation that promote an enhanced transcapillary fluid infiltration in the extravascular space, fluid accumulation and bleb formation under the tips of the villi, and subsequent rupture of the epithelial lining (14,15). Owing to the high regenerative capacity of the intestinal mucosa, the signs of mucosal injury disappear within a few days following alcohol abstinence (16).

III. INCREASED GUT PERMEABILITY

The marked mucosal injury induced by alcohol abuse in the stomach and upper small intestine is likely to be accompanied by a disturbance of the mucosal barrier, which leads to an enhanced permeability of the gut mucosa enabling bacterial and other toxins that normally cannot pass through the mucosa, or can only pass in trace amounts, to enter the systemic circulation more readily. Increased intestinal permeability to macromolecules following alcohol ingestion was first reported in animal experiments for hemoglobin, horseradish peroxidase, and polyethylenglycol (PEG) 1.500 Mr (17). Evidence of an increased intestinal permeability caused by alcohol intake was also reported in human subjects when smaller molecules (Mr $<$ 500) were used as a permeability probe (18–20). For example, a reversible increase in absorption of PEG 400 was observed after the oral intake of alcohol in healthy volunteers (18) and an increased intestinal permeability of 51^{Cr}-labeled EDTA was found in recently drinking alcoholics (19). On the other hand, a significantly increased intestinal permeability has been confirmed for macromolecules, such as PEG 4.000 Mr and 10.000 Mr, in recently drinking alcoholics without and with cirrhosis (21; Table 2). However, no significant changes of gut permeability were observed in chronic alcohol-abusing subjects in other studies when PEG Mr 400 (21) or molecules of similar masses, such as manitol and lactulose (20), were used as permeability probe.

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Table 2 Gut Permeability to Polyethylenglykol (PEG Mr 10,000) in Patients with Various Stages of Alcoholic Liver Disease

Subjects	*n*	PEG in urine positive, *n* (%)
Controls	34	0
Fatty liver	17	7 (41)**
Alcoholic hepatitis (no cirrhosis)	18	7 (39)**
Alcoholic cirrhosis	19	6 (31.5)*

* $p < 0.05$; ** $p < 0.001$.
Source: Data from ref. 21 with permission of the Publisher of *Journal of Hepatology*.

The hypothesis that the translocation of macromolecules through the intestinal mucosa might be enhanced following acute and chronic alcohol ingestion is supported by the observation of a transient endoxemia following acute alcohol consumption in healthy volunteers (22) and in patients with early forms of alcohol-related liver disease, such as fatty liver (23).

IV. BACTERIAL OVERGROWTH IN THE SMALL INTESTINE OF ALCOHOLICS

In studies using a specially devised tube, marked qualitative and quantitative alterations of the microflora of the jejunum have been documented in recently drinking alcoholics (71). Nearly 50% of the alcoholics had a distinct increase in the total number of bacteria per unit volume of jejunal juice ($> 10^5$ colony-forming units per milliliter) with a predominant increase of bacteria of the fecal flora (Table 3). An increased prevalence of bacterial overgrowth has also been

Table 3 Prevalence of Increased Numbers ($>10^5$/ml) of Colony-Forming Units (CFU) in Jejunal Juice Obtained from Alcoholics and Controls at Aerobic and Anaerobic Conditions

Type of bacteria	Controls ($n = 13$)	Alcoholics ($n = 25$)	*p*
Anaerobes	7.6%	48.1%	<0.001
Aerobes	23%	40.8%	<0.025

Source: Data from ref. 71.

reported when samples of duodenal juice obtained during gastroduodenoscopy in actively drinking alcoholics were studied (72). Indirect evidence of an increased prevalence of bacterial overgrowth in chronic alcohol abusers also stems from studies using the H_2 breath test after ingestion of lactulose (73) or glucose (74). Bacterial overgrowth in the small intestine might contribute to functional disturbances and mucosal damage in subjects chronically abusing alcohol. In addition, it might promote increased production of bacterial toxins, especially LPS, which in combination with the alcohol-induced mucosal damage might contribute to an enhanced LPS translocation from the gut lumen into the portal blood or the lymphatics.

V. ENDOTOXEMIA INDUCED BY ALCOHOL ABUSE

Despite good evidence that gut-derived LPS contributes to the development of various types of experimental liver disease, until the late 1970s studies on the role of LPS in the development and course of hepatic injury in humans were hampered by the fact that only a qualitative LPS test was available, which was found to lead to poorly reproducible results (9). Using this qualitative test, endotoxemia was detected in the venous blood of patients with cirrhosis in several studies (9,10,24). However, the observed prevalence of endotoxemia in these studies varied distinctly. More reliable results were obtained when a chromogenic assay for endotoxin was developed, which not only improved the sensitivity but also permitted quantitation of endotoxin (25).

A. Endotoxemia in Alcoholics With and Without Advanced Liver Disease

Endotoxemia was found significantly more frequently in patients with alcoholic cirrhosis than in patients with nonalcoholic cirrhosis (22). In a study using an improved chromogenic substrate assay with individual standard curves for each plasma sample, the mean LPS concentration was higher in patients with alcoholic cirrhosis than in those with nonalcoholic cirrhosis (17,23). In patients with cirrhosis endotoxemia was assumed to be mainly caused by impairment of the clearance of LPS in the liver due to intra- and extrahepatic portosystemic shunts and decreased phagocytic capacity of the hepatic reticuloendothelial system (9,24). Therefore, in cirrhotics, endotoxemia seems to be more a consequence of advanced chronic liver disease than a causative factor for the progression of the liver disease.

When considering the potential role of LPS for the initiation and progression of alcoholic liver disease, the occurrence of endotoxemia in the early stages might be more relevant. In fact, in actively drinking alcoholics without evidence of progressive liver disease distinctly elevated plasma LPS concentrations were

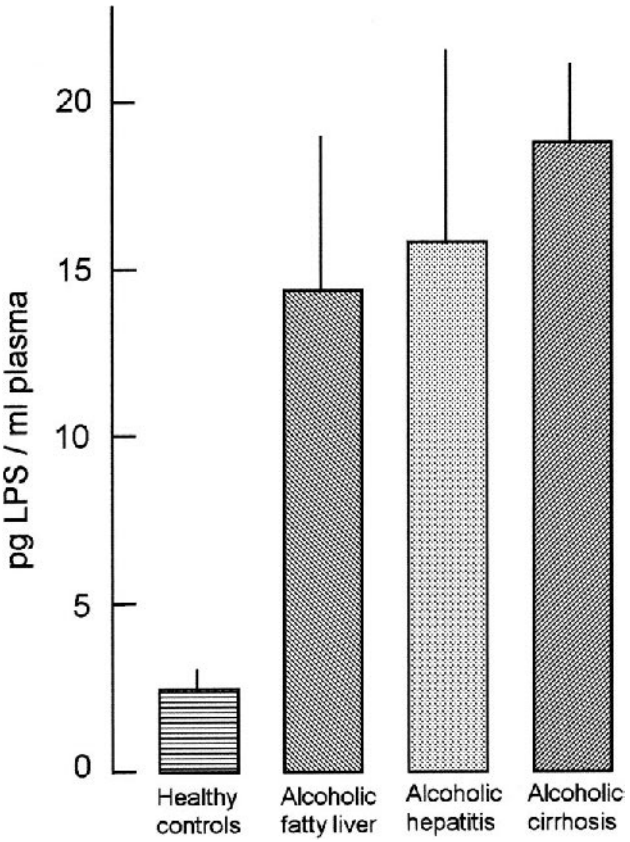

Figure 1 Plasma concentrations of LPS in patients with early stages of alcoholic liver disease and alcoholic cirrhosis compared to those in healthy controls. x ± SEM. (From ref. 23, with permission of the publisher.)

found (23; Fig. 1). A similar degree of endotoxemia was obtained in patients with mild forms of alcoholic hepatitis without fibrosis or cirrhosis. Endotoxemia was reversible in the majority of patients with alcoholic fatty and in about 50% of patients with mild alcoholic hepatitis within 1 week following cessation of alcohol intake (23). Further, transient endotoxemia was found in nonalcoholic subjects following acute alcohol intoxication (22). In the nonalcoholic subjects and in patients with alcoholic fatty liver portosystemic shunting is unlikely to play a role in the occurrence of endotoxemia. The observation of the reversible increase in LPS concentration in the peripheral venous blood following acute ingestion of large amounts of alcoholic beverages fits the hypothesis of an enhanced translocation of LPS from the gut mentioned earlier.

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B. Effect of Alcohol Consumption on LPS Inactivation and Binding of LPS to Plasma Factors

For more than two decades Kupffer cells have been known to play an important role in removing many toxic compounds from the portal blood that stem from the gut, including trace amounts of LPS, thereby avoiding a ''spillover'' of this compounds in the systemic circulation (9,24). The uptake of LPS by Kupffer cells is assumed to occur by absorptive pinocytosis (26). Within the Kupffer cells LPS is metabolized in such a way that detoxification occurs (27). Chronic alcohol abuse has been shown to impair the phagocytic activity of the hepatic reticuloendothelial system and to depress the clearance of gut-derived LPS by Kupffer cells (9,26).

A second effective system to neutralize LPS is the binding of this toxin to various plasma proteins that are rapidly produced in increased amounts during the ''acute-phase response'' induced by LPS. Quantitatively important binding of LPS has been reported for high-density lipoprotein (HDL) and albumin (28). The quantitative importance of this LPS binding to plasma constituents became evident when plasma samples of alcoholics with and without liver disease were treated either with a solvent for LPS (triethanolamine) or with a detergent (Tween 80). The extra fraction of LPS obtained following this treatment (''hidden LPS'') was shown to be about 2–4-fold higher than the LPS concentration obtained by the standard LPS assay (28). The increase in the concentration of hidden LPS in plasma samples of patients with early stages of alcoholic liver disease, such as fatty liver and mild alcoholic hepatitis, compared to that observed in healthy controls was even more pronounced (6–10-fold) than the increase of the LPS concentration observed when the standard LPS assay was used (28).

The concentration of hidden LPS in the blood of patients with alcoholic liver disease revealed a significantly negative correlation with the concentrations for albumin, HDL, and transferrin, which means that more LPS is bound to these proteins in the more advanced stages of alcoholic liver disease although the plasma concentration of these proteins is decreased if the liver function is reduced. Of interest in this context are the results of a study in which the overall LPS-binding capacity of the whole blood was determined. The binding capacity for LPS was markedly reduced in blood of patients with alcoholic hepatitis and/or alcoholic cirrhosis (29). The reduced capacity to neutralize LPS by binding to plasma proteins may enhance the adverse effects of endotoxins in these patients.

VI. GUT-DERIVED ENDOTOXIN IN EXPERIMENTAL ALCOHOLIC LIVER INJURY

In rats maintained on the Tsukamoto-French model (32) of alcohol-induced liver injury the plasma levels of LPS increased distinctly, and the levels correlated

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with the pathology scores of liver injury (30). Further evidence supporting the hypothesis that gut-derived bacterial toxins are important factors in the pathomechanism of alcohol-induced liver damage in rats was obtained in studies in which the bacterial flora of the gut was suppressed. Oral administration of broad-spectrum antibiotics (polymyxin B and neomycin) significantly reduced the endotoxin level in the blood and attenuated alcohol-induced liver damage measured by serum transaminase levels and the pathology score (31). In other experiments alteration of the intestinal flora by *Lactobacillus* feeding reduced endotoxemia and severity of experimental alcoholic liver disease (33). Administration of endotoxin via the portal vein seems to be important for the exacerbation of alcoholic liver damage. When LPS was administered by constant infusion into a peripheral vein in ethanol-fed rats, alcohol-induced liver damage was not potentiated despite markedly elevated plasma endotoxin levels, suggesting the development of tolerance to LPS in these animals (34). Taken together the results of the four studies mentioned above suggest that induction of alcoholic liver injury may require increased endotoxin levels in the portal blood as a mechanism of proinflammatory cytokine production by Kupffer cells (35,36).

Of interest are recently published results on distinct gender differences regarding the effect of alcohol on endotoxin levels in plasma. When rats were fed alcohol by using the Tsukamoto-French model of enteral alcohol feeding, levels of plasma endotoxin and parameters of hepatic damage (AST levels, neutrophil infiltration, and pathological score) were all increased significantly more in females than males (37).

VII. LPS BINDING PROTEIN (LBP) AND CD14

Activation of phagocytes, including Kupffer cells, by low concentrations of LPS (pg/ml to ng/ml range) depends on the expression of the CD14 receptor. This receptor exists in two forms, membrane-associated CD14 (mCD14) and soluble CD14 (sCD14; 38). Binding of LPS to mCD14 and activation of monocytes and macrophages depends strongly on the presence of LPS-binding protein (LBP; 39). The LPS-LBP complex then activates the macrophages by binding to mCD14 (Fig. 2). In recently drinking alcoholics, serum LBP concentrations were found to be distinctly elevated. Similar to the increase in LPS levels (23,28) a 2–3-fold increase in LBP concentrations was observed in patients with early stages of alcoholic liver disease without cirrhosis (40).

Soluble CD14 contributes to neutralize endotoxin but it is also required for binding of LPS to endothelial cells and to activate these cells (41). Again, in actively drinking alcoholics with fatty liver or mild alcoholic hepatitis sCD14

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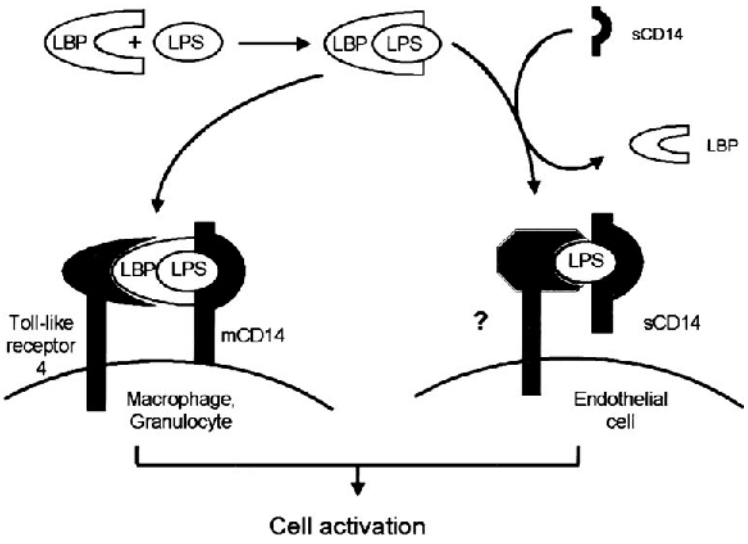

Figure 2 Involvement of LPS-binding protein (LBP), soluble and membrane-bound CD14, and the Toll-like receptor 4 in the LPS-dependent activation of macrophages and endothelial cells. (According to refs. 39 and 70.)

levels in plasma were significantly increased compared to nonalcoholic controls (40).

VIII. LPS- AND ALCOHOL-INDUCED RELEASE OF CYTOKINES AND OTHER MEDIATORS FROM KUPFFER CELLS AND OTHER MACROPHAGES

Kupffer cells, the resident macrophages of the liver, not only play an important role in host defense by phagocytizing bacteria and other foreign bodies, but also produce various potent mediators that participate in inflammation, immune responses, and modulation of liver metabolism (42). Some of the more important cytokines and other mediators that are produced by these cells in response to LPS are summarized in Figure 3. Alcohol-induced liver injury in rats was shown to be prevented by inactivation of Kupffer cells using gadolinium chloride, suggesting strongly that Kupffer cells participate in the early mechanism of alcohol-induced liver injury (34). Excessive production of proinflammatory cytokines, such as TNF-α and interleukin 1 (IL-1), reactive oxygen species, and other effectors have been implicated as casual factors in a wide spectrum of functional

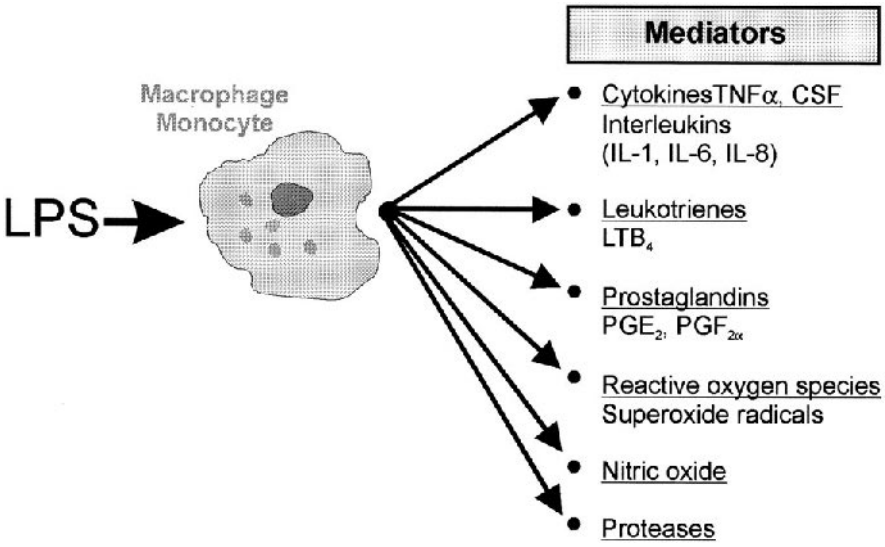

Figure 3 Schematic representation of the effects of LPS on mediator release from macrophages/monocytes. (From ref. 17, with permission of the publisher.)

alterations in the liver and liver damage (42,43). The discussion in the following section will be restricted to the effects of alcohol ingestion and LPS on TNF-α. Further discussion of the numerous studies on the effects of LPS and alcohol on other cytokines and additional mediators can be found in other reviews (17,44–46).

IX. TUMOR NECROSIS FACTOR-ALPHA (TNF-α) IN ALCOHOL-INDUCED LIVER INJURY

A. Animal Experiments

TNF-α is a central proinflammatory cytokine that is produced predominantly by the monocyte-macrophage lineage (47). During the past decade increasing evidence has accumulated that it is important in the development of alcohol-induced liver injury (Table 4). This assumption was supported by the finding that antibodies to TNF-α attenuate alcohol-induced liver injury in rats (37). In accordance with the latter results, blood TNF-α levels increased in acute endotoxin-induced liver injury in alcohol-fed rats (48) and in in vitro experiments using transfected liver cells (49). Direct evidence for this central role of TNF-α in the pathogenesis of alcohol-induced liver injury stems from recently published experiments using

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Table 4 Tumor Necrosis Factor Alpha (TNF-α) in Alcoholic Liver Disease (ALD): Important Results from Animal Experiments and Clinical Studies

Data from animal experiments
TNF-α is involved in alcohol-induced injury in transfected liver cells (49).
Antibodies to TNF-α attenuate alcohol-induced liver injury in rats (37).
Liver injury induced by chronic alcohol feeding in wild-type and TNF-α receptor 2 knockout mice was nearly completely abolished in TNF-α receptor 1 knockout mice giving support to the assumption that TNF-α plays an important role in the induction of ALD via the TNF-α 1 receptor pathway (50).
Acute and chronic alcohol administration to rats produce marked changes in both the affinity and capacity of TNF-α receptors of hepatocytes, Kupffer cells, and sinusoidal endothelial cells. LPS administration induced similar changes. The alcohol-induced changes are consistent with an increased sensitivity of these cells to the action of TNF-α (53).
Acute alcohol administration significantly increases the plasma clearance rate of TNF-α in rats in vivo (54).
Data from human studies
TNF-α plasma levels in patients with early stages of alcoholic liver disease have been found to be unchanged or increased (60,61).
In patients with advanced liver disease (cirrhosis) plasma levels of TNF-α are increased, independently of the etiology of the liver disease (17,59).
LPS-stimulated release of TNF-α from blood monocytes of patients with alcoholic liver disease is increased (61,63,64).
In a study investigating the frequency of two recently described polymorphisms of the TNF-α promotor the TNF-α allele associated with increased TNF-α expression (G_{-238}-A) was found in excess (66).

TNF receptor 1 (TNF-R1) and TNF receptor 2 (TNF-R2) knockout mice. Liver injury was induced by long-term enteral alcohol feeding in wild-type and TNF-R2 knockout mice but was nearly completely abolished if TNF-R1 knockout mice were fed in the same manner. It was concluded that the results gave solid evidence in support of the hypothesis that TNF-α plays an important role in development of early alcohol-induced liver injury via the TNF-R1 pathway (50). Of special interest is the observation that steatosis was also largely blocked in the TNF-R1-deficient mice. This finding was explained by the fact that TNF-α released by endotoxin-activated Kupffer cells stimulates lipids synthesis in the liver (51,52).

The effect of LPS and alcohol administration on the action of TNF-α is complicated by their pronounced effects on cell-surface receptors of liver parenchymal and nonparenchymal cells. Acute and chronic alcohol administration to rats produces marked changes in both the affinity and capacity of TNF-α receptors of hepatocytes, Kupffer cells, and sinusoidal endothelial cells (53). LPS administration induced similar changes. The changes in TNF-α cell-surface recep-

tors are consistent with increased sensitivity of the cells to the action of TNF-α (53). Further, acute alcohol administration significantly accelerates plasma clearance of TNF-α in rats in vivo (54). This effect might be important in humans after binge drinking. Finally, long-term alcohol consumption was shown to inhibit the regenerative effects of TNF-α (55).

In conclusion, the data from animal experiments (Table 4) strongly support the hypothesis that increased TNF-α release from Kupffer cells stimulated by gut-derived endotoxin plays an important role in the development of alcoholic liver disease. However, caution is necessary when the results are applied to the development of alcoholic hepatitis in humans because of the well-known marked differences in the types of alcohol-induced liver damage in rodents compared to alcoholic steatohepatitis and fibrosis in humans (1).

B. Association of TNF-α and Alcoholic Liver Disease in Humans

In patients with advanced stages of alcoholic hepatitis with or without cirrhosis plasma concentrations of TNF-α were found to be elevated (56,57). In another study of patients with severe alcoholic hepatitis, increased plasma levels of TNF-α were found to be associated with decreased long-term survival (58). However, marked increases in serum levels of TNF-α were also found in patients with nonalcoholic chronic liver disease, such as primary biliary cirrhosis, chronic hepatitis B and C, and autoimmune hepatitis (59). In the latter study serum levels of TNF-α were significantly higher in cirrhotics compared to noncirrhotics. It was concluded that elevated serum levels of TNF-α represent a consequence of advanced liver disease independently of the etiology of the underlying chronic liver disease (59).

Measurement of plasma TNF-α levels in patients with early stages of alcoholic liver disease, such as fatty liver and mild forms of alcoholic hepatitis, led to varying results. Both unchanged (60) and increased (61) plasma levels of TNF-α were found. In another study increased levels of soluble TNF-R1, which antagonizes the function of TNF-α, have been measured in serum of patients with alcoholic cirrhosis (62). The latter adds to the difficulty in interpreting the pathophysiological significance of increased plasma TNF-α concentrations in alcoholic liver disease.

In an attempt to obtain information about changes in the function of the monocyte/macrophage system induced by alcohol in humans, cytokine release in isolated blood monocytes has been repeatedly studied. In vitro investigation of LPS-stimulated monocytes from patients with advanced alcoholic liver disease resulted in both elevated (63,64) and reduced (65) release of TNF-α. Shortcomings in these studies were very high concentrations of the LPS stimulus, far above

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the relevant range found in patients with alcoholic hepatitis and cirrhosis (23). In a study in which markedly lower LPS concentrations were used for stimulation, a significantly enhanced release of bioactive TNF-α was observed from monocytes of recently drinking alcoholics with fatty liver and alcoholic hepatitis without cirrhosis (60). After 1 week of abstinence, the enhanced TNF-α release of these patients with early stages of alcoholic liver disease was partly reversible, while no decrease was observed in patients with alcoholic cirrhosis (61). An additional approach to test the hypothesis that TNF-α might be the ''final common pathway'' in the pathogenesis of alcoholic hepatitis in humans was a study in which the role of two polymorphisms in the TNF-α promoter were examined in determining susceptibility in alcoholic hepatitis (66). In this study the frequency of the two recently described polymorphisms of the TNF-α promoter, one at position −308 (67) and another at position −238 (68), in 150 patients with biopsy-proven alcoholic hepatitis and 145 healthy volunteers was investigated. A significant excess of the rare allele (TNF-α-A; G_{-238}-A) at position −238 was found in the group with alcoholic hepatitis compared with controls while no differences were observed for the polymorphism at position −308 (66). Since the change of the TNF-α allele at position −238 is associated with increased TNF-α expression it was hypothesized that this TNF-α allele might play a contributory role in determining the susceptibility to develop alcoholic hepatitis. In an editorial regarding this report, a number of critical questions were raised regarding the interpretation of the results and it was concluded that the interesting findings need confirmation in further investigation (69).

X. CONCLUSIONS

Various mechanisms have been proposed to be important for the pathogenesis of alcohol-induced liver injury. Among the most important are manifold metabolic alterations induced by alcohol oxidation, promotion of oxidative stress, direct toxic effects of acetaldehyde, and immunological mechanisms, especially those related to acetaldehyde-protein adduct formation (3–5). The findings reviewed here lend strong support to the hypothesis that gut-derived bacterial toxins are likely to play a key role in the development of progressive alcoholic liver disease, i.e., alcoholic hepatitis. The main sequence of events is as follows:

Alcohol abuse leads to mucosal damage in the upper gastrointestinal tract extending to erosive lesions in the stomach and duodenum, and to damage of the villi in the upper jejunum.

The mucosal injury induced by alcohol promotes an increase in gut permeability for macromolecules, enhancing the translocation of endotoxin and

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other bacterial toxins from the gut lumen to the portal blood and/or the lymphatics.

Bacterial overgrowth in the small intestine with predominant fecal flora, which is observed in about 30–50% of alcohol-abusing subjects, increases the production of endotoxin and other bacterial toxins.

The increased translocation of endotoxin from the gut to the blood vessels and/or the lymphatics leads to endotoxemia, which is already found following a single acute alcohol excess.

The intermittent increase in gut-derived endotoxin stimulates Kupffer cells and other macrophages thereby increasing the release of TNF-α (Table 4) and other potentially toxic mediators such as IL-1, IL-6, and reactive oxygen species.

Chronic overproduction of such mediators may induce the accumulation and activation of polymorphonuclear leucocytes (PMN), endothelial lesions, increased permeability of sinusoids, disturbed microcirculation, and other injurious events that finally lead to apoptosis or necrosis of hepatocytes, inflammatory infiltrates of PMNs, and deposition of collagen.

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19
Acetaldehyde and Liver Disease

Kalle Jokelainen
Helsinki University Central Hospital, Helsinki, Finland

I. ACETALDEHYDE METABOLISM

Acetaldehyde is a colorless, volatile liquid that is readily soluble in water and many organic solvents (1). Acetaldehyde is an intermediate in several biological processes and therefore occurs in nearly all living organisms. It has been identified as a natural constituent of many fruits and vegetables (2), and as a product of microbial alcoholic fermentation (3). In mammals, small amounts of acetaldehyde are produced by the normal flora of the gastrointestinal tract (4,5). Other endogenous sources of acetaldehyde, such as from the activities of deoxypentosephosphate aldolases, pyruvate dehydrogenase, and phosphorylphosphoethanolamine phosphorylase, also exist. In humans, however, acetaldehyde originates mainly from metabolism of ingested ethanol in alcoholic beverages (6). The bulk of ethanol oxidation in the body, up to 90%, takes place in the liver (7–10). In addition to the liver, almost all other organs are capable of oxidizing small amounts of ethanol, but the gastrointestinal tract is generally believed to be the major site for extrahepatic removal of ethanol. Three enzyme systems, namely the alcohol dehydrogenase (ADH) pathway, the microsomal ethanol oxidizing system (MEOS), and catalase, are involved in the initial oxidation of ethanol. All of these pathways produce acetaldehyde as the primary metabolic product. Ethanol-derived acetaldehyde is then readily oxidized further to acetate by ADH and, at least in part, by the MEOS. Acetate is almost completely converted to CO_2 and H_2O in the tricarboxylic acid cycle of peripheral tissues after reaction with coenzyme A to form acetyl-CoA (11). The remaining fraction of acetaldehyde is expired into the air (12) or excreted in the urine (13).

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A. Alcohol Dehydrogenase Pathway

The major pathway for ethanol oxidation involves ADH, a cytosolic enzyme that catalyzes the conversion of ethanol to acetaldehyde. The highest concentrations of ADH occur in the liver, but immunohistochemical studies have revealed that some ADH activity can be detected in all tissues investigated (14–16). Six major classes of ADH, arising from the association of different types of subunits into active dimeric molecules, have been postulated (17). Class I isoenzymes have low K_m (~1 mM) and high V_{max} for ethanol, and are consequently considered to be responsible for the majority of ethanol oxidation. Since the K_m for acetaldehyde is 0.6 mM, it could also act as a substrate for ADH in a reverse reaction (18). However, the rapid removal of acetaldehyde in somatic cells normally forces the reaction in a forward direction. Thus, in ADH-mediated oxidation of ethanol, hydrogen is transferred from the substrate to the cofactor nicotinamide adenine dinucleotide (NAD), converting it to its reduced form (NADH), and generating acetaldehyde.

This reaction is generally held to be the rate-limiting step in the total metabolism of ethanol. As a net result, the first step in the oxidation of ethanol generates an excess of reducing equivalents in the cell, primarily as NADH. The altered redox state, in turn, is responsible for a variety of metabolic abnormalities associated with alcohol consumption. The incidence of several ADH forms varies in different ethnic populations (17,19). ADH phenotypes have been correlated with the production of acetaldehyde and the extent of first-pass elimination of ethanol (16). More recently, some positive associations between ADH genotype and alcohol-related liver injury or even alcoholism have been reported (20–23).

B. Microsomal Ethanol Oxidizing System

A cytochrome P-450-dependent MEOS was first described by Lieber and DeCarli in 1968 (24). MEOS is located in the endoplasmic reticulum, and requires NADPH and oxygen. A specific ethanol-inducible isoenzyme of P-450 has been isolated from the livers of ethanol-treated rats and rabbits (25,26). The existence of the P-450 isoenzyme, with a high capacity to oxidize ethanol and other specific substrates, has also been confirmed in humans, and this ethanol-inducible P-450 has been designated CYP2E1 (27). The optimal pH for MEOS is within the physiological range, and the K_m for ethanol is 8–10 mM (28). The main characteristic of MEOS is that chronic ethanol consumption significantly increases MEOS activity (24,29). Accordingly, it may play a significant role in ethanol metabolism, at least when induced, and may account for up to 10% of total ethanol oxidation in heavy drinkers (30). In addition, there is recent evidence that P-450s other

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than CYP2E1, such as CYP1A2 and CYP3A4, may also significantly contribute to total ethanol oxidation by MEOS (31).

Recent studies have suggested that in vitro CYP2E1 can act as an additional pathway for acetaldehyde metabolism as well (32). It has been shown that small amounts of acetaldehyde are oxidized to acetate through MEOS (33,34). However, it may be of negligible quantitative importance since rat liver microsomes appear to oxidize acetaldehyde at a much lower rate than ethanol, and this acetaldehyde metabolism is strikingly inhibited by ethanol (32). Accordingly, because acetaldehyde production vastly exceeds its oxidation, the net result of MEOS activity is accumulation of this toxic metabolite.

C. Catalase

Catalase is a hemoprotein located in the peroxisomes of most tissues. Catalase is capable of oxidizing ethanol only in the presence of a hydrogen-peroxide-generating system. The reaction is limited by the rate of hydrogen peroxide generation from hypoxanthine, water, and oxygen by xanthine oxidase. The physiological rate of hydrogen peroxide production is small, suggesting that catalase does not play a significant role in ethanol metabolism. It has been proposed that no more than 2% of ethanol is oxidized by the catalase-mediated mechanism (35). However, rat gastric mucosa has recently been shown to possess significant catalase activity, suggesting that catalase may play a role in gastric ethanol metabolism, at least in vitro (36).

D. Aldehyde Dehydrogenase

Ethanol-derived acetaldehyde is readily oxidized further to acetate in a reaction catalyzed by aldehyde dehydrogenase (ALDH). The cofactor in this reaction is NAD, which is reduced to NADH (37). In comparison to ADHs, ALDHs are still a relatively understudied family of enzymes. They can, however, be grouped into four or five classes that consist of both constitutive and inducible isoenzymes. In humans, ALDH isoenzymes can be found in mitochondrial and cytosolic cellular fractions. The isoenzyme mainly responsible for the oxidation of acetaldehyde is mitochondrial *ALDH2*, which has a micromolar K_m for acetaldehyde. The enzyme is polymorphic, and two alleles, $ALDH2^1$ and $ALDH2^2$, have been recognized (38,39). Allele $ALDH2^2$ is relatively common in Asian populations and encodes a partially inactive enzyme subunit (40). Individuals carrying this allele accumulate acetaldehyde in their liver and blood during ethanol oxidation, which leads to alcohol-sensitivity symptoms, the so-called flushing reaction.

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II. HEPATOTOXICITY OF ACETALDEHYDE

Acetaldehyde possesses toxic properties that far exceed those of its parent molecules. The electrophilic nature of carbonyl carbon of acetaldehyde makes it susceptible to attachment to various nucleophilic groups in cellular macromolecules (41,42). As ingested ethanol is oxidized to acetaldehyde mainly in the liver, and detectable levels of acetaldehyde are found in the liver (43), acetaldehyde has long been linked to the development of alcoholic liver disease. Indeed, extensive evidence supporting a role of acetaldehyde in the detrimental actions of alcohol on the liver has been accumulated in recent years (for reviews, see refs. 44,45). A number of mechanisms by which ethanol-derived acetaldehyde may contribute to alcoholic liver disease have been suggested.

A. Acetaldehyde-Protein Adducts

Acetaldehyde has properties that make it very suitable for potential nucleophilic attacks (46). Since nucleophilic groups are present in proteins, they are natural targets for reactive acetaldehyde in different tissues (47). Theoretically, the α-amino groups of valine, ϵ-amino groups of lysine, OH groups of tyrosine, and thiol groups of cysteine could react with acetaldehyde. The reaction of free amino groups with acetaldehyde is a two-step, acid-catalyzed process resulting in the formation of Schiff bases (48). The products of this reaction are unstable adducts that serve as intermediates in stable adduct formation, and that can be stabilized by a reduction of ethyl groups (49). This stabilization can be achieved by reducing agents, such as NADH, found in large amounts in the liver during ethanol metabolism. It has been well established that acetaldehyde adduct formation occurs in the liver during ethanol oxidation in vivo in both experimental animals and humans (50–52). In addition, acetaldehyde has been shown to bind covalently to many cellular and extracellular proteins, such as tubulin (53), erythrocyte membrane proteins (54), albumin (55,56), hemoglobin (57), plasma proteins (58), microtubule proteins (59), hepatic proteins including CYP2E1 (55,60,61), ribonuclease (62), lipoproteins (63,64), and collagen (65,66). It appears that proteins contain lysine residues with varying relative reactivities toward acetaldehyde adduct formation and that certain proteins, e.g., α-tubulin, may serve as selective targets for adduct formation in cellular systems by virtue of containing especially reactive lysine residues (67).

To investigate the localization of acetaldehyde-protein adducts, immunohistochemical studies have been performed with specific antibodies. Adducts have been demonstrated mainly in the cytoplasm of perivenular hepatocytes, where acetaldehyde production is believed to be the highest (68,69). A more recent immunohistochemical study showed that acetaldehyde adducts can be detected in the areas of active fibrogenesis in the liver biopsy specimens from alco-

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holic patients (70). Recently, acetaldehyde adducts have been demonstrated in the rough endoplasmic reticulum (RER), and in some peroxisomes of hepatocytes, but also in myofibroblasts and Ito cells (71). In addition to intracellular acetaldehyde-protein adducts, hepatic acetaldehyde-modified epitopes have been found in the extracellular compartment (70).

Although covalent binding of acetaldehyde to hepatic proteins during ethanol metabolism has been well established, the precise role of acetaldehyde-protein adducts in the pathogenesis of alcoholic liver disease has not been clarified. One mechanism through which adducts could cause toxicity may involve the altered biological properties of adducted proteins. For example, covalent binding of acetaldehyde to enzymes has been shown to specially inhibit their activities (72). Acetaldehyde appears to inhibit the function of human DNA repair protein, O^6-methylguanine transferase, even at nanomolar concentrations (73). In addition, binding of acetaldehyde has been demonstrated to cross-link membrane proteins (54), and to interfere with the function of certain regulatory proteins such as calmodulin (74). Because of its high affinity for the sulfhydryl groups of cysteine, acetaldehyde readily binds to tubulin, thereby altering the capacity of tubulin to polymerize to form microtubules (46,75,76). This has been shown to impair microtubule formation and function (47,76). In addition, acetaldehyde-protein adducts may be recognized as neoantigens by the immune system, thus triggering potentially harmful immune responses directed against liver cells (77,78). It has been documented that patients with alcohol-associated liver disease have substantial abnormalities in both their humoral and cellular immune responses (78,79). The presence of circulating antibodies against acetaldehyde-protein adducts has been described in humans (77). Lymphocytes from patients with alcoholic liver damage appear to immunologically recognize their own hepatocytes and mount either cytotoxic or lymphoproliferative responses (80,81). As several observations indicate that immunological mechanisms are involved in alcoholic liver disease, the appearance of circulating antibodies against acetaldehyde-protein adducts may account for the development and progression of liver injury (82).

B. Promotion of Lipid Peroxidation

A free radical is generally defined as a molecule that contains one or more unpaired electrons. As the presence of unpaired electrons usually confers a large degree of chemical reactivity to the molecule, most free radicals are highly reactive, with a short half-life. Oxidizing free radicals (superoxide and hydroxyl radicals) may lead to cell injury by abstracting a hydrogen atom from a polyunsaturated fatty acid, initiating the degradative process known as lipid peroxidation.

Glutathione, a tripeptide consisting of glutamic acid, cysteine, and glycine, is present in all animal cells in high concentrations. One of its functions is the

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protection of cells from free radicals. As previously mentioned, free radicals can damage cellular components by reacting with lipids, proteins, nucleic acids, and lipoproteins. A severe reduction in the levels of glutathione has been shown to increase lipid peroxidation in vivo (83). As lipids are major components of biological membranes, it is likely that peroxidative processes on the membranes may lead to tissue injury.

Reactive oxygen intermediates are produced by the MEOS and xanthine oxidase pathway during ethanol oxidation (84). In agreement with this, enhanced lipid peroxidation, possibly mediated by acetaldehyde (85), was proposed as a mechanism for ethanol-induced liver injury several decades ago (86). Acetaldehyde has been suggested to contribute to the reduction in liver glutathione levels by binding with cysteine and/or glutathione after chronic ethanol feeding (87). The capacity of acetaldehyde to induce lipid peroxidation has been demonstrated in isolated perfused livers (88). In addition, incubation of rat liver supernatant with acetaldehyde was observed to result in the conversion of xanthine dehydrogenase to xanthine oxidase, an enzyme known to generate superoxide radicals (89). Whether ethanol administration enhances in vivo lipid peroxidation has long been debated (90–93). More recent studies, however, have supported this ability in laboratory animals (87) and in humans (94). Furthermore, high acetaldehyde concentrations administered to rats have been shown to result in the formation of free radical reactions in vivo (95). Taken together, ethanol-derived acetaldehyde may lead to a severe reduction in glutathione, which favors lipid peroxidation, and the damage is possibly compounded by the increased generation of active radicals through induced MEOS following chronic ethanol consumption. In addition, the peroxidation may be promoted by mobilization of iron from ferritin by NADH-dependent mechanism (96). Lipid peroxidation may play a prominent role in the carcinogenic process (97,98). Accordingly, reactive oxygen radicals generated during ethanol metabolism may also contribute to an increased risk of cancer associated with alcohol abuse.

C. Alterations of the Mitochondria

Electron microscopy has revealed marked morphological alterations, such as swelling and abnormal cristae, in the liver mitochondria of patients with alcoholism. The structural abnormalities are associated with functional impairments, e.g., decreased oxidation of fatty acids and acetaldehyde (99). Furthermore, a reduced mitochondrial respiratory capacity was observed (100,101). As chronic ethanol consumption suppresses a variety of important mitochondrial functions even at low acetaldehyde levels (102), acetaldehyde has been suggested to contribute to alcoholic mitochondrial injury (103). Functional changes in mitochondria may also be related to alteration of cellular membranes as a result of long-term ethanol consumption (104,105).

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D. Stimulation of Collagen and Cytokine Production

Acetaldehyde has also been incriminated as a pathogenetic factor contributing to alcohol-associated stimulation of collagen synthesis in cultured liver myofibroblasts (106), human fibroblasts (107), rat liver fat-storing cells, and Ito cells (108). Acetaldehyde has been demonstrated to increase the transcription rate of the type I collagen genes by mechanisms that are protein-synthesis-dependent (106,109). There is also evidence suggesting that part of the stimulation is exerted posttranscriptionally, because acetaldehyde can interfere with the feedback down-regulation of type I collagen synthesis by modifying its carboxy-terminal propeptide (110). In addition to type I collagen, acetaldehyde increases the expression of other extracellular matrix components, including type III collagen and fibronectin (109). Moreover, acetaldehyde has been demonstrated to decrease the expression matrix metalloproteinase-1 (MMP-1), an interstitial collagenase responsible for the degradation of type I collagen (111).

Cytokines are small peptides produced by many different types of cells, including monocytes, macrophages, endothelial cells, and various epithelial cells. Each cytokine binds to its receptor on the cell surface and has the potential to activate a number of transcription factors (112). Each of these transcription factors, in turn, can regulate the transcriptional activity of multiple different target genes. Emerging evidence suggests that ethanol, and its first metabolite acetaldehyde, influence cytokine production and activation of transcription factors (113). Serum concentrations of several proinflammatory cytokines, including tumor necrosis factor (TNF)-α, interleukins-1 (IL-1), 6 (IL-6), and 8 (IL-8) have been shown to be increased in patients hospitalized with alcoholic hepatitis (114–116). In such patients, cytokine concentrations have been demonstrated to correlate with the severity of the liver disease and normalize during recovery (114,115,117,118). Since cytokines, such as TNF-α, modulate the behavior of many cells by regulating the expression of a wide array of genes, it seems likely that one mechanism by which ethanol metabolism influences hepatocytes in liver injury is by changing the activity of cytokine-regulated transcription factors.

E. DNA Adducts

Acetaldehyde has been demonstrated to form DNA adducts (119), induce chromosomal abnormalities (120), and increase sister chromatid exchanges in numerous cell types (121–124). Accordingly, after chronic ethanol administration to mice, acetaldehyde-DNA adducts have been found in the liver (125). Because formation of DNA adducts is considered to be a critical initiating event in carcinogenesis (126), these results suggest that formation of DNA adducts and other chromosomal abnormalities caused by ethanol-derived acetaldehyde could be one

possible mechanism explaining the association between heavy drinking, alcoholic cirrhosis, and hepatic cancer.

III. OPEN QUESTIONS

Although extensive evidence supporting a role of acetaldehyde in the pathogenesis of alcoholic liver disease has been accumulated during recent years, there are, however, many open questions that have to be addressed in further studies.

A. Do Elevated Acetaldehyde Levels Exacerbate Alcoholic Liver Disease?

If acetaldehyde contributes to the pathogenesis of alcoholic liver damage, *ALDH2*-deficient individuals with the *ALDH2*^{2} allele (and, consequently, with higher hepatic acetaldehyde levels) should be the most likely candidates for development of ethanol-related liver diseases. However, *ALDH2*-deficient individuals with alcoholic liver disease are relatively rare, although variable incidences of *ALDH2* phenotypes in alcohol drinkers have been reported (127–129). In addition, association studies concerning *ALDH2* and *ADH2* (''superactive ADH'') gene polymorphism and alcohol-induced organ damages (130,131) have revealed some positive findings, but the mechanisms underlying these associations have remained speculative. It must be emphasized, however, that individuals with the *ALDH2*^{2} allele can be at lower risk for developing alcohol-induced liver damage simply because these individuals do not usually drink large amounts of ethanol in order to avoid unpleasant symptoms resulting from high blood acetaldehyde levels (132). Enomoto et al. (133) reported that rates of alcohol consumption among alcoholic liver disease patients with the mutant *ALDH2* gene were clearly lower than those among patients with the normal gene. However, the incidences of alcoholic fibrosis and cirrhosis were lower, whereas the incidence of alcoholic hepatitis tended to be higher among the *ALDH2*-deficient individuals as compared to the incidences of those among the individuals with normal enzyme activity. Although the differences did not reach statistical significance, the authors postulated that habitual drinkers who possess the mutant *ALDH2* gene may be at higher risk for alcoholic liver disease. In addition, this finding was recently challanged by Yang et al. (134), who reported that individuals with the normal *ALDH2* gene are more susceptible to develop alcoholic liver damage than those with the mutant allele, not only because they usually consume more alcohol, but also because they might suffer more severe hepatic injury when exceeding a certain level of alcoholic intake.

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In animal studies, the possible role of elevated acetaldehyde levels in the pathogenesis of alcoholic liver disease has been investigated in an intragastric feeding model where rats were fed a liquid diet containing fish oil and ethanol. Sustained elevation of liver acetaldehyde was achieved by daily treatment with two inhibitors of ALDH (135). It was demonstrated that treatment with the ALDH inhibitors led to increased acetaldehyde concentrations in the liver and blood but prevented hepatic necrosis and inflammation, whereas steatosis was not affected. This was accompanied by down-regulation of TNF-α and COX-2 in the inhibitor-treated groups. Taken together, these facts do not support the notion that acetaldehyde, a chemically more reactive substance than ethanol, promotes inflammatory changes in alcoholic liver disease.

B. What Is the Role of Intracellular Acetaldehyde-Protein Adducts in the Pathogenesis of Alcoholic Liver Disease?

Acetaldehyde-protein adducts have been demonstrated in the liver of alcohol-fed rats (136,137), micropigs (138), and patients with chronic alcohol abuse (139,140). Furthermore, acetaldehyde-protein adducts have been described in the centrilobular region of the liver of alcoholic patients, colocalizing with the areas of fatty infiltration, focal necrosis, and fibrosis (141). By ultrastructural immunohistochemistry, acetaldehyde adducts have been detected in the rough endoplasmic reticulum, in some peroxisomes, and in the cytosol of hepatocytes. Furthermore, in patients with steatofibrosis or cirrhosis, adducts in Ito cells were also observed (71). The presence of intracellular adducts in the different stages of liver disease has been considered to support the view that these modifications have a specific role in the pathogenesis of alcoholic liver disease. Interestingly, however, acetaldehyde-protein formation has been described to be independent of the noxious agent used to induce liver damage (142). Accordingly, acetaldehyde-protein adducts have also been demonstrated in patients with nonalcoholic liver disease (143). It has been suggested that the presence of adducts in nonalcoholic liver disease is the result of occasional ethanol intake, or that it can originate from physiological substrates or from ethanol produced endogenously by the gastrointestinal tract (4,144). Recently, Holstege et al. (70) reported that acetaldehyde-protein adducts are localized not only in the intracellular compartment but also in the extracellular matrix. Moreover, they found that no correlation existed between the immunohistochemical staining of the intracellular acetaldehyde-protein adducts and the histologically assessed severity of liver disease. In contrast, extracellular staining was often associated with an inflammatory cellular infiltrate and also seen in areas of histologically assessed active fibrogenesis. Furthermore, the presence of extracellular adducts significantly correlated with the progression of liver fibrosis. These results strongly suggest that intracellular acetaldehyde-

protein adducts do not exert any direct cytotoxicity, whereas extracellular adducts are associated with active inflammatory processes, collagen production, and, accordingly, more advanced liver disease.

C. What Is the Role of Acetaldehyde of Microbial Origin in the Pathogenesis of Alcoholic Liver Disease?

The gastrointestinal microflora is metabolically very active, and it is considered to be an important organ in its own right. Any compound taken orally, entering the intestine via biliary tract or the bloodstream, or any substance secreted into the lumen of the gut is a potential substrate for microbial transformation (145,146). The short generation time also allows the bacteria to adjust rapidly to any change in the environment (147). As a metabolically very active and flexible ''organ,'' microflora is therefore very likely to be involved not only in many physiological processes but also in many pathological processes of the gastrointestinal tract (148). Bacteria representing the normal colonic flora in humans have been demonstrated to posses significant ADH activities and, accordingly, generate high levels of acetaldehyde when incubated with ethanol in vitro (149). It has been shown that human colonic contents are capable of oxidizing ethanol to acetaldehyde in vitro at the comparatively low ethanol concentrations known to exist in the colon during social drinking (150). Furthermore, ingested ethanol has been shown to be effectively oxidized to acetaldehyde also in the colon in vivo, resulting in marked intracolonic acetaldehyde concentrations during normal ethanol metabolism (5). Colonic bacteria could thus provide a clinically significant source of acetaldehyde in the large bowel and this acetaldehyde may be absorbed from the colon to the portal blood, and, subsequently, be metabolized further in the liver and other tissues (151). High intracolonic acetaldehyde levels may also lead to the development of diarrhea, colorectal cancer, and colon polyps in heavy drinkers (152,153). Furthermore, intracolonically produced acetaldehyde may be an important determinant of blood acetaldehyde level and a potential hepatotoxin (153), possibly contributing to the formation of extracellular acetaldehyde-protein adducts.

IV. CONCLUSIONS

Acetaldehyde, the primary metabolite of ethanol oxidation, has long been suspected to be the pathogenetic factor behind ethanol-induced organ damages. Acetaldehyde has been shown to react with physiologically important proteins, such as albumin, collagen, tubulin, and lipoproteins, to form acetaldehyde adducts. Such acetaldehyde-protein adducts may interfere with the structural or enzymatic functions of the proteins. Furthermore, acetaldehyde-modified proteins may act

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as neoantigens, thus generating immune responses and initiating autoimmune processes. Acetaldehyde and its metabolism have also been demonstrated to enhance lipid peroxidation and alter mitochondrial functions, possibly sensitizing the mitochondria to the toxic effects of acetaldehyde. In addition, acetaldehyde has been shown to stimulate collagen production and has been suggested to enhance cytokine production and release. Recently, however, it was demonstrated that elevated liver acetaldehyde levels in rats and in humans do not result in exacerbation of alcoholic liver disease. Moreover, the lack of correlation between the immunohistochemical staining of intracellular acetaldehyde-protein adducts and histologically assessed severity of liver disease has challenged the role of intracellular adducts in the pathogenesis of ethanol-induced liver damage. It has recently been suggested that the intracolonic production of acetaldehyde from ingested ethanol by gastrointestinal bacteria may be associated with the development of extracellular acetaldehyde-protein adducts in the liver and increased permeability of the gut for bacterial endotoxins resulting in endotoxemia and Kupffer cell activation. Thus, it is conceivable that ethanol-derived acetaldehyde contributes to the pathogenesis of alcoholic liver disease but a more precise knowledge of the origin of acetaldehyde adducts, further characterization of cell types involved, and further innovative approaches to prove causal effects are required.

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The Role of Kupffer Cells in Alcoholic Liver Disease

Carl M. Oneta
University Hospital of Lausanne, Lausanne, Switzerland

I. INTRODUCTION

The pathogenesis of alcoholic liver disease, especially that of alcoholic fatty liver, can widely be explained by toxic effects due to ethanol metabolism in hepatocytes, and it is generally accepted that liver fibrosis is mainly mediated by hepatic stellate cells (1). However, a lot of evidence has emerged during the last 10 years that Kupffer cells may act as key cells in mediating especially the processes of alcohol-induced liver inflammation and necrosis, and furthermore, they are probably responsible for activation of hepatic stellate cells to transform into transitional and myofibroblast-like cells, which in turn produce extracellular matrix components including collagen (2–4). In response to alcohol, but also other factors, Kupffer cells become activated and produce a large array of different important mediators such as prostaglandins, reactive oxygen species, cytokines, nitric oxide, and different proteases. In this chapter, I will focus on the evidence that Kupffer cells play an important role in the pathogenesis of alcohol-induced liver injury and confine attention to experiments dealing primarily with alcohol as the toxic agent. Based on different experiments, several hypotheses about the role of Kupffer cells in alcoholic liver disease and the mechanisms of how they are activated have been carried out during the last 10 years. Some of them, which seem to be the most promising, will be presented and discussed in detail. All have one basic pathogenetic mechanism in commen, namely that Kupffer cells are directly or

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indirectly activated by alcohol to produce various mediators, which influence the function of other liver cells leading to liver injury (Fig. 1).

Kupffer cells are macrophages belonging to the reticuloendothelial system, which is recruited from the stem cells of the bone marrow. They are part of the so-called nonparenchymal cells of the liver, account for about 15% of the liver cell population, and comprise 80–90% of the body's resident macrophage population. They are predominantly found in the periportal region, but also, to a lesser extent, in the midzonal and pericentral regions of the hepatic lobules. They reside in the liver sinusoids in close relationship to the parenchymal (hepatocytes) and other nonparenchymal cells, which are sinusoidal endothelial cells, hepatic stellate cells, and pit cells (5–7). The main properties of Kupffer cells are endocytosis with destruction of ingested material, antigen presentation, and the secretion of biologically active mediators, as reviewed by Decker (5).

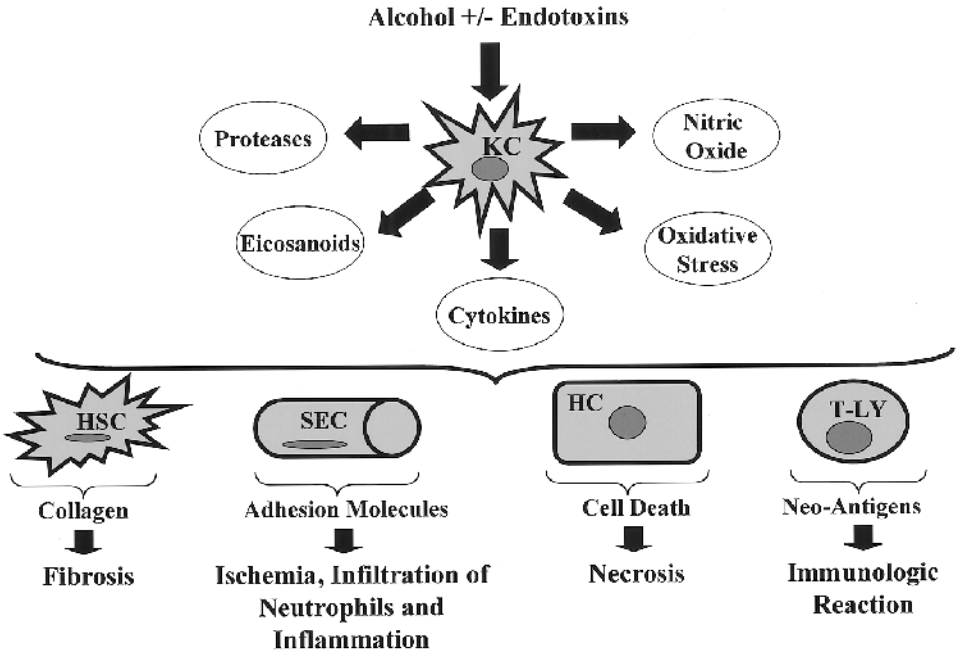

Figure 1 Simplified schematic representation of the involvement of liver cells in alcohol-induced liver inflammation, necrosis, and fibrosis showing Kupffer cells as key cells in these processes. HC = hepatocytes; HSC = hepatic stellate cells; KC = Kupffer cells; SEC = sinusoidal endothelial cells; T-LY = T lymphocytes.

II. EVIDENCE FOR THE INVOLVEMENT OF KUPFFER CELLS IN ALCOHOLIC LIVER DISEASE

On the one hand, in vitro and in vivo studies have demonstrated that alcohol impairs the phagocytic function of Kupffer cells, and therefore results in an enhanced exposure of the liver to endotoxins (8–11). On the other hand, there exists evidence that Kupffer cells can also be activated by ethanol to proliferate and to mediate liver injury (12–15). Karakucuk et al. were able to show that Kupffer cells are present in an increasing number in the portal tracts in humans with acute and chronic alcoholic liver disease by using immunohistochemical methods with macrophage-specific monoclonal antibodies (12), and Eguchi et al., using in vivo microscopy, demonstrated in mice that exposure to low doses of ethanol led to an increased number of Kupffer cells showing ultrastructural features of activation (13). Recently, Adachi et al. performed an elegant study to examine the effect of Kupffer cell inactivation on early events of alcohol-induced liver injury in rats using the Tsukamoto-French feeding model with and without ethanol during a 4-week period (14). Kupffer cell inactivation was achieved by intravenous administration of gadolinium-chloride ($GdCl_3$) twice weekly. $GdCl_3$ administration to alcohol-fed rats resulted in complete prevention of the gradually increased aspartate aminotransferase (AST) blood levels seen in alcohol-fed animals not treated with $GdCl_3$ and in a striking decrease in liver pathology scores, not only for necrosis and inflammation, but also for steatosis. Necrosis and inflammation were observed predominantly in pericentral regions of ethanol-fed rats, which led—in view of the fact that Kupffer cells are primarily located in the periportal regions—to the assumption that alcohol induces a hypermetabolic state in hepatocytes. Furthermore, because the inactivation of Kupffer cells prevented the so-called ''swift increase in alcohol metabolism'' in hepatocytes (16) in another study performed by Bradford et al. (17), it was hypothesized that Kupffer cells stimulate ethanol metabolism in hepatocytes and consequently cause pericentral hypoxia, probably by secreting mediators (vide infra). A study similar to that of Adachi et al. (14) has been published by Goldin et al. where the same effect of Kupffer cell inactivation on liver pathology was shown in mice treated with ethanol (18). However, this study failed to demonstrate an association between increased liver pathology and an enhanced cytokine production, measuring TNF-α levels in the serum of mice. Finally, it has recently been shown that inactivation of Kupffer cells prevents alcohol-induced liver injury in rats despite the induction of cytochrome P4502E1 (CYP2E1) in liver cells (19). This allows the assumption that the ethanol-induced activation of Kupffer cells is more important in causing hepatocyte damage and severe liver injury like inflammation and necrosis than the effects of ethanol metabolism through the CYP2E1 pathway in hepatocytes.

Therefore, it can be concluded that alcohol activates Kupffer cells to ac-

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tively participate in the pathogenesis of alcoholic liver disease by mediating hepatocyte damage and severe liver injury such as inflammation and necrosis.

III. ACTIVATION OF KUPFFER CELLS BY ETHANOL TO PRODUCE FACTORS THAT INFLUENCE OTHER LIVER CELLS, AND MECHANISMS OF ACTIVATION

One theory implies that Kupffer cells in chronically ethanol-exposed individuals are activated by gut-derived endotoxins (20). The latter are well known as major inducers of a large amount of different mediators in Kupffer cells (5). Especially one study performed by Thurman's group strengthens this theory by showing prevention of alcohol-induced liver injury in rats as a consequence of gut sterilization (15). The role of endotoxins in alcoholic liver disease is extensively discussed in the chapter by Bode (this volume); therefore, I do not go into further detail. Another theory, developed mainly by Lieber's group, implies that Kupffer cells might also be directly activated by ethanol itself or by its metabolite acetaldehyde (21). In reviewing alcohol's effects on Kupffer cells, it therefore seems to be important to know the experimental conditions by which Kupffer cell activation has been achieved. Of particular importance is the method by which animals are fed. In the study by Adachi et al. (15) as well as in other studies (see below), an intragastric animal feeding model (so-called Tsukamoto-French feeding model) was used (22). It allows on the one hand to applicate liquid diets by continuous intragastric infusion and therefore a better control of the diet consumed, and possibly an increase in the overall dose of ethanol administered as compared to the conventional Lieber-DeCarli method (23). On the other hand, the Tsukamoto-French feeding model has been criticized as ''unphysiological'' and, thereby, possibly exacerbating the extent of alcohol's effect on the liver (24). As demonstrated in the study by Adachi et al., feeding of animals with this method leads to endotoxemia, which probably worsens the ''simple'' effect of alcohol (15). In contrast, endotoxinemia probably does not occur when using the ''more physiological'' and less aggressive Lieber-DeCarli feeding model (25). Based on several in vitro and in vivo studies, there exists a lot of evidence that Kupffer cells can directly or indirectly (via endotoxins) be activated by ethanol to produce important factors that influence the function of other liver cells. In the following, I try to present mainly evidence for direct ethanol effects on Kupffer cells with special regard to the mechanisms of Kupffer cell activation.

A. Activation of Kupffer Cells to Produce Cytokines

The classic experiments, showing that Kupffer cells produce cytokines in vitro in experimental alcoholic liver disease using the Tsukamoto-French feeding

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model, were published by Matsuoka and Tsukamoto in 1990 (26,27). They have been able to demonstrate that Kupffer cells derived from alcohol-fed animals express mRNA for TGF-β_1 and release active and latent forms of this cytokine leading to proliferation of hepatic stellate cells in vitro and to an increased collagen production by these cells. Endotoxins have not been measured in the different study groups. This study provided a direct link between the activation of Kupffer cells by ethanol and/or endotoxins and the activation of hepatic stellate cells with TGF-β_1 as the responsible mediator of this intercellular communication. In contrast, Earnest et al. used the Lieber-DeCarli method to feed their rats (25). Ethanol treatment resulted not only in an increased expression of mRNA for TNF-α in isolated Kupffer cells and in an increased release of this cytokine into the culture medium, but also in a stimulation of their phagocytic and metabolic activity. And, as stated by the authors, these changes in Kupffer cell activity were probably not due to an increased absorption of endogenous intestinal endotoxins since blood levels of endotoxins were not elevated and did not change during the study. Therefore, it seems that these Kupffer cells have been directly activated by ethanol. The increased production of TNF-α in alcoholic liver disease is an important finding, since TNF-α plays a key role as mediator of inflammation and cell death (28,29). In addition, the importance of TNF-α as a mediator of ethanol-induced hepatotoxicity is emphasized by the experimental finding of a total blockage of the increase in AST-blood levels in rats receiving an alcohol diet for 4 weeks in the presence of anti-TNF-α antibodies (30). Treatment with anti-TNF-α antibodies also resulted in a significant attenuation of liver inflammation and necrosis, whereas no significant attenuation of the rate of steatosis was noted. This can be explained by the fact that steatosis is probably more a consequence of direct ethanol metabolism in hepatocytes rather than of paracrine actions of TNF-α or of Kupffer cell activation.

Recently, the coordinate induction of TNF-α, TGF-β_1, and IL-6 mRNA expression by Kupffer cells has been shown in progression of experimental alcoholic liver disease by Kamimura and Tsukamoto again using the intragastric feeding model (31). Interestingly, IL-6 mRNA expression in Kupffer cells was only slightly induced after 10 weeks of alcohol administration compared to the other two cytokines, whereas it showed a remarkable up-regulation after 17 weeks. Because this up-regulation of IL-6 mRNA expression coincided with alcoholic liver fibrogenesis, it was argued that IL-6 might also have a fibrogenic role in alcoholic liver disease.

In conclusion, chronic exposure of Kupffer cells to alcohol activates them to produce important amounts of cytokines with cytotoxic, chemotactic, and fibrogenic properties that actively influence the function of parenchymal and/or other nonparenchymal liver cells. The initiation of Kupffer cell activation in alcoholic liver disease is most likely mediated by direct alcohol effects, by endogenous endotoxins (see chapter by Bode, this volume), or by both factors at the same time.

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B. Activation of Kupffer Cells to Produce Eicosanoids

Kupffer cells are the major producers of eicosanoids in the liver (2,5). They are made from arachidonate by two pathways, one involving cyclooxygenase, the other lipoxygenase. The main products of the former are prostaglandins (PGD_2, PGE_2, PGF_2), prostacyclin (PGI_2), and thromboxanes (TXA_2, TXB_2), and of the latter—among others—leukotrienes (5).

1. Activation of Kupffer Cells to Produce Thromboxanes

It has been shown by Nanji et al. that lipids enriched in polyunsaturated fatty acids modulate the severity of alcohol-induced liver injury (32). The polyunsaturated fatty acid responsible for this promoting effect is linoleic acid (33). It has been hypothesized that the metabolism of linoleic acid to arachidonic acid and the selective utilization of arachidonic acid for the synthesis of eicosanoids could be a possible mechanism by which alcohol causes liver injury (34). Recently, Kupffer cells from ethanol-fed rats have also been identified as the most likely source of the enhanced synthesis of TXA_2, the unstable precursor of TXB_2, by immunohistochemical methods evaluating thromboxane synthase mRNA in the individual liver cell types (35). Endotoxins have been proposed as stimulus for the enhanced synthesis because they have been found to be increased in rats fed corn oil and ethanol (36). In addition, in vitro studies on Kupffer cells have demonstrated that both endotoxins and ethanol increase the secretion of the major prostaglandins and free arachidonic acid into the culture medium (37). Endotoxins probably cause the synthesis of the inducible cyclooxygenase-2 (COX-2) in Kupffer cells, whereas ethanol exerts its effect through the constitutive cyclooxygenase-1 (COX-1) in part by increasing the free arachidonic acid concentration (37).

Earlier studies performed by Nanji et al. demonstrated that the production of TXB_2 by liver nonparenchymal cells was increased in rats treated with corn oil (containing linoleic acid) and ethanol, and that the plasma TXB_2 levels correlated significantly with the severity of liver pathology (38). Moreover, application of thromboxane synthase inhibitors to these animals resulted in attenuation of necroinflammatory changes caused by ethanol as well as in a reduction of the severity of fatty liver (39). This reduction in inflammatory changes was accompanied by a decrease in mRNA for TNF-α, a cytokine, which has been shown to play an important role as a mediator of cytotoxicity (28,30,40). Therefore, mediating TNF-α activity is one possible mechanism by which thromboxanes could contribute to liver injury. On the other hand, TXA_2, which is known to be a potent vasoconstrictor and a platelet aggregatory agent, could lead to vasoconstriction of the hepatic sinusoid with impairment of oxygen consumption and to the release of secretory products causing cell injury, whereas TXB_2 may provoke bleb formation in hepatocytes (39). The actions of TXA_2 are believed to be mediated by

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specific TXA_2 receptors (41). These TXA_2 receptors have been found to be upregulated in all cell types of the liver with largest increase in Kupffer cells permitting the assumption that they may function as autocrine and paracrine regulators of cytokine synthesis (39). Finally, treatment with thromboxane synthase inhibitors also resulted in a significant decrease of lipid peroxidation (conjugated dienes and 8-isoprostane) (39). The coincidence with a decrease of the severity in fatty liver and the fact that inhibition of cytochrome P-450 is also accompanied by a decrease in the severity of fatty liver and lipid peroxidation (42) allows the assumption that thromboxane inhibitors may also inhibit cytochrome P-450 activity and, therefore, diminish lipid peroxidation.

It has been shown, mainly by Nanji and co-workers, that Kupffer cells produce thromboxanes in response to ethanol in the presence of the polyunsaturated fatty acid linoleic acid. These thromboxanes play an important role as mediators of alcohol-induced cytotoxicity by causing cytokine production (TNF-α) and lipid peroxidation.

2. Activation of Kupffer Cells to Produce Prostaglandins

Thurman and collaborators also see an important role for eicosanoids in the pathogenesis of alcoholic liver injury, but in contrast to Nanji, they favor PGE_2 as a major mediator for toxic effects (43,44). Furthermore, they assume that hypoxia is a primary pathophysiological mechanism of liver injury. It has been shown that both chronic and acute ethanol consumption result in a hypermetabolic state, which causes an increased ethanol metabolism coupled with an increased oxygen uptake (16,45). The hypermetabolic state after acute ethanol administration has been called ''swift increase in alcohol metabolism'' (SIAM) (16). Furthermore, it has been demonstrated by Arteel et al. that this hypermetabolic state leads to hypoxia at cellular levels in pericentral regions of rat liver tissue in vivo by using a 2-nitroimidazole hypoxia marker, and that this effect was mediated mainly by Kupffer cells (46). Another study performed by Qu et al., where the addition of PGE_2 stimulated the oxygen uptake of parenchymal cells isolated from normal rats to the same extent as conditioned medium from Kupffer cells in a dose-dependent manner (47), led then to the assumption that ethanol first stimulates Kupffer cells to produce PGE_2, which sequentially elevates oxygen consumption in parenchymal cells. It was demonstrated in the same study that the incubation of Kupffer cells with indomethacin (inhibitor of cyclooxygenase) and nisoldipine (calcium channel blocker) blocked the stimulation of oxygen consumption of parenchymal cells due to conditioned media from Kupffer cells. The inhibitory effect of nisoldipine, the fact that Ca^{2+} activates phospholipases to produce an increased amount of eicosanoids (5), as well as earlier findings by Goto et al. that chronic alcohol exposure of Kupffer cells makes their calcium channels easier to open (48), allow the hypothesis of the involvement of calcium ions in the mecha-

nism of Kupffer cell activation to produce eicosanoids. The mechanism of how ethanol opens calcium channels and elevates PGE_2 synthesis is still unknown. However, according to the results of Adachi et al., where antibiotic treatment of rats fed alcohol led to a diminuation of the hypermetabolic state in vivo (15), alcohol-induced endotoxemia could be the cause. This hypothesis is supported by the finding of increased expression of the CD14 endotoxin receptor protein on the surface of rat Kupffer cells as well as elevated levels of lipopolysaccharide-binding protein (LBP) mRNA in hepatocytes due to chronic ethanol exposure of rats (49,50).

Taken together, the studies performed by Thurman's group demonstrate the induction of a hypermetabolic state in hepatocytes in response to ethanol, which is, at least partly, dependent on PGE_2 produced by Kupffer cells. Endotoxins seem to play an important role in mediating ethanol toxicity, probably by acting on calcium channels.

C. Activation of Kupffer Cells to Produce Oxidative Stress (Evidence for Ethanol Metabolism in Kupffer Cells)

As already stated, Kupffer cell activation in alcoholic liver disease is most likely mediated by endogenous endotoxins, by direct alcohol effects, or by both factors at the same time. A lot of evidence exists on how endotoxins might activate Kupffer cells (see chapter by Bode, this volume), whereas their activation by ethanol and/or its metabolites is much less investigated. Kupffer cell activation could happen in response to acetaldehyde, reactive oxygen species, and/or lipid peroxidation products resulting from direct ethanol metabolism in Kupffer cells themselves. Although hepatocytes are ideal sources of all these metabolites, these cells probably do not produce these products in significant amounts extracellularly (51,52). Therefore, we should first concentrate on ethanol metabolism in Kupffer cells.

It is mainly Wickramasinghe and co-workers who have elaborated the ethanol metabolism in Kupffer cells. They have incubated Kupffer cells with various inhibitors of ethanol metabolism in vitro, and then measured the effect on acetate production (53). Carbon monoxide, metyrapone, and tetrahydrofurane, which are important inhibitors of cytochrome P-450 activity, caused a marked inhibition of ethanol metabolism in these cells ($>50\%$), whereas pyrazole and other substances known to block alcohol dehydrogenase (ADH) activity caused only a slight inhibition of ethanol metabolism ($<10\%$). Therefore, ethanol metabolism in Kupffer cells is mainly ADH independent, which is in striking contrast to ethanol metabolism in hepatocytes, where the ADH pathway predominates. Recently, important cytochrome P-450 activity was determined in Kupffer cells from chow- and chronically ethanol-fed rats and identified as CYP2E1 (21). In this experiment, ethanol treatment caused a sevenfold increase in CYP2E1 content in both Kupffer

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cells and hepatocytes, but CYP2E1 content was about 10 times lower in Kupffer cells than in hepatocytes. With respect to the results of Wickramasinghe et al. (53), the ethanol-inducible CYP2E1 may be the major pathway of ethanol metabolism in Kupffer cells, which might theoretically have two consequences: first, the relative contribution of CYP2E1 to overall acetaldehyde production in Kupffer cells is probably much higher than in hepatocytes (21), especially after chronic alcohol administration, leading eventually to a relatively higher increase of intracellular acetaldehyde levels in Kupffer cells after chronic ethanol exposure compared to hepatocytes. Second, as already stated, it seems that Kupffer cells, in contrast to hepatocytes, have a much lower capacity to inactivate acetaldehyde to acetate (51). This might be another cause of its enhanced accumulation and release into the hepatic sinusoid with the production of deleterious effects such as the formation of free oxygen radicals, lipid peroxidation, and acetaldehyde-protein adducts.

Oxidative stress has been demonstrated to play an important role in the pathogenesis of alcoholic liver disease (54–57). Kupffer cells seem to be the major source of extracellular oxygen-derived free radicals (58) and acetaldehyde (51) in the liver. Some of this acetaldehyde may also be formed extracellularly near the surface of the macrophages via the action of macrophage-derived superoxide anion radicals on ethanol (59). It has been shown that Kupffer cells as well as blood macrophages significantly enhance their basal release of superoxide anion after chronic alcohol feeding (60), whereas depletion of Kupffer cells attenuates superoxide anion release into the hepatic sinusoid (61,62). The mechanisms of the formation of free oxygen radicals in response to alcohol are not fully understood. There are several hypotheses: (1) Enhanced phosphatidyl inositol turnover leads to the release of secondary messengers that modulate protein kinase C activity. The latter enzyme induces the translocation of NADPH oxidase to the plasma membrane leading to superoxide anion generation (60). (2) Because acute ethanol administration to rats results in two phases of reactive oxygen radical formation, which means in a second peak after alcohol withdrawal (63), and because Ca^{2+} is known to be a regulator of superoxide anion generation in phagocytes (64), an up-regulation of Ca^{2+} influx in hepatic nonparenchymal cells could also play a role in this process (60). The finding, that the administration of a calcium channel blocker prevents the progression of experimental alcoholic liver disease (65), supports this hypothesis or, at least, corroborates a role of Ca^{2+} in this process. (3) Another source of free oxygen radicals might be direct metabolism of ethanol through the induced CYP2E1 pathway. It is well known from experiments in hepatocytes that the oxidation of ethanol by CYP2E1 results in an enhanced formation of free oxygen radicals that have the capability to initiate lipid peroxidation (66,67). A strong correlation between hydroxyl free radical formation and the stimulation of lipid peroxidation in association with increased liver injury has been shown in liver microsomes by Albano et al. (68). Further-

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more, the same authors were able to demonstrate that the decrease of CYP2E1 content in rat liver due to treatment with diallyl sulfide and phenylethyl-isothiocyanat—both suppressors of the CYP2E1 induction by ethanol—even led to a decreased hydroxyethyl radical generation, peroxidative damage, and alcohol hepatotoxicity. Recently, Takeyama and collaborators were able to demonstrate in chronically ethanol-fed rats a correlation between an enhanced generation of free oxygen radicals by Kupffer cells and increased malondialdehyde contents in liver microsomes (69). Therefore, free oxygen radicals together with lipid peroxidation products might be major stimuli for an up-regulation of inflammatory cytokines.

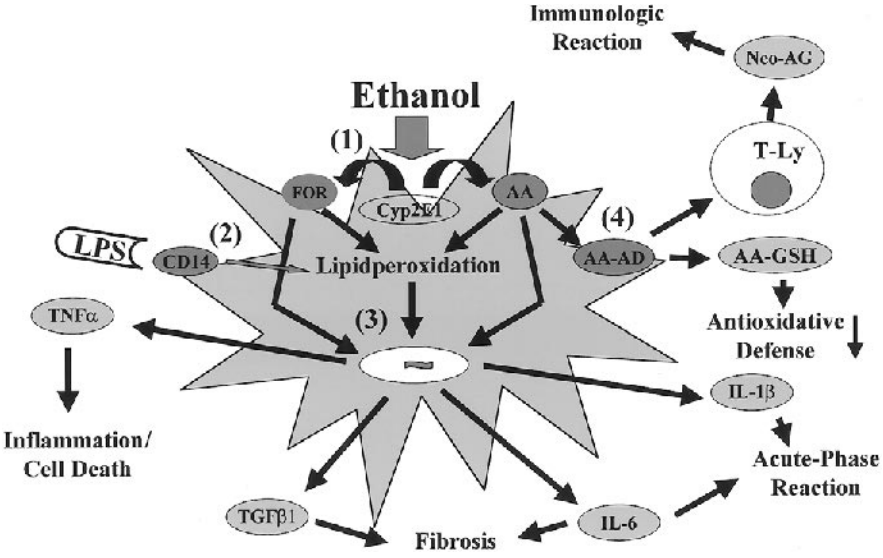

Figure 2 Hypothesis of direct activation of Kupffer cells by ethanol via the cytochrome P4502E1 pathway (1) or indirect activation by lipopolysaccharides (LPS) via LPS receptors (CD14) on the cell surface (2). Both mechanisms are important producers of oxidative stress through the production of free oxygen radicals (FOR), acetaldehyde (AA), and lipid peroxidation. Lipid peroxidation, as well as free oxygen radicals and acetaldehyde, are triggers for an enhanced expression of mRNA for different cytokines in the nucleus (3), whereas the formation of acetaldehyde protein adducts (AA-AD) leads on the one hand to an immunological reaction via the production of neoantigens (Neo-AG) by T lymphocytes (T-Ly), and on the other hand to an impairment of the antioxidative defense system by building adducts for example with glutathione (AA-GSH) (4). TNF-α = tumor necrosis factor α; TGFβ_1 = tumor growth factor β_1; IL-1β = interleukin 1β; IL-6 = interleukin 6.

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The up-regulation of cytokines is most probably mediated by transcription factors like activating protein 1 (AP-1) and nuclear factor-κB (NF-κB), which have recently been shown in HepG2 cells to be activated by ethanol as well as by acetaldehyde through distinct mechanisms (70). The coordinate induction of superoxide anion and the cytokines TNF-α and IL-1 has also recently been demonstrated in chronically alcohol-fed rats (60). In this study, endotoxemia was significantly enhanced in chronically ethanol-fed animals compared to controls. Therefore, endotoxins might also have been responsible for the enhanced cytokine production, or at least, they could have contributed to this effect by inducing lipid peroxidation or second messengers like prostaglandins or thromboxanes, again followed by activation of the above-mentioned transcription factors (71). Furthermore, TNF-α has recently been shown to prime hepatic nonparenchymal cells for enhanced superoxide anion release in vitro (72). It seems that TNF-α may maintain its own induction via an autostimulatory loop. Finally, ethanol metabolism in Kupffer cells through the CYP2E1 pathway results in generation of substantial quantities of intra- and extracellular acetaldehyde (vide supra), which may exert its toxicity mainly by the formation of acetaldehyde-protein adducts (51). Several target proteins of acetaldehyde have been described, for example, hemoglobin, tubulin, calmodulin, collagen, and glutathione (4). On the one hand, the formation of acetaldehyde-glutathion adducts may result in glutathione inactivation with impairment of the antioxidative defense system; on the other hand, it may induce the production of antibodies by T lymphocytes (1). The involvement of immune mechanisms against acetaldehyde protein adducts in alcohol-induced hepatitis and fibrosis has recently been demonstrated (73,74).

In conclusion, Kupffer cells are the major source of extracellular oxygen-derived free radicals and acetaldehyde, both probably being generated, at least in part, via ethanol metabolism through the CYP2E1 pathway. Both free oxygen radicals and acetaldehyde exert their toxic activity by inducing lipid peroxidation and probably also by inducing cytokine expression in the nucleus. The formation of acetaldehyde-protein adducts contributes importantly to liver damage (Fig. 2).

IV. THERAPEUTIC OPTIONS IN PREVENTING KUPFFER CELL ACTIVATION

Several strategies have been directed against Kupffer cells in animal models of various liver diseases, and most of them are effective in attenuating liver injury. Although the inactivation and/or destruction of Kupffer cells by gadolinium chloride, liposomal dichloromethylene diphosphonate, and methyl palmitate is commonly used in animal experiments to investigate the role of these cells in liver disease, this is not a viable therapeutic approach for humans, because the elimina-

tion of Kupffer cells bears the risk for severe infections. Most of these strategies are therefore directed against mechanisms or factors that activate Kupffer cells (antibiotics, antibodies to endotoxins, calcium channel blockers, blocking of complement activation) or against cellular products of already activated cells like proteases (antiproteases), cytokines (antibodies to cytokines, receptor antagonists), reactive oxygen species (antioxidants), and against other known mediators of liver injury like eicosanoids (thromboxane inhibitors, nonsteroidal anti-inflammatory drugs). As already indicated, these therapeutic approaches work well in animal experiments, but usually fail to show significant effects in humans. On the other hand, their efficacy on Kupffer cells in animal models underlines the key role of these cells in the pathogenesis of liver injury, and furthermore, helps us to better understand pathophysiological pathways. For example, the attenuation of alcohol-induced liver injury by the application of antibodies to TNF-α (30) or thromboxane inhibitors (39) demonstrates the importance of these Kupffer-cell-derived mediators in the development of alcoholic liver injury. The study by Iimuro et al. showed furthermore in response to anti-TNF-α a total blockage of liver macrophage-inflammatory protein-2 (MIP-2) (30), which is an important chemotactic factor for neutrophils known to play an important role in alcoholic hepatitis, whereas the treatment of animals with thromboxane inhibitors was associated with a decreased TNF-α and TGF-β_1 mRNA expression in liver cells (39), demonstrating that thromboxanes may play a role in cytokine activation. Both, anti-TNF-α antibodies and thromboxane inhibitors could theoretically be suggested for use in the treatment of alcoholic liver disease. It should be emphasized that Kupffer cell activation or inactivation has not been directly proven in both of the two above-mentioned studies as well as in the study performed by Adachi et al. (15), in which gut-derived endotoxins have been assumed as triggers for Kupffer cell activation in alcoholic liver disease. Sterilization of the gut by antibiotics resulted in a marked attenuation of liver injury, probably due to a marked diminution of endotoxin delivery to the liver with consequent reduction of Kupffer cell activation.

Another approach to treatment of alcoholic liver disease is modification of various dietary factors. In vivo animal studies have demonstrated that several nutritional factors may influence the development and subsequent course of alcohol-induced liver injury. As recently shown by Nanji et al., alcohol-induced liver injury may, for example, be potentiated by linoleic acid, which seems to serve as a substrate for the formation of free oxygen radicals and eicosanoids (32,75). Furthermore, an increased iron accumulation in the liver, possibly associated with increased lipid peroxidation (76), and the presence of malnutrition (77) have also been implicated in the pathogenesis of alcoholic liver damage. Finally, other nutrients such as S-adenosyl-L-methionine, glutathione (regarded as the body's most important factor in the antioxidative defense system), and polyunsaturated phosphatidylcholine play an important role in alcohol-induced liver injury, most

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likely by promoting lipid peroxidation as a consequence of their depletion (1).

Supplementation of chronically alcohol-fed animals with polyenylphosphatidylcholine (PPC) has been shown to protect against liver fibrosis and cirrhosis (78–80). The possible mechanisms of action of PPC are extensively reviewed by Lieber in this book. Recently, it was also shown that dilinoleoylphosphatidylcholine (DLPC), constituting the active component of PPC (80), selectively modulates the lipopolysaccharide (LPS)-induced activation of Kupffer cells by decreasing the production of cytotoxic TNF-α, while potentiating the release of the protective IL-1β (81). This dual action may provide a potent mechanism against liver injury. In addition to the reduction in TNF-α, the enhanced release of IL-1β from Kupffer cells could oppose the hepatotoxicity of TNF-α, either directly or indirectly through an IL-1β-related increase in the tolerance to TNF-α-mediated toxicity (81). IL-1β has been shown in vitro to reduce phenobarbital-induced cytochrome P-450 mRNA levels in hepatocytes, including that of CYP2E1 (82). Therefore, it could be hypothesized that the DLPC-mediated increase in IL-1β activity may provide a further mechanism against alcohol-induced liver injury, namely by reducing CYP2E1 activity leading consequently to a reduction of oxidative stress. The mechanism of action of DLPC is unknown. However, according to recent results by Lieber's research group showing important antioxidant properties of DLPC (83,84), a diminution of lipid peroxidation as well as a reduction of membrane damage by the replenishment of phosphatidylcholine in the membranes could be hypothesized as one possible mechanism (Fig. 3).

V. SUMMARY

Several animal experiments during the last 10 years have clearly documented that Kupffer cells play an important role in the pathogenesis of alcohol-induced liver disease, particularly in mediating inflammation, necrosis, and fibrosis. They are key cells in the progression of alcoholic liver injury, which influence the function of other liver cells by the production of important key factors. Cytokines like TNF-α, TGF-β1, IL-6, and IL-1β seem to be of major importance. TNF-α exhibits mainly cytotoxic activity, whereas TGF-β1 and IL-6 have been shown to induce and maintain fibrotic processes by activating hepatic stellate cells to produce extracellular matrix components. The role of IL-1β is controversial. However, evidence exists that this cytokine may have protective properties by increasing tolerance to TNF-α-mediated toxicity.

How Kupffer cells are activated is still poorly understood. However, present evidence favors two main mechanisms that are probably working together: endotoxins and ethanol metabolism in Kupffer cells via inducible CYP2E1 representing the main pathway for ethanol in these cells. Endotoxins may activate

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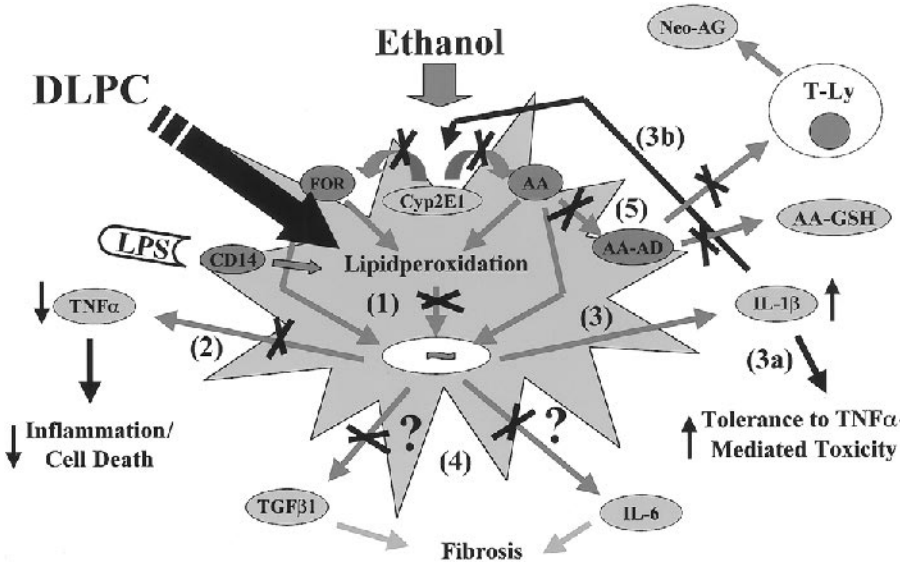

Figure 3 Hypothesis of how dilinoleoylphosphatidylcholine (DLPC) may modulate Kupffer cell activation and of the effects produced by this modulation. DLPC may probably act as an antioxidant by diminishing lipid peroxidation (1), eventually by the replenishment of phosphatidylcholine in the membranes. Decreased lipid peroxidation results in decreased tumor necrosis factor α (TNF-α) release with attenuation of cell death and inflammation (2), probably supported by the increased release of Interleukin 1β (IL-1β) (3), which potentiates the tolerance to TNF-α-mediated toxicity (3a). The increased release of IL-1β may additionally suppress cytochrome P4502E1 (CYP2E1) activity and, therefore, consequently diminish the production of free oxygen radicals (FOR) and lipid peroxidation, as well as acetaldehyde (AA) (3b). An effect on cytokines other than TNF-α or IL-1β has not yet been demonstrated (4). Finally, as a result of the increased production of IL-1β and the consecutive suppression of cytochrome P-450 activity, other pathomechanisms could be attenuated or suppressed, for example, the production of acetaldehyde protein adducts (AA-AD) with all its consequences (5).

Kupffer cells via mediators like eicosanoids, but also—like ethanol metabolism via CYP2E1—by the production of oxidative stress triggering several pathomechanisms as an increased mRNA expression for cytokines or the formation of acetaldehyde protein adducts. DLPC may—owing to its antioxidant properties—be an important tool to reduce Kupffer cell activation, and therefore to stop the course of important pathophysiological processes at an early stage of a cascade of deleterious events.

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Retinoids and Alcoholic Liver Disease

Xiang-Dong Wang
Jean Mayer USDA Human Nutrition Research Center on Aging, Tufts University, Boston, Massachusetts

I. INTRODUCTION

The critical event in early alcohol-induced hepatic damage is an impaired nutritional status of retinoids (retinyl esters, retinol, and retinoic acid) and proliferative activation of hepatic cells. Vitamin A is an essential nutrient for all mammals since it cannot be synthesized in the body. The most important physiologically active derivative of vitamin A is retinoic acid, since this molecule fulfills the vitamin A requirements for growth and maintenance of normal epithelial function and plays an important role in controlling the progression to early carcinogenesis in a variety of cancers. The discovery of retinoic acid receptors provides a mechanistic basis for understanding possible actions of vitamin A against alcohol-related diseases. In this chapter, recent studies that demonstrated that chronic alcohol intake can interfere with hepatic retinoic acid metabolism, diminish retinoid signaling, and enhance Jun N-terminal kinase (JNK) pathway in liver are reviewed, with emphasis on the retinoid-related biochemical and molecular mechanisms whereby ethanol ingestion results in hepatic cell proliferation and promoting fibrogenesis and carcinogenesis (Fig. 1).

II. ETHANOL AND RETINOID METABOLISM

A. Ethanol Decreases Hepatic Levels of Both Retinol and Retinyl Esters, Which Are Precursors of Retinoic Acid

In the liver, retinoids are stored primarily in hepatic stellate cells. Impaired nutritional status of vitamin A has been reported in alcoholics; i.e., reduced levels of

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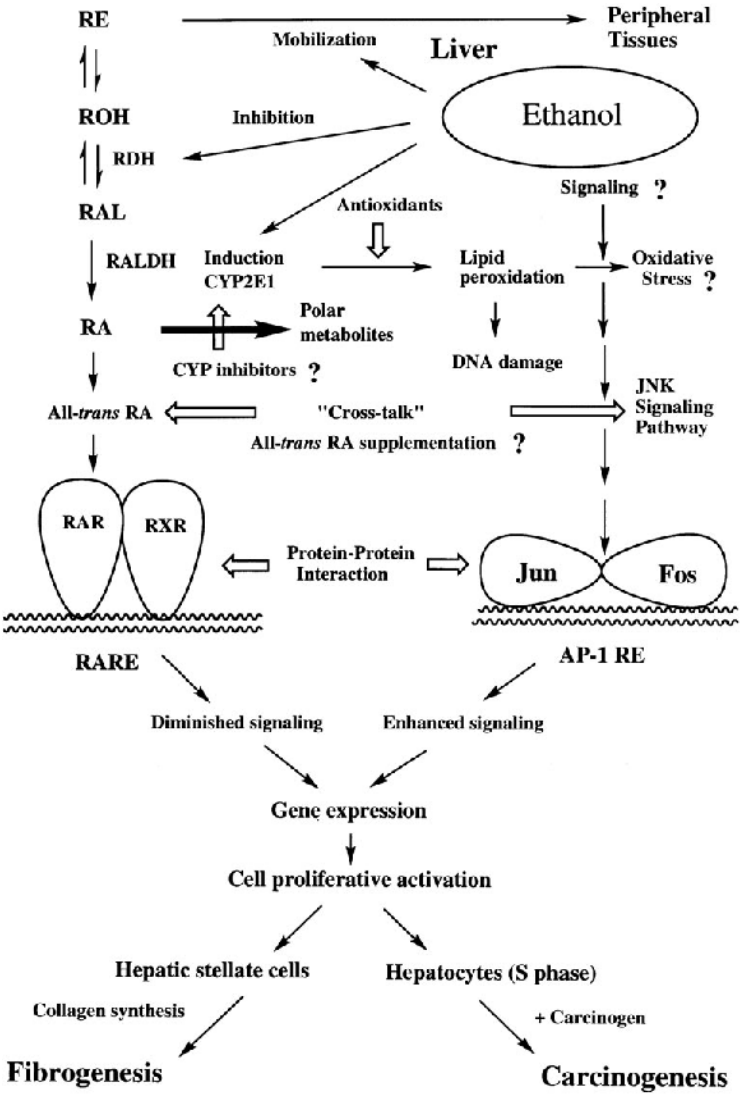

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vitamin A have been found in serum of alcoholics with and without liver disease and in liver biopsies from alcoholics (1). For example, hepatic stellate cells isolated from rats after chronic alcohol feeding contain only 15% of retinyl palmitate level of that in pair-fed control animals (2). Decreased vitamin A uptake and enhanced degradation of vitamin A by cytochrome P-450 (CYP) in the liver after chronic alcohol consumption has been demonstrated (1,3,4). Vitamin A can also be mobilized from the liver to other organs after chronic alcohol consumption (5,6), although the mechanism(s) for this vitamin A mobilization is still unclear. Regardless the mechanism(s) involved, these alcohol-induced changes result in decreased hepatic levels of both retinol and retinyl esters, which are precursors of retinoic acid (Fig. 1).

Figure 1 Simplified schematic illustration of possible interactive metabolic pathways of ethanol related to retinoid signal transduction and AP-1 (c-*Jun* and c-*Fos*) nuclear complex. Retinoid signaling occurs through nuclear receptors, which appear to act as transcription factors. Two families of nuclear receptors (RAR-α, -β, -γ and RXR-α, -β, -γ) have been cloned and have been shown to be active in receptor-mediated regulation of gene transcription by binding as dimeric complexes to specific DNA sites, the RAREs (retinoic acid response elements), which are located in the 5′ promoter region of susceptible genes. The products of the two proto-oncogenes, c-*Fos* and c-*Jun*, form a complex in the nucleus, termed activator protein-1 (AP-1), which binds to a DNA sequence motif not recognized by retinoid receptors and is referred to as the AP-1 response element (AP-1 RE). By binding to this sequence, AP-1 mediates signals from growth factors, inflammatory peptides, oncogenes, and tumor promoters, usually resulting in cell proliferation. Ethanol or acetaldehyde, an oxidative metabolite of ethanol, can induce AP-1 (c-*Jun* and c-*Fos*) transcription. Although the mechanism as to how alcohol generates a signal transduction cascade is unknown, oxidative stress caused by alcohol has been proposed. All-*trans* retinoic acid (RA) may act as a negative regulator of AP-1 responsive genes via protein-protein interactive inhibition or ''cross-talk'' inhibition with Jun N-terminal kinase (JNK) signaling pathway. Thus, AP-1 activity resulting in proliferation is inhibited. Owing to the inhibition of retinoic acid biosynthesis (e.g., via the competition of retinol (ROH) and retinal (RAL) oxidation), increased retinoic acid catabolism (e.g., via cytochrome P4502E1 (CYP2E1)), and the mobilization of vitamin A (retinyl esters (RE)) from the liver to other organs by chronic alcohol intake, low levels of retinoic acid in the liver may occur and interfere with normal retinoid signal transduction (e.g., functional down-regulation of RAR activity due to lack of the ligand). Since AP-1 and RARs can inhibit each other's activity, diminished retinoid signaling and increased AP-1 activity after alcohol exposure could cause either activation of hepatic stellate cell (increased collagen synthesis and fibrogenesis) and proliferation of hepatocytes (increased proliferating hepatocytes in the S phase, which are sensitive targets for initiating events during carcinogenesis). These mechanisms, in part, may be responsible for alcohol-induced cell injury, hepatic fibrogenesis and malignant transformation. (Modified from ref. 49.)

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B. Ethanol Acts as a Competitive Inhibitor of Retinol Oxidation in Both Liver and Other Tissues

Ethanol metabolism undergoes a two-step process: first, it is oxidized to acetaldehyde, and then acetaldehyde is oxidized to acetic acid. Similarly, retinol is converted first to retinal, and then retinal can be oxidized to retinoic acid. The reversible oxidation of retinol to retinal, by both cytosolic and microsomal retinol dehydrogenase (RDH), is the rate-limiting step in retinoic acid biosynthesis (7). Ethanol was shown long ago to be a competitive inhibitor of retinol oxidation in both liver and other tissues, and more supporting evidence has been provided recently (8–16). Human class I alcohol dehydrogenases (ADH, EC 1.1.1.1), are mainly located in liver and may function as retinol dehydrogenases, although there is controversy on this point (7,12). A recent study by Han et al. (13) showed clearly that ethanol was a competitive inhibitor against retinol for class I, II, and IV ADHs with apparent inhibition constants ranging over 0.037–11 mM, indicating that retinoic acid synthesis through the ADH pathways can be blocked during heavy drinking. In this study, the retinol-oxidizing activity of human class I ADH was 90% inhibited by 5 mM ethanol (blood ethanol levels of 5–20 mM are usually reached after social drinking), and the retinol-oxidizing activity of some forms of human class II and III ADH was 60–80% inhibited by 20 mM or 50 mM ethanol (seen only in heavy drinking). Allali-Hassani et al. (14) showed that ethanol inhibits both all-*trans*-retinol and 9-*cis*-retinol oxidation by class IV ADH with K_1 values of 6–10 mM (in the range of blood ethanol concentrations after social drinking). Furthermore, Kedishvili et al. (15), showed that the contribution of ADH isozymes to retinoic acid biosynthesis depends on the amount of free retinol in cells, and that physiological levels of ethanol can substantially inhibit the oxidation of retinol by human ADHs. It has been postulated that the ethanol-induced craniofacial defects in the fetal alcohol syndrome are due to an ethanol-induced reduction in retinoic acid caused by inhibition of retinol oxidation catalyzed by class IV ADH (16). The interaction of ethanol and vitamin A as a potential mechanism for the pathogenesis of fetal alcohol syndrome is gaining more experimental support (17). In one in vivo study (19) treatment with high-dose ethanol led to a significant reduction of the retinoic acid concentration in the liver, as compared to animals pair-fed an isocaloric control diet containing the same amount of vitamin A (Fig. 2). Quantitation of retinoic acid in the liver

Figure 2 HPLC analysis showing the inhibitory effect of ethanol on the biosynthesis of retinoic acid in rat liver. (Upper panel) Chromatogram of extract of rat liver from the control group. (Lower panel) Chromatogram of extract of rat liver from the ethanol-fed group. Peak identification: 1, retinoic acid; 2, retinol; and 3, retinyl acetate as internal standard. (Adapted from ref. 19.)

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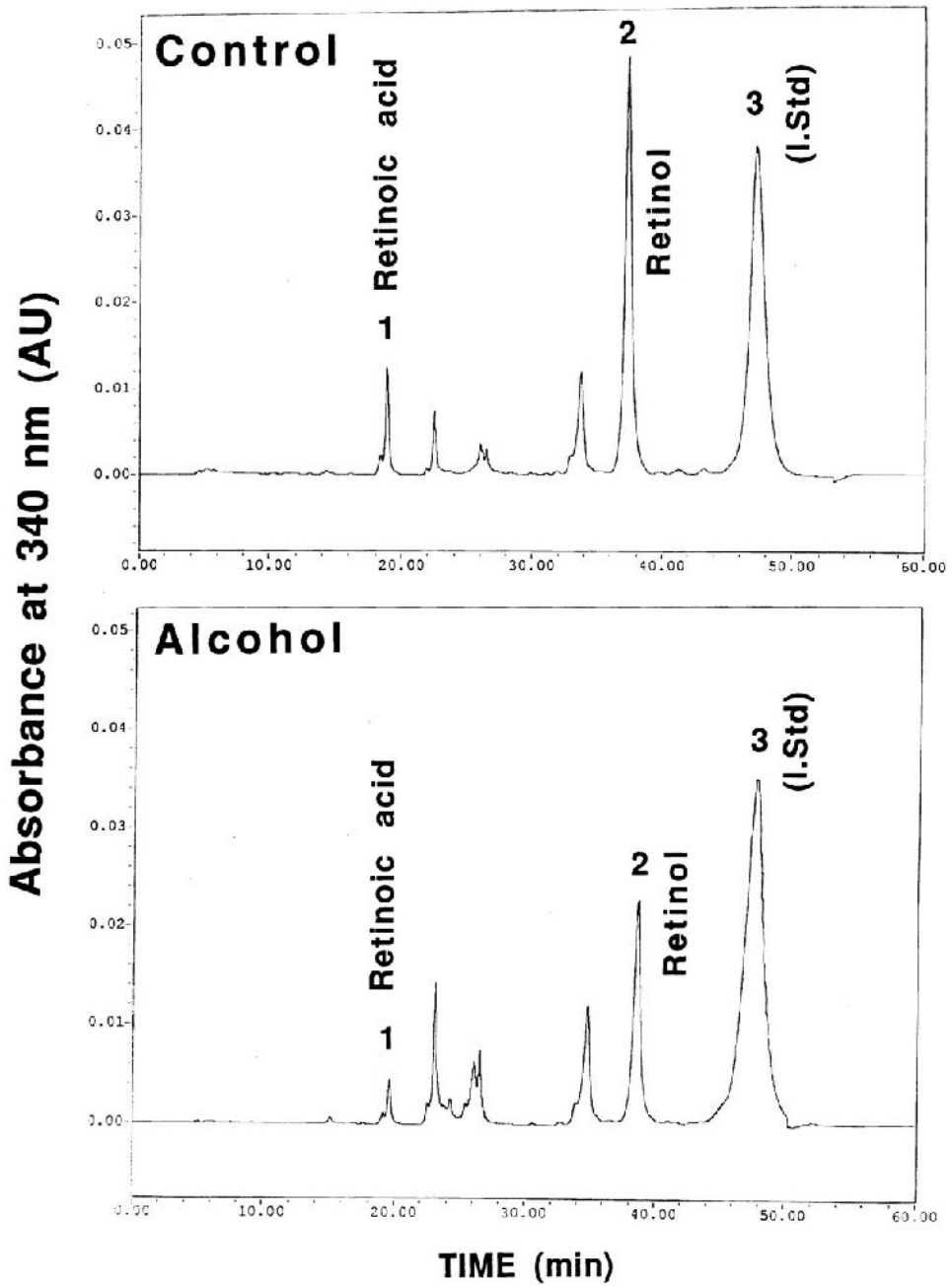

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showed that the retinoic acid concentration was significantly lower (11-fold reduction) as compared with the pair-fed control animals. Both the retinol and retinyl palmitate levels in the liver were also lower in the alcohol-fed group (6.5-fold and 2.6-fold reductions, respectively), as compared with the pair-fed control animals. Similar to the reduction of liver retinoic acid in the rats treated with high doses of ethanol, plasma retinoic acid levels in the rats treated with high doses of ethanol were also significantly lower (8.5-fold reduction) as compared with the pair-fed control animals (Fig. 3). These studies indicate that alcohol causes a local deficiency of vitamin A (a precursor of retinoic acid) in the liver, resulting in a decreased biosynthesis of retinoic acid (Fig. 1).

C. Ethanol Enhances Retinoic Acid Metabolism into Polar Metabolites via Induction of Cytochrome P-450 Enzymes

Increased catabolism of retinoic acid into more polar metabolites, e.g., 4-oxo-retinoic acid and 4-hydroxyl-retinoic acid, in the liver has been shown after alcohol treatment (3). The generation of polar metabolites of retinoic acid is proposed to be a cytochrome P-450 (CYP)-dependent process (3,4,18).

The main alcohol-inducible CYP enzyme is CYP2E1, which has high oxidizing activity for the metabolism of a number of compounds. Chronic ethanol treatment in rats results in a threefold increase in the rate of microsomal NADPH consumption, which corresponds to an increase in rat liver microsomal CYP2E1 (20). Furthermore, alcohol feeding in the rats caused a five- to 10-fold increase in CYP2E1 protein with a corresponding rise in mRNA (21,22). The increase of CYP2E1 protein may also be due to stabilization of the protein by ethanol treatment (23). To provide evidence that alcohol drinking can result in increased catabolism of retinoic acid in rat liver by induction of CYP2E1, we carried out an in vitro incubation of retinoic acid with microsomal fractions of liver tissue and various inhibitors of CYP2E1 (24). Chlormethiazole, a compound derived from the thiazole moiety of thiamine, effectively decreases the rate of the CYP2E1-dependent reactions in microsomes, but not the rate of other CYP isozyme-dependent reactions (e.g., CYP1A1, CYP1A2, CYP2B1, and CYP3A1) (25,26). Using the inhibitory property of chlormethiazole on CYP2E1 in our in vitro incubation study, we showed that the enhancement of the catabolism of retinoic acid by alcohol is reduced (threefold) by chlormethiazole (Table 1). This is in agreement with our Western blot analysis, which showed that CYP2E1 protein increased fourfold after administration of alcohol for 1 month in vivo (24). Since the hepatic distribution of CYP2E1 is localized mainly to the centrilobular region of the liver (21), the local concentration of CYP2E1 protein would be greater than measured, since our microsomal fractions were prepared from whole-liver homogenates. In

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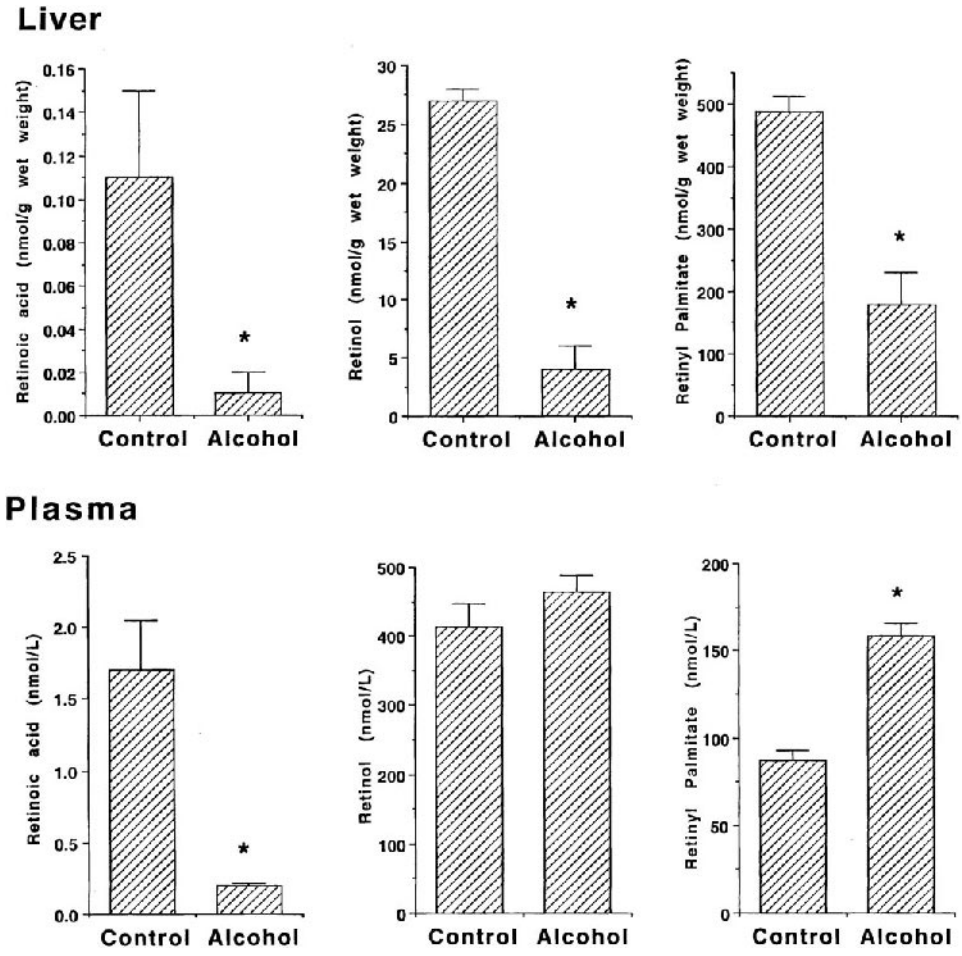

Figure 3 The concentrations of retinoids in rat liver and plasma after 1 month of treatment with or without alcohol. Values are expressed as mean ± SEM ($n = 10$). *Significantly different at $p < 0.05$. (Adapted from ref. 19.)

our study, the apparent K_ms are the same for 18-hydroxy-retinoic acid formation from retinoic acid oxidation in the microsomal fractions from alcohol-fed rats with or without chlomethiazole (Fig. 4). This suggests that inhibition by chlomethiazole is noncompetitive. More evidence that CYP2E1 is involved in retinoic acid catabolism is that pretreatment with CYP2E1 antibody led to a similar reduc-

Table 1 Recovery of Retinoic Acid (RA) and Production of 18-Hydroxy- and 4-Oxo-RA in Alcohol-Fed Rat Liver Microsomal Fractions after 1 μM RA (1000 pmol/ml) Incubation With or Without Cytochrome P-450 Inhibitors

Treatment	RA recovered (pmol)	18-hydroxy-RA (pmol)	4-oxo-RA (pmol)
Non–alcohol fed	715 ± 22[a]	6.9 ± 1.5[a]	6.1 ± 1.6[a]
Alcohol fed	613 ± 15[b]	33.0 ± 2.8[b]	29.9 ± 2.5[b]
Plus CYP2E1 specific inhibitors (10 μM)			
Chlormethiazole	689 ± 14[a]	12.1 ± 1.1[d]	11.1 ± 1.8[d]
Allyl sulfide	693 ± 18[a]	20.1 ± 1.6[d]	9.4 ± 0.8[d]
Plus CYPs selected inhibitors (10 μM)			
Troleandomycin	631 ± 18[b]	29.8 ± 2.6[b]	26.2 ± 1.8[b]
Resveratrol	626 ± 11[b]	32.8 ± 1.5[b]	30.4 ± 1.5[b]
α-Naphthoflavone	627 ± 12[b]	31.1 ± 1.1[b]	28.1 ± 0.9[b]
Plus CYPs nonspecific inhibitors (10 μM)			
Liarozole	684 ± 20[a]	ND	3.6 ± 1.1[c]
Disulfiram	697 ± 14[a]	ND	ND

Values (mean ± SEM) are expressed in pmol/2 mg protein/ml. ND: Not detected. Data represent three to five determinations. Different superscript letters for a given column indicate values that are statistically significantly different from each other ($p < 0.05$). *Source*: Adapted from ref. 24.

tion of the alcohol-enhanced formation of 18-hydroxy-retinoic acid and 4-oxo-retinoic acid from retinoic acid after incubation, as compared with the inhibitory effect of CYP2E1 inhibitor (24).

It is interesting that there was 73% (but not total) inhibition of enhanced retinoic acid catabolism due to alcohol by both specific CYP2E1 antibody and specific inhibitors against CYP2E1, which implies that other CYP enzymes are also involved in retinoic acid metabolism. To confirm our hypothesis, we used both disulfiram and liarozole, which are nonspecific inhibitors of CYP (27,28) (and liarozole has been shown to be a retinoic acid catabolism-blocking agent) (29), in our incubation experiments. The results from the studies clearly show that the formation of polar metabolites (18-hydroxy-retinoic acid and 4-oxo-retinoic acid) of ethanol-fed rats can be blocked almost completely by either inhibitor (Table 1). The small amount of 4-oxo-retinoic acid that was still detected in the presence of liarozole may be due to additional inhibition of liarozole on the metabolism of 4-oxo-retinoic acid (30). The finding that the inhibitory levels of disulfiram and liarozole on the catabolism of retinoic acid in alcohol-fed rats were even below the levels in the non-alcohol-treated animals (Table 1) is due to the fact that both compounds inhibit multiple CYP enzymes, both alcohol-induced and non-alcohol-induced.

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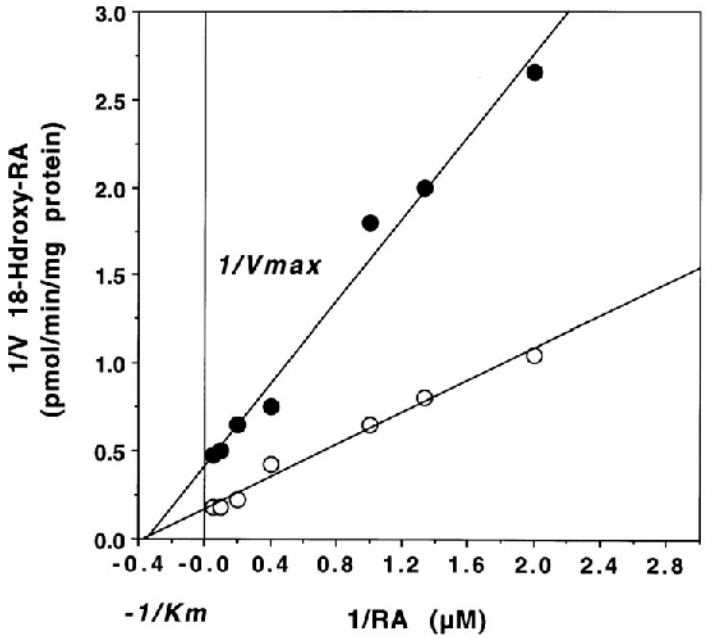

Figure 4 Inhibitory effects of chlomethiazole on retinoic acid metabolism in alcohol-fed rat microsomal fractions. Lineweaver-Burk plots of 18-hydroxy-retinoic acid formation from retinoic acid oxidation in alcohol-fed rat microsomal fractions with or without chlomethiazole. The data are plotted as a reciprocal of the reaction rate (pmol/min/mg protein) and as a reciprocal of the retinoic acid concentration (μM). The plot of enzymatic activity versus retinoic acid concentration in the presence (●) and absence (○) of chlomethiazole (100 μM). The apparent K_m and V_{max} are, respectively, 2.94 μM and 5.9 pmol/min/mg protein for 18-hydroxy-retinoic acid formation from retinoic acid oxidation in alcohol-fed rat microsomal fractions without chlomethiazole. The apparent K_m and V_{max} are, respectively, 2.98 μM and 2.4 pmol/min/mg protein for 18-hydroxy-retinoic acid formation from retinoic acid oxidation in alcohol-fed rat microsomal fractions with chlomethiazole. (Adapted from ref. 24.)

It has been reported that a series of microsomal CYP isozymes (31,32), CYP1A2 and CYP2B4 in rabbits (31), CYP2C7 (33,34) and CYP3A (35) in rats, and CYP26 (36,37), CPYf (38), and CYP2C8 (39,40) in humans, are involved in the metabolism of retinoic acid. Although most of these enzymes are induced by retinoids, we examined whether some of these enzymes could contribute to the catabolism of retinoic acid in the alcohol-fed rats. We found that preincubation of antibodies against CYP1A1, CYP1A2, CYP2C12, and CYP3A1 in the incubation mixture of retinoic acid with the alcohol-induced microsomal fraction of liver

had no appreciable inhibitory effect on the formation of 4-oxo-retinoic acid or 18-hydroxy-retinoic acid from retinoic acid (24). Similarly, there was no appreciable inhibition of alcohol-enhanced retinoic acid metabolism by adding three selective inhibitors [resveratrol for CYP1A1 (38), α-naphthoflavone for CYP1A2, and troleandomycin for the CYP3A family (39)] to the incubation mixture (Table 1). These data show that CYP2E1 is the major CYP responsible for the catabolism of retinoic acid in hepatic tissue after treatment with alcohol. Since the catabolism of retinoic acid was measured and the CYP2E1 protein level in the hepatic microsomal fraction of the alcohol-fed rats after 12 hr of fasting was still increased fourfold, compared with the control rats, it is probable that alcohol-induced hepatic CYP2E1 continues to metabolize retinoic acid even after the ethanol has been cleared from the body. Therefore, alcohol-enhanced retinoic acid catabolism may be the major factor responsible for the decrease in retinoic acid levels seen after alcohol treatment. This may explain some of controversies on whether ethanol can competitively inhibit retinol oxidation in vivo (7,16). That is, if ethanol did inhibit retinol oxidation, retinoic acid formation would be impaired. However, once alcohol is cleared from the body, retinoic acid formation could once again take place. In contrast, in chronic intermittent drinking, CYP enzymes induction would continue to be a factor in destroying retinoic acid, even after alcohol was cleared from the body. This hypothesis is supported by our recent demonstration that treatment of ethanol-fed rats in vivo with chlormethiazole as a specific inhibitor for CYP2E1 can restore both plasma and hepatic retinoic acid concentrations to normal levels (24).

The striking enhancement of catabolism of retinoic acid to polar metabolites by the microsomes of alcohol-treated rats, the inhibition of microsomal retinoic acid catabolism by both specific inhibitors and antibody against CYP2E1 and the restoration of both plasma and hepatic retinoic acid concentrations to normal levels in the ethanol-fed rats by chlormethiazole treatment offer a biochemical mechanism for the reduction in both plasma and hepatic retinoic acid concentrations seen with chronic alcohol treatment in vivo (19,24). This observation also provides a possible explanation for why chronic and excessive alcohol intake is a risk not only for hepatic but also for extrahepatic cell proliferation and carcinogenesis since it has been reported that CYP2E1 is also present and inducible by alcohol in the esophagus, forestomach, and surface epithelium of the proximal colon (40). The modulation of experimental alcohol-induced liver disease by a CYP2E1 inhibitor has been reported (41).

III. ALCOHOL AND RETINOID SIGNALING PATHWAY

A. Retinoid Signal Transduction Pathway

Retinoic acid is the most physiologically active derivative of vitamin A. It is well known that retinoids exert profound effects on development, cellular growth, and

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differentiation. Retinoid signaling occurs through nuclear receptors, which appear to act as transcription factors (Fig. 1). Two families of nuclear receptors (RAR-α, -β, -γ and RXR-α, -β, -γ) have been cloned and have been shown to be active in receptor-mediated regulation of gene transcription (42,43). RAR binds all-*trans*-retinoic acid and the closely related isomer 9-*cis*-retinoic acid, whereas RXR binds only 9-*cis*-retinoic acid. Retinoid receptors regulate gene expression by binding as dimeric complexes to specific DNA sites, the RAREs (retinoic acid response elements), which are located in the 5′ promoter region of susceptible genes. The RARE of retinoid-responsive genes consist of direct repeat of the sequence 5′-AGGTTCA-3′ separated by two or five base pairs. The RXR response element (RXRE) is a direct repeat of the same sequence separated by one base pair. High-affinity binding of an RAR to any of these RAREs in vitro requires heterodimerization with an RXR (44–46). The identification and cloning of target retinoid-responsive genes and their regulatory DNA sequences support the concept that retinoic acid exert its biological effects through RARs and RXRs. Mice carrying mutations in RARs or RXRs have provided proof that these receptors indeed control retinoid signaling, since the defects observed in such mice mimic essentially all the those previously seen during vitamin A deficiency (47). RARs function as ligand-dependent transcription factors, and down-regulation (loss or low expression of specific RARs) or ''functional'' down-regulation (lack of the ligand) of retinoic acid receptors could interfere with retinoid signal transduction, resulting in cell proliferation, and enhancing malignant transformation (48,49).

B. Molecular Mode of Action of Retinoic Acid

Retinoic acid plays an important role in controlling the progression to early carcinogenesis in a variety of cancers (48). The chemoprotective effects of retinoids are thought to be mediated through proliferation control. The molecular mode of action of retinoic acid, the most active form of vitamin A, may involve several mechanisms:

A first mechanism is transactivation through direct binding to RARE in target gene promoters, thereby transcriptionally activating a series of genes with distinct antiproliferative activity (50). Some of the retinoid-inducible genes can account for certain actions of retinoic acid. For example, RARβ transcription that can be induced by retinoic acid is down-regulated in tumor cells compared with normal human mammary epithelial cells (51,52). Conversely, introduction of RARβ2 into RARβ-negative HeLa cells inhibits cell proliferation in vitro in a retinoic-acid-dependent manner (53). These data support a role for RARβ as a tumor suppresser gene, which has been proposed by several other investigators (48,52). To date, a series of human tumor suppressor genes has been identified throughout the human genome, e.g., P53. Tumor suppressor genes function by inhibiting the ability of normal cells to proliferate, which are known to act at

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different control points in the cell cycle. It is possible to envision the life cycle of a normal cell as a balance of positive and negative growth effects. Loss of tumor suppressor function, by mutation or transcriptional repression, leads to enhanced cell proliferation and potentially to tumor formation.

A second mechanism is transrepression of activator protein-1 (AP-1) involving inhibition of AP-1 protein induction (42,43). The products of the two proto-oncogenes, c-*Fos* and c-*Jun*, form a complex in the nucleus, termed AP-1, that binds to a DNA sequence motif referred to as the AP-1 response element (AP-1 RE). By binding to this sequence, AP-1 mediates signals from growth factors, inflammatory peptides, oncogenes, and tumor promoters, usually resulting in cell proliferation. It has been reported that RARα acts as a negative regulator of AP-1 responsive genes (54). Retinoid receptors and the transcription factor AP-1 (Jun/Fos) can inhibit each others activities. Recently, a protein-protein interaction mechanism for an antiproliferative effect of retinoic acid receptors has become of interest, as suggested in a recent study by Kamei et al. (55), who showed that the interaction between RAR and CBP (cAMP response element-binding protein) is responsible for some forms of repression of AP-1 activity. A recent study has also revealed a novel posttranscriptional mechanism by which all-*trans*-retinoic acid antagonizes ultraviolet activation of AP-1 by inhibiting c-Jun protein induction (56). In recent studies, Lee et al. (57) provide the first evidence that all-*trans* retinoic acid suppress Jun N-terminal kinase (JNK) activity by inhibiting JNK phosphorylation. As AP-1 sites are found in a number of genes that are important in the control of cell proliferation, this type of interaction (or ''cross-talk'') is responsible for certain of the antiproliferative and anticancer properties attributed to retinoic acid. However, little information is available for the role of retinoic acid signal transduction pathways on alcohol-induced AP-1 activity.

A third mechanism to explain the chemopreventive effects of retinoids is regulation of apoptosis (58). Apoptosis is a biochemical and morphological event leading to a cell death that is different from necrosis. It is a form of regulated cell death to eliminate unwanted or superfluous cells from the organism (59). It plays an indispensable role in embryogenesis and in adult tissue homeostasis, but can also contribute to the pathogenesis of a number of human diseases when deregulated. It is controlled by multiple signaling pathways that mediate active responses to external growth or death factors. Several cell cycle regulators have been shown to be involved in responses that lead to apoptosis, such as tumor suppressor p53 and Rb, cyclins, Cdk inhibitor p21, and c-*myc*. Cell cycle checkpoint controls are linked to apoptotic enzymes cascade, and the integrity of these links can be genetically compromised in many diseases, such as cancer. In addition to their effects on embryonic tissues, retinoids have been implicated in the induction of cell death in many tumor-derived cultured cell system (such as myeloid leukemia cell, breast, tracheal epithelial cell, cervical cancer, etc.). Reti-

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noids have been shown to regulate the expression of regulatory factors of apoptosis such as p21 (60) and Bcl-2 (61), as well as effector enzymes tissue transglutaminases (TGase), which results in the extensive cross-linking of intracellular protein during the apoptotic program (62,63). A recent study by Hsu et al. (64) demonstrates that retinoic acid treatment results in the induction of p21 and Bax protein expression concomitant with Cdk1 kinase activation, Rb2 protein phosphorylation as well as cell death in human hepatoma Hep3B cells. However, little information is available on the role of retinoid signal transduction pathways in alcohol-induced hepatocellular apoptosis. Retinoic acid has been shown to be of potential clinical interest in cancer chemoprevention and treatment; therefore, understanding the role of retinoic acid on cell cycle events has obvious therapeutic importance.

C. Effects of Ethanol on Retinoid Signaling and Activation of JNK Pathways

Expression of RARs in rat liver has been demonstrated (65,66). It has been suggested that increased hepatic stellate cell proliferation and collagen synthesis may result, in part, from decreased hepatic stellate cell retinoid responsiveness, e.g., the loss or decrease of nuclear RARβ gene expression (65). Ohata et al. (66) reevaluated this hypothesis and confirmed that diminished retinoic acid signaling occurs in hepatic stellate cells derived from cholestatic rats with liver fibrosis. This decreased signaling appears to result from retinoic acid deficiency and suppressed expression of RARβ and RXRα. Recently, we have shown that the lower retinoic acid level and down-regulation of specific RAR (RARβ) gene expression causes squamous metaplasia in the lung tissue of ferrets (67). In our recent study (19), treatment of rats with chronic ethanol led to a significant decrease in retinoic acid concentrations in both the liver and plasma (Fig. 2). However, the expression of RARs (α, β, and γ) in the liver, especially RARβ, which has RARE in its promoter and which is induced by retinoic acid, was unaffected by ethanol treatment over a 1-month period (19). This result is similar to other reports (68,69), although the retinoic acid concentration was not reported in previous studies.

It is probable that gene expression of RARβ is not affected unless severe retinoic acid deficiency occurs, or may undergo some posttranscriptional modification. However, it is possible that even moderate lack of ligand (retinoic acid) could result in a ''functional'' down-regulation of the retinoic acid receptor and thus interfere with retinoid signal transduction, despite normal RAR expression. Since RARs function as ligand-dependent transcription factors, in which RAR transcriptional activation occurs with the binding of either all-*trans*-retinoic acid or 9-*cis*-retinoic acid, and play an important role in retinoic-acid-mediated cell differentiation, this ''functional'' down-regulation of retinoic acid receptor could

potentially interfere with retinoid signal transduction, causing cell proliferation, and enhancing malignant transformation (Fig. 1).

Recent studies have demonstrated that the mouse tissue transglutaminase (TGase II) promoter is activated by ligand activation of either RAR-RXR heterodimers of RXR-RXR homodimers, and is associated with a complex retinoid response element located 1.7 kb upstream of the transcription start site (58). To test our hypothesis that retinoid signaling is functionally ''defective'' in alcohol-fed animals, we conducted studies to examine the expression of retinoid controlled TGase II in the liver tissues of alcohol-fed rats and control rats (70). The results showed that chronic alcohol feeding of rats leads to a functional down-regulation of retinoid signaling (lack of ligand), as evidenced by a decreased expression of a retinoid controlled gene, TGase II (Fig. 5), without altering retinoic acid receptors. Since all-*trans*-retinoic acid is a potent inducer of TGase II in rat tracheobronchial epithelial cells (71), and rats rendered vitamin A–deficient have a market depression in the level of TGase II activity in many tissues, which is rapidly reversed by administration of all-*trans*-retinoic acid, it will be interesting to investigate whether the down-regulated TGase II in the livers of chronically alcohol-fed rats can be prevented or up-regulated by retinoic acid supplementation.

Although the mechanism as to how alcohol generates a signal transduction cascade is unknown, recent evidence has accumulated supporting a role for reactive oxygen species caused by ethanol in the regulation of AP-1 (c-*Jun* and c-*Fos*) gene expression. Since the earlier effect of chronic alcohol feeding on AP-

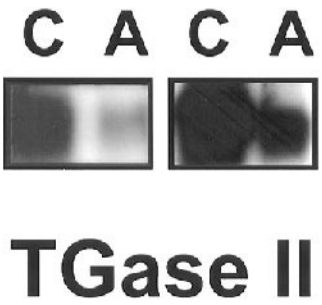

Figure 5 Effect of ethanol on the expression of TGase II in rat liver. Representative Northern blot for TGase II in liver samples from two pair-fed rats (C, control; A, alcohol) are shown. TGase II was detected in both the control and the alcohol-fed groups. However, TGase II was decreased at least twofold by Northern blot analysis in the livers of alcohol-treated rats, as compared with control rats. These data support our hypothesis that ''functional'' down-regulation of retinoic acid receptor can potentially interfere with retinoid signal transduction and activation of target genes (Data from ref. 70.)

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1 expression in an in vivo animal model has not been explored previously, we examined AP-1 (c-*Jun* and c-*Fos*) expression in rat liver after 1 month of alcohol feeding (19). In this study, expression of both c-*Jun* and c-*Fos* genes in rat liver was enhanced 7–8 fold (Fig. 6) by chronic alcohol feeding, despite a significant decrease in retinoic acid concentrations in both the liver and plasma (Fig. 3). It has been shown that enhanced expression of AP-1 binding proteins in hepatic stellate cells correlates with cell activation and overexpression of collagen type I (72); therefore, it is possible that AP-1 regulates the expression, at least in part, of the type I collagen gene (through binding to the AP-1 binding-site present in the enhancer of the first intron) in the stellate cell, which is a major collagen-producing effector cell responsible for hepatic fibrogenesis (72–74). Ohata et al. (66) demonstrated diminished retinoic acid signaling and a reciprocal increase in AP-1 activity in the in vivo activated hepatic stellate cell, suggesting the role

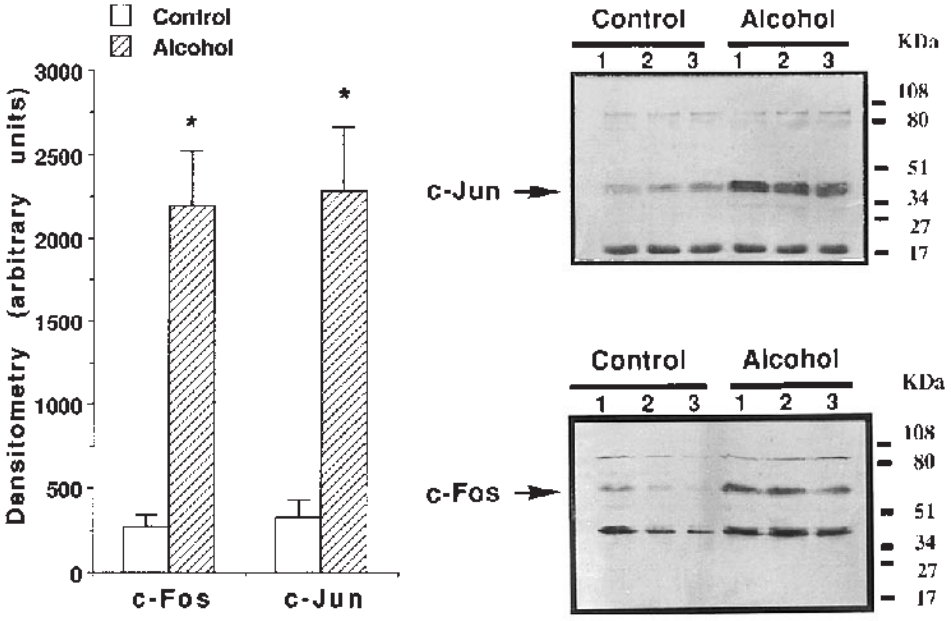

Figure 6 Effect of ethanol on the expression of AP-1 (c-*Jun* and c-*Fos*) in rat liver. Densitometry analysis followed by Western blotting analysis. Data represent the mean ± SE ($n = 10$). Using densitometry analysis, c-*Jun* expression was sevenfold greater in the rat livers of the alcohol-fed group, as compared with the control group. Similar to c-*Jun* expression, c-*Fos* was detected in both groups, but was eightfold greater in the rat livers of the alcohol-fed group, as compared with the control group. (Adapted from ref. 19.)

of the former in mediating the latter for hepatic stellate cell activation in liver fibrogenesis. Taken together, these studies suggest that down-regulation of RARs and up-regulation of AP-1 gene expression by ethanol may contribute to hepatic stellate cell activation in liver fibrogenesis (Fig. 1).

It has recently been reported that c-*Jun* is required for progression through the G1 phase of the cell cycle by a mechanism that involves direct transcriptional control of the cyclin D1 gene (75). It is conceivable that the overexpression of c-*Jun* by chronic and excessive alcohol intake may cause abnormal cell cycle regulation and drive cells into premature S phases, resulting in aberrant mitotic process. We hypothesize that the up-regulation of c-*Jun* by ethanol may be an important mechanism for causing cell proliferation and promoting carcinogenesis by alcohol, and dietary retinoic acid supplementation may inhibit c-*Jun* overexpression and, therefore, alcohol-induced hepatocellular proliferation (49). Recently, we have found that dietary retinoic acid supplementation in alcohol fed-rats can restore normal hepatic retinoic acid concentrations and inhibit alcohol-induced c-*Jun* overexpression as well as phosphorylation of JNK (76). Therefore, retinoid signaling restored by retinoic acid supplementation may control alcohol-induced hepatocellular cell proliferation by inhibition of alcohol-induced c-*Jun* expression. However, the molecular regulatory mechanisms of hepatocellular proliferation by retinoic acid, which may be central to elucidating the pathophysiology of hepatic cell proliferation, remains to be understood.

IV. RETINOIDS IN THE PREVENTION AND TREATMENT OF ALCOHOLIC LIVER DISEASE

A. Carcinogenesis

A number of epidemiological studies have indicated that chronic and excessive alcohol consumption is a significant risk factor for cancer including cancer of the liver, oral cavity, oropharynx, esophagus, colorectal, and breast (77). It has been reported that the risk of liver cancer in alcoholic cirrhosis is higher than in any other type of cirrhosis (78). Although the exact mechanisms by which alcohol ingestion promotes carcinogenesis are not known, malnutrition in alcoholic patients due to poor diet, malabsorption, interferences with nutrient metabolism, and energy and protein wastage may be significant factors (79). Other proposed mechanisms, including the generation of acetaldehyde (77) and reactive radicals (80), the formation of DNA strand breaks (81), and the decreased ability of the liver to metabolize dietary nitrosamines (82), have also been invoked.

Chronic alcohol intake could induce a number of biochemical and molecular alterations and result in an environment (such as interference of retinoid metabolism and signaling) that may contribute to hepatocellular cell proliferation and carcinogenesis (49). Cell proliferation has essential roles in hepatic carcino-

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genesis including initiation and promotion processes, particularly when chemical carcinogens are involved. Several carcinogens induce tumors in various organs, but not in the liver unless they are associated with a proliferative stimulus (83). It has been shown that proliferating hepatocytes in the S phase (after partial hepatectomy) are sensitive targets for chemical initiation of carcinogenesis (83). One of the chemoprotective effects of retinoids is thought to be mediated through control of proliferation via delaying progression of damaged cells into the S phase, which would allow more DNA repair thereby reducing the risk of carcinogenic initiation. The observation that retinoid (retinol and retinyl esters) concentrations are decreased in both cancerous and surrounding noncancerous liver tissues of alcoholic patients suggests a role for retinoid depletion in hepatocarcinogenesis of these patients (84). However, it is not known whether chronic alcohol-induced hepatocellular cell proliferation may convert hepatocytes from a state of resistance to carcinogen to a state of high susceptibility due to alcohol-impaired retinoid metabolism and signaling. This question is essential for studying the chemopreventive effect of retinoids in alcohol-promoted carcinogenesis (liver as well as peripheral organs).

Although the mechanisms are unclear, it has been reported that chronic ethanol feeding increases hepatocellular apoptosis and proliferation (85,86). In ethanol-fed rats for 6 weeks with Lieber-De Carli liquid diets, bromodeoxyuridine (BrdU)-labeled hepatocytes and apoptosis increased 2.5- and fivefold, respectively, compared with the pair-fed controls (85). Halsted et al. (86) demonstrated that chronic ethanol feeding for 12 months results in abnormal methionine metabolism in micropigs and the number of apoptotic bodies and proliferating cell nuclear antigen (PCNA)-positive cells cycling in S phase in livers increased by three- and sixfold from ethanol-fed minipigs, respectively, compared with the control group. These investigators suggest that increased apoptosis and cell proliferation may reflect a regenerative proliferative response to maintain cell number homeostasis. However, it is possible that under certain risk conditions, such as diminished hepatic retinoids signaling by chronic alcohol intake, apoptosis may be deregulated; therefore, the proliferative response induced by chronic alcohol intake may promote genomic instability and neoplasia. However, it is not known whether retinoic acid treatment, with or without an inhibitor of cytochrome P-450 enzymes, can suppress both alcohol-induced cell hyperproliferation and alcohol-promoted hepatocellular carcinogenesis.

The administration of retinoids, such as retinyl esters, can enhance alcohol-induced liver toxicity, and the use of nonhepatotoxic synthetic retinoids may be beneficial against hepatocarcinogenesis. It has been reported that there was a significant reduction after treatment with the acyclic retinoid polyprenoic acid in the incidence of second primary tumors in patients who have previously undergone resection of hepatomas (87). In the recent follow-up analysis (62 months) for this study, a significant difference in survival (74% vs. 46%, $p = 0.04$) was

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also found (88). This is the first report on the activity of a novel retinoid in the prevention of hepatocellular carcinoma. Retinoic acid is currently used in the treatment of several types of cancers, including acute promyelocytic leukemia, squamous cell carcinoma of the head and neck, oral and cervical premalignant lesion, and skin cancer (89). However, more understanding of the molecular mechanism(s) is needed for the use of retinoids in prevention and treatment of alcohol-induced liver injury.

B. Fibrogenesis

Hepatic fibrosis is a common response to liver injury from chronic excessive alcohol in both humans and baboons (90,91). Hepatic stellate cells are sinusoidal cells localized within the space of Disse of the liver and are thought to be the major source of extracellular matrix synthesis in chronic alcoholic liver disease (92). An early event in alcohol-induced hepatic fibrosis is an alcohol-induced activation (cell proliferation and increased fibrogenesis) of the hepatic stellate cell, and deposition of collagen in the space of Disse and around the hepatocytes represents a characteristic feature of alcohol-induced liver fibrosis (93). Hepatic stellate cells isolated from animal models of liver fibrosis have been shown to produce more collagen than those from control animals (94). Moreover, hepatic stellate cells, but not hepatocytes, produce significant amounts of type I collagen after acetaldehyde treatment of primary cell cultures (95). During hepatic fibrosis, hepatic stellate cells gradually lose their vitamin A in parallel with their increased proliferation, increased collagen synthesis, and transformation into myofibroblastlike cells (96), although these events do not necessarily predict subsequent myofibroblastic activation (97).

Baboons with alcoholic liver fibrosis show morphological evidence of reduced vitamin A–containing lipid droplets in hepatic stellate cells (93). Conversely, hepatic stellate cells treated in vitro with vitamin A show suppressed proliferation (98), decreased collagen synthesis (99), and amelioration of liver fibrosis (96). Moreover, in vivo intraperitoneral injections (1 mg/kg body weight) of retinoic acid into rats caused a marked reduction in the abundance of type I collagen mRNA in both total hepatic and purified hepatic stellate cell RNA (98). Other studies showed similar findings; e.g., vitamin A treatment of rats prevented the development of CCl_4-induced liver fibrosis and hepatic stellate cell proliferation in cell cultures (100,101). These studies suggest that the maintenance of hepatic retinoid levels blocks the fibrogenic response, and that retinoids may serve as regulators of hepatic stellate cell proliferation and collagen synthesis. Retinoic acid is 100–1000 times more potent than retinol with respect to inhibition of hepatic stellate cell proliferation (98). However, the molecular mechanisms of type I collagen gene regulation by retinoic acid and the role of retinoic acid supplementation in blocking fibrogenesis remain to be understood. It has

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been reported also that chronic ingestion of huge amounts of vitamin A stimulates fibrogenesis and results in a disorder of hepatic function resembling cirrhosis (102). Thus, both deficiency of vitamin A and toxicity due to vitamin A stimulate fibrogenesis, although the mechanisms may be different or dose-dependent. A recent study showed that increased 9,13-di-*cis*-retinoic acid exacerbated liver fibrosis, at least in part, by inducing the activation and production of latent TGF-β in hepatic stellate cells (103).

V. CONCLUSION

Cell proliferation plays a central role in hepatic carcinogenesis in both the initiation and promotion stages. Retinoic acid plays an important role in controlling carcinogenic progression in a variety of cancers. One of the chemopreventive effects of retinoids is thought to be mediated through control of proliferation by delaying progression of damaged cells into S phase, which would allow more DNA repair and thereby reduce the risk of carcinogenic initiation. However, chronic and excessive alcohol intake can cause retinoic acid deficiency in liver tissue by either the inhibition of retinoic acid biosynthesis and/or enhancement of retinoic acid catabolism via the cytochrome P-450 enzyme system. This local hepatic deficiency of retinoic acid may cause defective retinoid signaling pathway or ''cross-talk'' with other signaling pathways (e.g., MAP kinase pathway, hepatic growth factor, or transforming growth factor-β). The diminished retinoid signaling and enhanced AP-1 activity after alcohol exposure may contribute to alcohol-induced hepatocyte hyperproliferation. However, it is not known whether chronic alcohol-induced hepatocellular cell proliferation may convert hepatocytes from a state of resistance to carcinogen to a state of high susceptibility due to alcohol-impaired retinoid metabolism and signaling. This question is essential for studying the chemopreventive effect of retinoids in alcohol-promoted carcinogenesis (liver as well as peripheral organs).

Carcinogen-initiated hepatocellular neoplasia occurs in the presence or absence of fibrogenesis. However, alcohol especially increases the risk of hepatic carcinogenesis in the present of cirrhosis. Most studies have focused on a possible protective effect of vitamin A (retinol and retinyl esters) against stellate cell activation and fibrogenesis. However, the molecular mechanisms of type I collagen gene regulation by retinoids and the role of retinoic acid supplementation in blocking fibrogenesis remain to be understood. Little evidence is available regarding underlying mechanism(s) by which ethanol plays a role in proliferative activation of hepatic stellate cells or hepatic fibrogenesis and the potential protective role of retinoic acid-mediated signal transduction. Both deficiency of vitamin A and toxicity due to vitamin A stimulate fibrogenesis, although the mechanisms may be different or dose-dependent. Further understanding of these mechanisms

is important in the prevention and treatment of alcohol-induced fibrosis by retinoids.

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22
Treatment of Alcoholic Liver Disease

Helmut K. Seitz and Tatjana Arslic
Salem Medical Center, Heidelberg, Germany

I. INTRODUCTION

The major purpose of this chapter is to summarize clinically established treatment forms of alcoholic liver disease (ALD). In addition, new experimental approaches in the therapy of ALD will also be discussed. Two of them, namely the action of *S*-adenosyl-methionine (SAM) and of polyenylphosphatidylcholine (PPC), are discussed in detail by Lieber in this book.

Four therapy modalities can be distinguished: (1) abstinence, (2) nutrition therapy, (3) drug therapy, and (4) liver transplantation.

Early diagnosis of alcoholic liver disease is important in the attempt to retard the progression of the disease and to improve prognosis (1). It is important to differentiate alcohol-related liver disease from nonalcoholic liver disease, and to determine the type of alcoholic liver disease (fatty liver, alcoholic hepatitis, cirrhosis) and its severity. Thus, liver biopsy is an important prerequisite to treating the disease successfully.

II. ABSTINENCE

The treatment of choice in ALD is abstinence (1–4). It has been shown that abstinence, regardless of the severity of alcoholic cirrhosis, results in a significant improvement of the 5-year survival rate (5,6). Although abstinence is essential, it is often difficult to achieve. In this process the physician who is primarily responsible for the patient's care plays an important role. He should diagnose the disease early, should encourage the patient to abstain from alcohol, and inform the patient about all treatment modalities offered for alcoholism. All other treat-

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ment forms have to be compared to the success rate related to abstinence. This is especially relevant with respect to the outcome in clinical trials using different drugs. Thus, it is mandatory that alcohol intake during such trials is monitored, since the result of the trial depends not only on the drug administered, but also on whether alcohol is continuously consumed or the patient is abstinent. Clinical trials without monitoring alcohol consumption are useless.

III. NUTRITIONAL THERAPY

It is well known that severe ALD develops only in a subset of drinkers, implying the role of some selective, probably genetic, predisposing factors. Perhaps, as simultaneous infection with hepatitis C may aggravate ALD, nutrition also may fit into such a modifying role (7–9). It is generally accepted that alcohol per se is the major cause of alcoholic liver injury. However, it is also generally accepted that malnutrition, especially of the protein-calorie type, is of great importance in the development of ALD (2,9). This theory is supported by several studies in rodents and humans.

Moreover, moderate to severe malnutrition is common in patients with severe alcohol abuse who need hospitalization. There are many reasons for malnutrition. The most important is decreased intake of nutrients (primary malnutrition). Many studies have shown that, in patients with heavy alcohol abuse, calories derived from alcohol (so-called ''empty calories'') constituted about 50% or more. Thus, protein and carbohydrate intake is low. Secondary malnutrition due to either malabsorption or maldigestion is also of importance. Subsequently, hepatic storage and processing of nutrients, including those of vitamins, are impaired. Thus, intermediary hepatic metabolism is greatly disturbed (1,9–12). Anorexia with diminished food intake, decreased digestion and intestinal absorption of dietary constituents, and the abnormal metabolism of dietary constituents are all affected by development of ALD. In addition, deficiencies of micronutrients including folate, zinc, and selenium may influence processes of liver injury such as DNA-methylation reactions, hepatocellular apoptosis and repair, cellular immune response and antioxidative defense.

At least four mechanisms of malnutrition contribute to ALD. First, some nutrients may be capable of supporting some defense mechanisms against the effect of excess alcohol. For example, cysteine, a key amino acid, is essential to maintain physiological glutathione levels that may protect the liver against ethanol-induced peroxidative stress (2,9,12). Moreover, methionine or its precursors may be important to generate *S*-adenosyl-methionine and, in turn, phospholipids for membrane integrity (2,13). Reduced levels of vitamin E and other antioxidants in alcoholics with or without liver disease may also play a role in

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the pathogenesis of alcohol-induced liver injury (14,15). Second, poor nutrition may impair immune response with consecutively increased susceptibility to infections. Third, hepatic regeneration, which depends on a cascade of events involving the substrates and machinery for protein synthesis, may suffer from combined alcohol-induced liver damage and poor nutrition. Finally, postoperative mortality is likely to be increased in patients with ALD and malnutrition because of poorer wound healing and sepsis (16,17).

A. Calories and Protein

Almost all patients with advanced ALD show moderate to severe marasmus; moreover, there is some evidence that the prognosis of patients with severe liver disease decreases with malnutrition (17–19). Approximately 20 controlled prospective studies of the effect of nutritional supplementation on mortality of ALD have been carried out (for review see refs. 7,8,18,20). Although these studies vary considerably (number of patients, severity of disease, type, time course, amount of nutritional support, etc.), some general conclusions can be drawn: enteral or parenteral supplementation of nutrients, especially of the protein-calorie type, does improve nutritional status and liver function of patients with severe liver disease. No significant side effects were noted. The supplements did not exacerbate encephalopathy or azotemia. Fluid overload did not occur. Liver morphology was modestly improved in the two studies in which hepatic biopsies have been taken. However, only two studies demonstrated an improved survival rate (21,22). In one study by Mendenhall et al. a significant correlation of mortality at 1-, 6- and 12-month follow-up with degree of protein-calorie malnutrition was found (19). It seems that possibly a more selected group of patients with malnutrition, but still acceptable liver function, might benefit best from nutritional therapy. Indeed, in a recent study of Mendenhall et al. a significant improvement in survival rate by nutritional supplementation was shown in moderately malnourished patients with ALD, but not in patients with severe malnutrition (in this study oxandrolone was also administered; see below) (23). Thus, although nutritional supplementation is of objective benefit in patients with severe ALD in itself, it seems not to improve short-term or long-term survival.

It can be concluded that a general oral, enteral, or parenteral nutritional supplementation with a high-protein-calorie diet is of benefit in malnourished patients with ALD, especially in selected groups, without any side effects. Calories should provide 1.2 times the required resting energy expenditure (>30 kcal/kg body weight) and protein 1.0–1.5 g/kg body weight to prevent catabolism. Branched-chain amino acids should be reserved for patients with severe refractory encephalopathy. In most patients standard amino acid solutions given enterally are satisfactory.

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B. Vitamins and Trace Metals

In addition to protein-calorie malnutrition, patients with ALD may also suffer from deficiencies of vitamins and trace elements, mainly due to decreased intake and/or absorption (2,9,10,12).

1. Folate, Thiamine, Pyridoxine, Riboflavin, Nicotinic Acid

Most common is a deficiency of folate, which is reflected by macrocytosis. Moreover, in some patients there may be an additional deficiency of thiamine, pyridoxine, riboflavin, and nicotinic acid. Deficiency of B vitamins may manifest as peripheral neuropathy and Wernicke's and possibly Korsakoff's encephalopathy (9). Thus, supplementation of the above-mentioned vitamins in excess should be provided in addition to the protein-calorie diet. No side effects of an excess administration of these vitamins have been noted.

2. Vitamin A

Vitamin A is decreased in ALD and this may lead to night blindness and sexual dysfunction. Chronic ethanol consumption results in hepatic vitamin A depletion due to enhanced mobilization and metabolism of the vitamin (24,25). Hepatic vitamin A depletion is associated with many adverse effects that may advance liver injury. Thus, vitamin A supplementation should be given to alcoholic patients to correct its deficiency. However, vitamin A should be given carefully, since excessive amounts are hepatotoxic. This hepatotoxicity of vitamin A is due to the enhanced generations of toxic intermediates by cytochrome P4502E1 in microsomes (26). When vitamin A is taken together with alcohol it may potentiate fibrogenesis (27). In this context it is interesting that it has recently been shown that even β-carotene may be deleterious for the liver when administered with alcohol (28). Furthermore, administration of β-carotene and ethanol has been shown to be carcinogenic (29). It has been shown recently that chronic ethanol ingestion in rats stimulates the expression of AP-1 gene (c-*fos* and c-*jun*), an early event in carcinogenesis (30).

3. Vitamin E and Selenium

Vitamin E (α-tocopherol), as well as selenium, is part of the antioxidant defense system and is decreased in patients with alcoholic liver disease (14,15,31). Vitamin E is considered to be ''the last line of defense'' against membrane lipid peroxidation. Studies have shown that the combination of ethanol and decreased intake of vitamin E results in a depletion of α-tocopherol in the liver. This makes the liver more susceptible to the attack of free radicals. Further, selenium is an essential component of the tetramer glutathione peroxidase and of the monomer

phospholipid hydroxyperoxide-gluthatione peroxidase, which act as a repair system of peroxidized membrane phospholipid fatty acids. In one study by Wenzel et al. 56 patients with alcoholic hepatitis and cirrhosis (Child B and C) were treated with supplements of α-tocopherol, selenium, and zinc or placebo. Patients who received antioxidants showed not only a significant improvement of serum bilirubin, ammonium, and malondialdehyde, but also a decreased mortality (32). However, because neither compliance nor alcohol intake was recorded, the results have to be questioned.

4. Zinc

Zinc deficiency is common in heavy drinkers, mostly because of decreased intake and absorption and increased urinary excretion. It may be associated with night blindness, acrodermatitis, hypogonadism, altered immune response, mental changes, and diarrhea (9,31). It could be shown that low plasma levels of zinc may be responsible for the increased production of interleukins by Kupffer cells that are stimulated by ethanol and its metabolites. In severe alcoholics zinc should be supplemented (1,18,31).

Special nutritional therapy with ''supernutrients'' such as *S*-adenosylmethionine (SAM) and polyunsaturated phosphatidylcholine (PPC) is possibly beneficial in ALD. Animal studies have been performed (33,34) and clinical studies (13,35) are being conducted for further information.

5. S-Adenosyl-Methionine

It has been shown that chronic alcohol consumption leads to a decrease of activated methionine. Several mechanisms contribute to the low levels of SAM including decreased production of methionine, due to folate- and B_{12} deficiency (9) as well as vitamin B_6 deficiency (36), low intake of methionine itself, decreased activation of methionine to SAM due to an inhibition of the responsible enzymes (35,37).

In any case, low SAM leads to decrease in methylation. Such methylation is important with respect to DNA stability and membrane function. Approximately 6% of cytosine bases in the DNA are methylated. In addition, disturbed methylation of phosphatidylethanolamine results in consequent decrease of phosphatidylcholine. This is important with respect to the integrity of membrane function. It had been shown in baboons that dietary supplementation with SAM improves liver morphology (38). More recently a randomized, placebo-controlled, double-blind trial has also shown that the administration of SAM in patients with alcoholic liver cirrhosis leads to a decrease in mortality and in liver transplantation (39).

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6. Polyenylphosphatidylcholin

As pointed out earlier, the prerequisite to creating a normal concentration of PPC is availability of sufficient amounts of SAM. Since this is not the case, PPC is also decreased in ALD and after chronic ethanol consumption. This will be discussed in more detail in Lieber's article in this book. Briefly, PPC has various effects on the liver including metabolic, antifibrotic, and antioxidative effects. For example it has been shown in baboons that administration of PPC in diets for a long period prevents the occurrence of advanced liver disease including cirrhosis (34). The omission of PPC from a diet, on the other hand, accelerates the development of cirrhosis. On the basis of these studies, a huge placebo-controlled, randomized, double-blind multicenter trial has been performed in the United States and the results will be available soon.

IV. DRUG THERAPY

On the basis of the pathophysiology of alcoholic liver disease drug therapy may include antioxidative, antifibrotic therapy, suppression of the immune system by corticosteroids, anticytokine-therapy including the administration of TNF-α antibodies, and a decrease of endotoxins possibly best accomplished by administration of antibiotics. Here only established drug therapy will be reported and experimental drug therapy will be addressed only briefly. A variety of drugs have been used to treat ALD. Unfortunately, most of the drug trials were negative. Because of their proved uselessness and/or side effects, compounds such as insulin and glucagon, D-penicillamine, and antioxidants such as (+)-cyanidanol-3 or thioctic acid (alpha lipoic acid) cannot be recommended for treatment of ALD (4,40,41). Thus, only a few compounds have been shown to have some beneficial effect in ALD.

A. Corticosteroids

As a rationale for the therapy of alcoholic hepatitis (AH) with corticosteroids it is believed that altered immune response may be important in the initiation and perpetuation of AH. Moreover, acetaldehyde forms adducts with macromolecules in the hepatocyte that may act as neoantigens provoking an immune response (42), and there is evidence that cytokines may also play a role in the pathogenesis of AH (43).

Twelve studies with corticosteroids (44–55) in a dose between 30 and 40 mg/day in AH have been included in a meta-analysis (56). These studies had to fulfill the following criteria: they had to be randomized, only patients with AH had to be included, they had to be published in an article, and they had to be

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analyzed according to the intention-to-treat method. Thus, altogether, 304 patients treated with corticosteroids and 304 patients treated with placebo were included in this meta-analysis. Of the 12 trials five reported a statistically significant reduction in short-term mortality due to corticosteroids. Overall, the reduction of mortality due to corticosteroids was 17% ($p < 0.01$). In patients with encephalopathy the mortality reduction was 27% ($p < 0.0001$), while in patients without encephalopathy no differences in mortality occurred. In patients without gastrointestinal bleeding mortality reduction was 26% under corticosteroids ($p < 0.01$), whereas in patients with gastrointestinal bleeding this difference was no longer statistically significant. In patients without renal insufficiency there was a significant reduction in mortality by 35%($p < 0.01$), which was no longer significant if patients with renal insufficiency were included.

A recent study of Mathurin et al. (57) has investigated long-term survival and prognostic factors in patients with severe biopsy-proven AH treated with prednisolone. A significant reduction of 1-year mortality has been shown, which lost its statistical significance after 2 years. Furthermore, these authors found independent prognostic factors such as increased blood neutrophilia and marked liver neutrophil infiltrate, which are useful to identify patients who benefit more from treatment with corticosteroids. It can be concluded that corticosteroids are of benefit in patients with severe AH because they reduce short-term mortality and possibly also 1-year mortality. In addition, factors such as absence of gastrointestinal bleeding, absence of renal failure, presence of encephalopathy, increased blood neutrophilia, and marked liver neutrophil infiltrate result in a better response to corticosteroids.

B. Anabolic Steroids (Oxandrolone)

Oxandrolone showed no positive effect on short-term mortality in earlier studies (58). However, Mendenhall et al. were able to demonstrate that in moderately malnourished patients with AH the combination of vigorous correction of nutritional status and simultaneous administration of oxandrolone results in decreased mortality (23). In contrast, oxandrolone has no such effect on mortality rate in patients with severe malnutrition (23). Despite the favorable effects of oxandrolone on metabolism and survival, it has been withdrawn at least from the U.S. market because of widespread abuse by athletes and bodybuilders (7).

C. Antioxidant Drugs

It has been shown that during ethanol oxidation a variety of electrophilic intermediates are produced that are able to react with cell macromolecules, leading to lipid peroxidation. Among those intermediates are oxygen- and carbon-centered radicals (59–66). Radicals such as the hydroxyethyl radical can form adducts,

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resulting in an autoantigen response (67,68). Furthermore, chronic ethanol consumption results not only in increased generation of reactive oxygen species, but also in a severe alteration and reduction of the antioxidant defense system, which includes, among others, hepatic glutathione, vitamin E, selenium, and related compounds (1,9,69–71). On the basis of these pathogenetic aspects, it is not surprising that antioxidative therapies have been investigated in the treatment of ALD (72). Among these drugs, (+)-cyanidanol-3 (catechin) and silymarin have been investigated experimentally in detail and a number of clinical trials have been performed. Owing to a side effect of autoimmune hemolytic anemia, (+)-cyanidanol-3 has been withdrawn from the market, at least in Germany.

1. Vitamin E and Selenium

Both tocopherol and selenium belong to the antioxidant defense system and the levels of both compounds are decreased in alcoholism and ALD. Selenium is an essential component of the tetramere glutathione peroxidase and of the monomere phospholipid hydroxyperoxide glutathioneperoxidase, and they act as a repair system for peroxidized membrane phospholipid fatty acids. Because of this, these compounds have been used in a clinical trial (32). Fifty-six patients with alcoholic hepatitis and cirrhosis of the liver (Child class B and C) received either 600 mg alpha-tocopherol, 200 μg selenium, and 12 mg zinc daily or placebo. In the antioxidant group, a significant improvement in serum bilirubin, ammonia, and malondialdehyde was observed. Mortality of the control group was 40%, as compared to 6.5% in the antioxidant group, a statistically significant difference. Again, neither compliance nor alcohol intake was recorded; therefore, the results have to be questioned.

Most recently, the effect of tocopherol supplementation on ethanol-induced organ damage has been investigated in the rat. Morphological and functional changes in the heart and skeletal muscle induced by ethanol could not be prevented by alpha-tocopherol supplementation (Preedy and Seitz, unpublished data). Changes in hepatic protein metabolism (e.g., changes in albumin and activities of hepatic arginyl aminopeptidase) could not be prevented by alpha-tocopherol supplementation (Preedy and Seitz, unpublished data). However, ethanol-induced cellular hyperproliferation of colorectal mucosa could be partly inhibited by alpha-tocopherol (73). It is of interest that Eskelson et al. (29) reported an inhibitory effect of tocopherol on ethanol-stimulated, nitrosamine-induced esophageal carcinogenesis (74).

2. Thioctic Acid

Thioctic acid (alpha-lipoic acid) has antioxidant properties in addition to an anti-inflammatory effect via its action on cyclooxygenase. Furthermore, thioctic acid is a cofactor in pyruvate dehydrogenase and alpha-ketoglutarate dehydrogenase

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and might therefore prevent changes in citric acid cycle activity that accompany ethanol oxidation in the liver. However, one randomized, double-blind trial of 300 mg thioctic acid versus placebo in 40 patients with precirrhotic ALD over 6 months did not show any beneficial effect of the drug (75).

3. Silymarin

The hepatoprotective properties of silymarin (SYM), which consist of silybinin, silychristin, and silydianin, have been claimed for many years. The effect of SYM and its main constituent, silybinin, on experimentally induced liver toxicity has been investigated intensively. Although in animal studies using galactosamine, CCl_4, paracetamol, phalloidin, glutathione-depleting agents, and ischemia a beneficial effect of SYM could be documented (76–79), the mechanism of action of this drug is not completely clarified.

It has been clearly shown that SYM is a scavenger of oxygen radicals, including OH and HOCl (80,81), but not of O_{2-} and possibly not of H_2O_2, although some controversy exists. Furthermore, it has been claimed that silybinin is a weak iron chelator (80). Several studies have clearly identified silybinin as a protective agent against lipid peroxidation (82,83). In one study, SYM protected against paracetamol-induced lipid peroxidation and liver damage, possibly by its antioxidant properties as a free radical scavenger (76). It was interesting to note that glycogen was restored much faster under SYM. In another experimental study, SYM prevented membrane alterations in acute CCl_4 liver damage—again through its antioxidant properties by modifying the plasma membrane phospholipid content (77).

The fact that SYM possesses membrane-stabilizing properties has been recognized for a long time and therefore intravenous SYM is used in *Amanita phalloides* poisoning to prevent the uptake of the toxin by hepatocytes.

In addition, SYM also increases protein biosynthesis by stimulating rRNA polymerase, leading to an unspecific increase in hepatic protein (84). Furthermore, silybinin decreases ethanol metabolism in rats and it was thought that this effect is due to an inhibition of the microsomal ethanol-oxidizing system (MEOS). It was proposed that this inhibition of MEOS by silybinin is also related to its antioxidant properties as a scavenger of free radicals (85).

On the basis of these experimental data obtained in cultures and in the rat model, SYM was also used in patients with various types of liver diseases. Salmi and Sarna (86) performed a prospective, randomized, double-blind trial over 4 weeks with 420 mg SYM daily versus placebo in 97 patients who had persistent elevation of serum transaminase activities. The majority of these patients drank alcohol regularly and liver biopsies were performed in all patients. The SYM group showed a significantly greater decrease in serum transaminase activities. Eleven of 15 patients who were rebiopsied had an improvement of liver histology

under the drug. However, drinking behavior of the patients during the trial was not monitored. Salvagnini et al. (87) treated 122 alcoholic patients with either 420 mg SYM or placebo for 6 weeks and found an improvement of serum aspartate transaminase activity in the treated group. Velussi et al. (88) investigated the effect of 600 mg SYM on diabetes caused by alcoholic liver cirrhosis for 6 months. In SYM-treated patients, mean fasting blood glucose level, glycosylated hemoglobin, daily insulin requirements, fasting insulinemia, blood malonaldehyde, and basal as well as glucagon-stimulated C-peptide were all significantly lower than in untreated patients and lower than at baseline (88). These results indicated that SYM reduces lipid peroxidation of liver cell membranes also in cirrhotic patients with diabetes and increases the sensitivity of the insulin receptor, among others.

Two long-term, double-blind, placebo-controlled trials in patients with alcoholic cirrhosis of the liver have been performed. Ferenci et al. (89) randomized 170 cirrhotic patients (61% with alcohol abuse) to either 420 mg SYM per day or placebo over 2 years. The 4-year survival rate of the patients of the SYM group was 58%, compared to 39% in the placebo group ($p = 0.04$). A detailed analysis of the study showed that only patients with alcoholic cirrhosis and patients with Child A classification had a significant benefit from SYM treatment. Again, drinking history was not recorded in this study, which makes it difficult to draw precise conclusions.

More recently, Parés et al. (90) published another double-blind, randomized, multicenter trial comparing 450 mg SYM with placebo over 2 years in biopsy-proven alcoholic cirrhotics. Survival was similar in patients receiving SYM or placebo. SYM did not have any significant effect on the cause of the disease. In this study, alcohol consumption was monitored by measuring urinary alcohol every 3 months and/or by questioning the patients and their relatives about the amount of alcohol consumed. It was important to note that patients who had an additional hepatitis C viral infection had a better outcome under SYM. Preliminary results of a double-blind, placebo-controlled, multicenter 4-year study with SYM in Germany did not show any effect of the drug on survival.

D. Colchicine

Colchicine has been introduced as a therapeutic agent for the treatment of gout and familial Mediterranean fever. Colchicine inhibits granulocyte migration and interferes with the degradation of polymorphonuclear leukocytes (PMN). Since PMN accumulation in the liver is one characteristic feature of AH, and since PMN-derived cytokines possibly mediate some clinical symptoms in AH, colchicine was tried as a therapeutic agent. In addition, colchicine has other effects that may be beneficial in alcoholic liver disease, including inhibition of fibrogenesis

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(91,92). Colchicine reduces collagen synthesis and increases collagenase activity. Theoretically, the drug is able to inhibit, prevent, or even reverse fibrosis. Since colchicine affects the microtubular system in the hepatocytes, it interferes with transport and secretion of macromolecules such as procollagens (93,94). In CCl_4-treated rats, colchicine reduced the degree of fibrosis and improved liver function (95). Furthermore, colchicine also partially prevented the toxic effect of D-galactosamine on rat liver. Since lipid peroxidation may be a prerequisite for galactosamine-induced hepatic damage, the protection by colchicine may be related to its capacity to inhibit lipid peroxidation (96).

Since colchicine inhibits MEOS by approximately 30% in the rat, it could theoretically prevent the production of free radicals generated via this pathway (97).

Clinically, various trials have been performed with colchicine as therapeutic agent. Akriviadis et al. performed a randomized, placebo-controlled trial in 72 patients with severe alcohol hepatitis by giving them either 1 mg of colchicine daily or placebo over 30 days. The mortality was 19% versus 17%, which was not statistically significant (98). Similar negative data were collected by Trinchet et al. in a randomized, placebo-controlled trial in 67 patients with biopsy-proven AH. Repeated biopsies were taken during the trial, and alcoholic drinking behavior was recorded. No significant effect was observed in clinical, laboratory, or histological variables between the two groups. However, improvement of biopsy scores at 3 months was greater in the colchicine-treated group (99).

Since colchicine, as already pointed out, has antifibrotic effects in the rat model, it was also used in cirrhosis. A double-blind, controlled trial of 43 cirrhotic patients was published in 1979 by Kershenobich et al. (100). After treatment for up to 4 years the colchicine group (1 mg/day) showed greater clinical improvement and a reduction of fibrosis in histology. However, there was no statistically significant difference in mortality between the two groups. Later the same investigators reported an extended trial including 100 patients with cirrhosis: 45 of them had AC, 41 had posthepatitic cirrhosis, and 14 had cirrhosis of various causes (101). All patients except one were classified as Child A or B. The patients were followed up over 14 years and sequential liver biopsies were performed. The overall mean survival time in the colchicine group was 11 years and in the control group 3.5 years ($p = 0.001$). The cumulative 5-year survival rate was 75% in the colchicine group and 34% in the placebo group. The corresponding 10-year survival rates were 56% and 20%. In nine of 30 patients having received colchicine histological improvement was seen. No histological improvement was seen in 14 patients of the control group who had two or more biopsies. Although these data seem impressive at first glance, a variety of problems exist in this study: there were a number of differences in the two groups including the presence of esophageal varices and laboratory values influencing the Child classification,

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such as serum albumin. In addition, alcohol consumption during the trail was not recorded, and compliance was not monitored. Thus, at the present time more trials are certainly necessary to evaluate the effect of colchicine, especially on AC of the liver, before recommending such medication.

E. Propylthiouracil

There is substantial experimental evidence that chronic ethanol exposure may increase hepatic demand for oxygen (102) and that the early (predominant) hepatic lesions in this disorder are centrolobular, where oxygenation is lowest. Propylthiouracil (PTU), theoretically, can reduce oxygen demand by decreasing the hypermetabolic state and, indeed, accomplishes this in animals with experimentally induced centrolobular oxygen lack (103). Studies in patients are contradictory. One report cited improvement in liver tests (104); another showed no benefit (105). The beneficial study correlated improvement with low T_3 on admission and no reported significant side effects (106,107). Further data are needed to define the short-term effects of PTU on alcoholic liver disease.

Apparently only one long-term study of PTU in this disorder has been done (107). This report cited a 60% reduction in mortality from alcoholic liver disease over 2 years. The greatest benefits were seen in the most severely ill patients with only moderate continuing alcohol intake (108). A statistical reanalysis of this study seemed to confirm the validity of the design (108).

In view of the interesting theoretical basis for this drug's action, the experimental data, and the apparent long-term benefits without significant toxicity, we believe that PTU should undergo further clinical testing.

V. LIVER TRANSPLANTATION

Liver transplantation is a successful procedure in end-stage alcoholic liver cirrhosis. One-year survival is approximately 80% and 5-year survival is almost 70% (109). A careful selection of patients is mandatory to avoid relapse into alcoholism after transplantation. Thus, psychological assessment is required and various alcohol prognosis scales have been published based on established prognostic criteria (110). In the past, abstinence of 6 months or more was considered a reasonable criterion. Overall relapse rates under these conditions were between 11 and 22%. However, when patients with less than 6 months of sobriety were transplanted, relapse rates were as high as 95%. It has been shown that the serum concentration of carbohydrate-deficient transferrin (CDT) is a good marker to diagnose relapse into alcoholism following liver transplantation (111).

At present we are studying a series of 50 patients 7 years following transplantation with a survival rate of 59% and relapse rate of 12% (112). In most

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transplantation centers approximately 10–15% of all patients are transferred for liver transplantation or transplanted because of alcoholic end-stage liver cirrhosis.

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Alcohol and Cancer

The Role of Acetaldehyde

Nils Homann
Medical University of Lübeck, Lübeck, Germany

I. INTRODUCTION

Alcohol is, together with tobacco smoke, the main cause for upper-gastrointestinal (GI)-tract cancer in industrialized countries. It is also a risk factor for development of liver, breast, and colorectal cancer. However, the tumor-promoting effects of alcohol intake are poorly understood and alcohol is not carcinogenic in the animal model.

There is increasing evidence that alcohol metabolism, rather than alcohol itself, is generating carcinogenic and cell-toxic compounds. Acetaldehyde, the main, first metabolite of ethanol, is highly toxic and mutagenic and it has been shown to be carcinogenic in the animal model. Polymorphisms and/or mutations in the genes coding for enzymes responsible for acetaldehyde accumulation and detoxification have been associated with an increased cancer risk in humans.

Acetaldehyde can also be produced by the physiological microflora deriving from ethanol metabolism. High microbial acetaldehyde levels after ethanol intake have been described in the upper gastrointestinal tact and colorectum, both sites known to represent organs susceptible for ethanol-associated carcinogenesis.

In the recent experimental and epidemiological data, there is increasing scientific evidence that the major part of the carcinogenic effect of ethanol is exhibited by acetaldehyde.

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II. ALCOHOL AND CANCER: EPIDEMIOLOGY

A. Upper Gastrointestinal Tract

In the beginning of the nineteenth century, Lamu reported that absinth drinkers have an increased risk of developing esophageal cancer (1). Since then, a great number of epidemiological studies have demonstrated a significant correlation between alcoholism and the development of upper alimentary and laryngeal cancer (2). These studies clearly showed that all types of alcoholic beverages are associated with an increased cancer risk, suggesting that ethanol itself is the common ingredient causing that effect.

The various studies showed different attributable cancer risks for a certain amount of alcohol. Assuming the relative risk (RR) for a person with a daily alcohol consumption of 25 g to be 1, Tuyns and co-workers found a controlled RR of 12.5 for hypopharynx carcinoma, 10.6 for epipharynx carcinoma, 2.0 for supraglottic larynx carcinoma, and 3.4 for glottic and subglottic larynx carcinoma when 121 g of alcohol were consumed daily (3). Another European group, Brugere and co-workers, found significantly higher RRs of 13.5 for oral cancer, 15.2 for oropharyngeal carcinoma, and 28.6 for hypopharyngeal carcinoma, when 100–159 g of alcohol were consumed daily (4). It is noteworthy that even with those high daily alcohol doses the alcohol-associated cancer risk is not saturable. If alcohol is consumed excessively with more than 160 g/day, there is a further increase in cancer risk (oral cancer RR = 70, oropharyngeal cancer RR = 70, hypopharyngeal cancer RR = 143). In general, American studies often showed lower RR, a fact that could not be explained satisfactory. A good overview of the different epidemiological studies is given in an International Agency of the Research on Cancer Report (2).

With respect to upper-GI-tract cancer, alcohol and tobacco act together in a more multiplicative rather than an additive manner and seem to have synergistic tumor-promoting effects. In summary, the various studies clearly show that alcohol is the main cause of cancer of the oral cavity, the pharynx, and the esophagus and, together with tobacco smoke, the main risk factor for laryngeal cancer (5).

It has also been estimated that up to 80–90% of these cancers can be avoided just by abstaining from these two risk factors.

B. Liver

In contrast to the well-performed and numerous epidemiological studies in the upper alimentary tract, data for the association between cancer risk in the liver and alcohol consumption are much more rare and indefinite. This is mainly due to the confounding factors of viral infection and preexisting viral or alcoholic liver cirrhosis.

Cirrhosis of the liver is the major prerequisite for the development of hepa-

tocellular cancer. Since infection with hepatitis B and C virus also leads to cirrhosis of the liver followed by an increased occurrence of hepatocellular cancer and since alcoholics are often infected by those viruses, the exact risk of alcohol as compared to hepatitis B and C etiology in the development of hepatocellular cancer is still not exactly defined. Almost all prospective and retrospective case-control studies in Western countries indicate that the incidence of hepatocellular cancer among alcoholics is above the expected level (6) and deaths from liver cancer are reported to be increased in alcoholics by about 50%. However, variable prevalence of hepatocellular cancer in alcoholic cirrhosis has been reported. With some exceptions generally lower incidence rates have been reported in Western countries (<15%), which showed some increased trends within the last two decades, while in Japan the prevalence of hepatocellular cancer in alcoholic cirrhosis increased at the rate of 1.0% per annum during 1976 to 1985, reaching a 25% incidence rate (7). The higher prevalence in Asia may be linked to the increased concomitant viral infection.

C. Breast

The data on breast cancer and alcohol consumption have been discussed controversially for years. Recently, a large meta-analysis of all available data was performed, showing a 1.2–2.0-fold relative risk (8). Strong evidence was found of a dose-response relationship in case-control and follow-up studies. The relative risk of breast cancer at an alcohol intake of 24 g of absolute alcohol per day relative to nondrinkers was found to be 1.4 in the case-control and 1.7 in the prospective studies. The risk is increased with higher doses, but in contrast to the upper GI tract the attributable risk seems to be saturable and no higher relative risks than up to 2.0 are reported.

As there is today no doubt about the association of breast cancer risk and alcohol consumption in moderate and heavy drinkers, a recent reevaluation of data from the original Framingham study supported no evidence for an association of breast cancer with only low alcohol intake as compared to nondrinkers (9). However, given the high incidence of these tumors, every effort should be made to reduce alcohol intake in women who are at risk to develop breast cancer, since a recent study showed that even in patients with familial breast cancer, the risk is still increased if alcohol is consumed.

D. Colon and Rectum

Numerous epidemiological studies including case-control studies and prospective cohort studies have identified the rectum as a site of enhanced alcohol-associated cancer development. In a meta-analysis from 1992 seven correlational studies, 34 case-control studies, and 17 prospective cohort studies were reevaluated (10).

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An association was found in five of the seven correlational studies and in half of the 34 case-control studies. In the majority of the case-control studies (10 of 12) using community controls such a correlation was found, suggesting that the absence of an association when hospital controls were used is due to a high prevalence of alcohol consumption/alcohol-related diseases in the hospital controls. Eleven of the 17 cohort studies also demonstrate a positive association with alcohol. A positive trend with respect to dose-response was found in five of the 10 case-control studies and in all prospective cohort studies in which this factor had been taken into consideration. Later studies confirmed those results in general and found attributable relative risks of approximately two- to threefold. While a dose-response relationship is apparent, the relative risks seem to be saturable, while an increased risk is seen in most of the studies at a daily consumption of approximately 20–40 g/day.

Several prospective studies have also found an association between colon polyps, precursor lesion for malignant transformation, and high alcohol consumption (11). Recently, it was shown that alcohol also increases the risk for developing liver metastases in colorectal carcinoma (12). Thus alcohol obviously interferes and influences in different phases in the multistep process of colorectal cancer.

In conclusion, epidemiological data are still somewhat controversial, but it seems that chronic ethanol ingestion, especially consumed as beer, results in a 1.5–3.5-fold risk of rectal and to a lesser extent colonic cancer in both sexes, but predominantly in men. Recently, these data have been reviewed in detail by a panel of European experts at the WHO Consensus Conference on Nutrition and Colorectal Cancer in Stuttgart, Germany and it was stated that more than 20 g of alcohol per day increases the risk of colorectal cancer (13).

E. Other Organs

Alcohol has been suspected to play a causal role in the development of stomach, pancreatic, bladder, and other cancer, but the overall incidence of available studies suggests that alcohol is not a risk factor for these cancers (5). However, in a subset of patients with deficient aldehyydedrogenase-2, leading to an accumulation of the metabolite acetaldehyde, an increased risk for developing stomach cancer has been reported (14).

III. PATHOMECHANISMS OF ETHANOL-ASSOCIATED CARCINOGENESIS IN THE UPPER GI TRACT

Alcohol itself is not carcinogenic. However, it has various tumor-promoting effects. Alcohol promotes carcinogenesis in the upper GI tract locally via swal-

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lowing or redistribution in the water phase after absorption and/or via different systemic pathways. Given the extraordinarily high attributable cancer risks in the upper GI tract in contrast to the weak to moderate cancer risks in other organs with ethanol-associated carcinogenesis, such as liver, breast, and colon, it is clear that local alcohol effects might be of particular importance. This is supported by the fact, that the endolarynx, which has no direct contact with alcohol during drinking, shows the lowest alcohol-related cancer risks of all anatomical subsites in the upper GI tract (2).

Systemic alcohol-promoting effects include displacement of potential cancer-protective nutrients in the diet, inhibition of detoxification of carcinogenic compounds due to inhibition of liver enzyme function, an increase in the oxidative exposure, alterations in the hormonal status, and suppression of immune function (5).

Moreover, chronic alcohol leads to the induction of cytochrome P-450 2E1, an enzyme that is important also for the toxification and detoxification of certain compounds. Accordingly, the induction of CYP 2E1 may lead to increased activation of procarcinogens that are mainly inhaled by smoking.

When alcohol is present, nitrosamine metabolism in the liver is also inhibited. Dimethylnitrosamine undergoes a first-pass metabolism in the liver. When alcohol is given concomitantly, the first-pass metabolism of dimthylnitrosamine in the liver is partly blocked due to competitive inhibition with alcohol in the microsomal enzyme system and, as a result, more nitrosamines can bypass the liver (15). Subsequently, activation and enhanced exposure of the nitrosamine occurs in extrahepatic organs such as the upper GI tract.

Vitamin deficiencies such as those of riboflavin and zinc may be of additional importance, moreover, as severe drinkers frequently suffer from malnutrition (16).

Local alcohol effects can be caused while drinking, and especially beverages with a very high alcohol content such as spirits are suspected to cause severe local damage. Ethanol may facilitate the uptake of environmental carcinogens, especially from concomitantly inhaled tobacco smoke, through cell membranes that are damaged and changed in their molecular composition by the direct effect of alcohol. Furthermore, it is postulated that alcohol acts as a solvent that enhances the penetration of carcinogenic compounds (e.g., tobacco smoke) into the mucosa (5). Both factors may be relevant in the upper GI tract, particularly since chronic alcohol abuse leads to atrophy and lipomatic metamorphosis of the parenchyma of the parotic and submandibulary gland and this morphological alteration results in functional impairment including reduction of saliva flow and increased viscosity of saliva (17). Thus, the mucosa surface will be insufficiently rinsed. Therefore, higher concentrations of locally effective carcinogens, in addition to a prolongation of the contact time of those substances with the mucosa, can be observed.

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Moreover, direct mucosal damage by high alcohol concentrations of more than 20% can occur (18). This has been proposed to cause a repair mechanism of the mucosa with accelerated cell division, which, in the case of chronic alcoholism, leads to an abnormally increased regeneration. This hyperproliferation itself causes an increased susceptibility toward concomitantly inhaled or ingested carcinogens. A permanent hyperproliferation is also a first step in the multistep process of malignant transformation (19,20).

Other local cocarcinogenic effects may be enhanced intake of possible carcinogenic congeners in alcoholic beverages. For example, high concentrations of nitrosamines have been found in whiskey and beer (21). Another factor may be impaired motility of the esophagus due to alcohol and the enhanced gastroesophageal reflux leading to esophagitis and Barrett's esophagus, a precancerous lesion of the distal esophagus.

However, two epidemiological findings cannot be explained sufficiently by these possible local alcohol effects. First, every alcoholic beverage, and not only high-alcohol-content spirits or certain nitrosamine-rich liquids, cause cancer. Second, cancer also occurs in well-nourished, nonsmoking subjects without additional procarcinogen intake. Thus, additional, direct, local carcinogenic effects of alcohol must exist. This leads to the intermediate of alcohol metabolism, acetaldehyde.

IV. ACETALDEHYDE: TOXICITY AND CARCINOGENEITY

A. Acetaldehyde as a Chemical Substance

Acetaldehyde is a volatile, colorless mobile liquid with a pungent odor. It evaporates quickly from aqueous solutions owing to its low bowling point of 20.2°C. It is miscible with water and most common organic solvents, and, thus, can easily pass organic membranes in the human body. It is highly reactive and undergoes numerous chemical reactions such as condensation, addition, and polymerization reactions. In the human body, it is a metabolic intermediate, with alcohol oxidation being the major source.

B. Natural Occurrence

On the other hand, acetaldehyde is not just one compound deriving from ethanol metabolism. Exposure to humans is widespread and occurs regularly. Acetaldehyde is emitted in the air due to residential external combustion of wood. It is also a natural component in the leaves of several plants and, as an aldehyde, found in the essential oil of many different spices and fruits. It has been detected

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in many animal feeds and in food such as cheese, heated skim milk, and cooked beef (22).

Acetaldehyde is also a chemical intermediate for many different biochemical and chemical reactions and, thus, used in the industrial synthesis of many chemicals (acetic acid, pyridine and pyridine bases, polyols, various acetals used as flavor and fragance additives, plastics, synthetic rubber) and is produced as a by-product in industrial combustion (22).

Acetaldehyde is also detected in the smoke of various tobacco products in high concentrations (22).

However, the main human exposure derives from the intake of alcoholic beverages. Although acetaldehyde can be produced as an intermediate in the reduction of carbohydrates to alcohol in microbial metabolism in the gut, the major metabolic pathway, if alcohol is consumed, is oxidation from alcohol to acetaldehyde, both in microbes and in somatic cells.

C. The Metabolic Pathways in Alcohol Metabolism

Alcohol is metabolized mainly by various alcohol dehydrogenases. Enzymatic production of acetaldehyde from ethanol by the microsomal enzyme system (MEOS), cytochrome P-450 IIE1, and catalase plays only a negligible role. Alcohol dehydrogenases (ADH) are present in different genetic forms. Four classes of ADH exist with various kinetic properties (ADH1–4). ADH2 and ADH3 exhibit genetic polymorphism and it is known that ADH2-2 and ADH3-1 are involved in alcoholism and alcohol-associated organ damage (23). Both enzymes produce more acetaldehyde than their corresponding isozymes.

Acetaldehyde, as a toxic compound, is usually rapidly further metabolized to acetate, mainly by different aldehyde dehydrogenases, for which, again, four different isozymes exist. ALDH2 is the most important, as owing to its low K_m, it is capable of rapidly detoxifying even very small concentrations of acetaldehyde. Again, a genetic polymorphism exists, and patients with homozygous deficient forms of ALDH2 are unable to tolerate alcohol owing to acetaldehyde accumulation (so called ''Oriental flushers''; 14). The major organ of alcohol metabolism is the liver, where more than 90% of the alcohol is metabolized. However, various tissues such as the kidney, lungs, stomach, and bone marrow cells can metabolize alcohol, and in the mucosa of the whole gastrointestinal tract, alcohol-metabolizing enzymes are expressed. Recently, it has been described that aerobic, facultative anaerobic, and microaerophilic bacteria and yeasts, which colonize the whole GI tract, harbor enzyme systems capable of metabolizing alcohol and acetaldehyde. Although the enzymatic background of these pathways is still poorly understood, the higher detoxification capability to metabolize acetaldehyde of these enzyme systems with respect to somatic cells is obvious. Thus, much higher concentra-

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tions of acetaldehyde can be achieved in fluids with microbial colonization as compared to somatic cells (24–26).

D. Acetaldehyde: Mutagenicity and Genotoxicity in Microbial and Eukaryotic Systems

Acetaldehyde, the first intermediate of alcohol metabolism, is a highly toxic and volatile compound. It interferes at many sites with DNA synthesis and repair (27). Numerous in vitro and in vivo experiments in pro- and eukaryotic cell cultures have strikingly shown that acetaldehyde has well-known direct mutagenic and carcinogenic effects. It causes point mutations in certain genes, induces sister chromatide exchanges and gross chromosomal aberrations, and induces micronuclei formation (28–30). It induces inflammation and metaplasia of tracheal epithelium, delay in cell cycle progression, stimulation of apoptosis, and enhanced cell injury associated with hyperregeneration.

In all the different cell systems used, a strong dose dependency was observed and the induced damages occurred after acetaldehyde treatment with acetaldehyde concentrations ranging from 40 to 1000 μM and incubation times from 1 to 90 hr.

Available evidence suggests that acetaldehyde produces similar cytogenetic effects in vivo. It is noteworthy that 15 years ago Obe and colleagues showed that the metabolism of ethanol leads to a compound that induces mutagenic properties and they stated that acetaldehyde but not ethanol is mutagenic and carcinogenic (30).

E. Acetaldehyde: Other Tumor-Promoting Pathomechanisms

It has also been shown that acetaldehyde interferes with the DNA-repair machinery. Acetaldehyde, and not ethanol, directly inhibits O^6-methylguanine transferase, an enzyme important for the repair of adducts caused by alkylating agents (31).

Acetaldehyde also binds rapidly to cellular proteins and to DNA, which results in morphological and functional impairment of the cell. Covalent binding to DNA and the formation of stable adducts is one mechanism by which acetaldehyde could trigger the occurrence of replication errors and/or or mutations in oncogenes or tumor suppressor genes (32). The occurrence of stable DNA adducts has been shown in different organs of alcohol-fed rodents (33). Moreover, it has recently been shown that the major stable DNA adduct, N^2-ethyl-guanosine, can indeed efficiently be utilized by eukariotic DNA polymerases (34). In addition, acetaldehyde adducts represent neoantigens leading to the production of

specific antibodies and to the stimulation of the immune system, possibly leading to a cytotoxic immune response (35).

Acetaldehyde has also been shown to destroy folic acid in vitro (36). Folic acid is an important cofactor in restoring *S*-adenosylmethionine and, consequently, the C1-transmethylating pool. Hypomethylation of certain functional genes has been shown to occur in an early step of cancer development. Thus,it is not surprising that a low-folate diet is associated with an increased cancer risk in humans (37). Accordingly, the cleavage of folate might be another tumor-promoting effect of acetaldehyde.

F. Acetaldehyde: Carcinogenicity Studies in the Animal Model

In an inhalation study in hamsters, it has been shown that acetaldehyde at concentrations of 2750 mg/m^2 caused nasopharyngeal and laryngeal carcinoma (38).

In a carcinogenicity study in rats, again the larynx and the nose (which is the major site in inhalation, as all rodents are nose breathers) were the major target sites of acetaldehyde (39). Severe hyperplastic and metaplastic changes such as malignant tumors were observed, and in a recovery study, preneoplastic changes were shown to possibly progress to maligant tumors despite ending of treatment.

Oral studies failed to show definite tumor development. However, again hyperplastic and metaplastic alterations of the upper GI tract were observed (40).

In conclusion, according to the International Agency for Research on Cancer, there is sufficient evidence to identify acetaldehyde as a carcinogen in animals (27).

V. ACETALDEHYDE: EVALUATION OF ITS ROLE IN ALCOHOL CONSUMPTION AND HUMAN CANCERS

No direct studies or epidemiological data are available with respect to the direct teratogenicity, mutagenicity, or carcinogenicity of acetaldehyde to humans. However, indirect evidence is reported that acetaldehyde might be involved in the carcinogenesis of alcohol-related cancer. DNA-acetaldehyde adducts, which possibly might cause DNA damage due to deficient repair, DNA strand breaks, and/or point mutations, have been reported in granulocytes of alcoholics; however, no data are available for target tissues of alcohol-associated carcinogenesis such as the upper GI tract, liver, and colorectum (33).

Recent and striking evidence for a causal role of acetaldehyde in ethanol-associated carcinogenesis derives from genetic linkage studies in alcoholics.

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Individuals who accumulate acetaldehyde due to polymorphism and/or mutation in the genes coding for enzymes responsible for acetaldehyde accumulation and detoxification have been shown to have an increased cancer risk (14,23).

Acetaldehyde is produced by mucosal alcohol dehydrogenases (ADH) and detoxified by mucosal aldehyde dehydrogenases (ALDH). The main mucosal ADH belong to the class I ADH, especially ADH3 for which a polymorphism exists. Recently, it has been shown that individuals with ADH3∗1, which has a significantly higher V_{max} compared to ADH3∗2, leading to an enzyme expression that rapidly converts ethanol to acetaldehyde, are at an increased risk for esophageal cancer when consuming more than 60 g ethanol per day (23).

Acetaldehyde is further metabolized and detoxified by various ALDH. This mitochondrial ALDH-2, owing to its low K_m, is capable of detoxifying even very small acetaldehyde levels. Individuals who lack ALDH-2 activity cannot metabolize acetaldehyde adequately and thus accumulate acetaldehyde. Indeed, a Japanese group demonstrated that individuals with heterozygous mutations in the ALDH-2 gene, leading to decreased enzyme activity, have a much higher risk of developing a malignant tumor in the upper GI tract than nonmutant controls, if high alcohol amounts are taken (14).

These recent findings raised the idea that mainly local acetaldehydes trigger the tumor-promoting effects. However, recent findings showed that the major metabolic source of local acetaldehyde might be the microbial production of acetaldehyde from ethanol by the oral microflora.

A. The Microbial Production of Acetaldehyde

It is well-known from alcoholic fermentation that microbes have the possibility to produce energy by metabolizing sugar to alcohol. This occurs also in the human body and ethanol can be produced by endogenous conversion of different carbohydrates to ethanol by the intestinal microflora. Patients with a ''blind-loop'' syndrome and a consequential bacterial overgrowth in the gut have been shown to have measurable amounts of blood alcohol deriving from this microbial production of alcohol (41). The last biochemical step is reduction from acetaldehyde to ethanol via microbial ADH.

Like almost every enzymatic reaction, the direction of this reaction depends on the circumstances, e.g., excess of the substrate and the presence/absence of oxygen, and is, in principle, reversible. Thus, it has been described that microbes can produce alcohol to acetaldehyde, a finding described first by Lieber and colleagues in the oropharynx and the bronchopulmonary tract (42,43). They showed that acetaldehyde was produced from ethanol in bronchopulmonary washings of humans and concluded that in addition to mucosal production, acetaldehyde is also produced by microbes (43). The first in vivo evidence for a direct link to bacteria was revealed by mouth rinses with ethanol in human volunteers. Local

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acetaldehyde production in the oral cavity from ethanol was almost totally abolished after the volunteers rinsed their mouth with a local antiseptic, indicating that these acetaldehyde levels were of microbial origin (42).

Seitz and colleagues were the first to show experimental evidence that this bacterial acetaldehyde might possibly be involved in ethanol-related carcinogenesis. In their animal model, they showed that ethanol feeding in rats led to an abnormally increased proliferation in the rectum of rats, a condition that has been linked to increased cancer development. This hyperregeneration was directly associated with the measured mucosal acetaldehyde levels and they concluded that the acetaldehyde levels were mainly of microbial origin (44). The existence of the microbial oxidation of ethanol in humans is considered to be so efficient that disturbances in the fecal microflora can alter ethanol metabolism (45).

In the human body, acetaldehyde is quickly detoxified by ALDH. Its major kinetic form is ALDH2, which, owing to its high K_m, is capable of metabolizing even small amounts of acetaldehyde very efficiently. This ALDH is expressed in a variety of cells in the human body and in the mucosa. Thus, systemic acetaldehyde levels are usually very low. In contrast, bacteria seem to lack sufficient ALDHs for the quick conversion of small amounts of acetaldehyde (46). Thus, very high acetaldehyde levels can occur in human fluids with physiological bacterial colonization in case of microbial conversion from ethanol to acetaldehyde. Jokelainen et al. were able to show very high acetaldehyde production from ethanol in human feces in vitro (24). They also reported extremely high acetaldehyde levels in vivo in the gut of piglets after ethanol administration, leading to concentrations of up to 1 mmol/L (25). These acetaldehyde levels could have been significantly inhibited by ciprofloxacin, indicating that mainly the aerobic and facultative anaerobic flora is involved in microbial acetaldehyde production. This was supported by recent findings of Tillonen et al., who showed that treatment with metronidazole, reducing the amaerobic flora of the gut, leads to an increase in microbial acetaldehyde production (unpublished results, personal communication).

Ethanol is present in saliva in concentrations comparable to blood ethanol levels after the consumption of alcoholic beverages (47). Recent research revealed a substantial production of acetaldehyde of up to 140 μM in the saliva of volunteers consuming moderate amounts of alcohol (0.5 g/kg body weight). This acetaldehyde production was significantly decreased after treatment with the antiseptic chlorhexidine (26). Salivary acetaldehyde levels have been shown to be significantly increased in heavy drinkers and smokers (48). Smoking showed a positive linear correlation and it can be estimated that a smoker with a daily consumption of approximately 20 cigarettes has an increased salivary acetaldehyde production of about 50–60%. This implies that smokers, even after moderate alcohol intake, produce much higher levels of carcinogenic acetaldehyde in the oral cavity than nonsmokers. Alcohol seems to interact and increase salivary

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acetaldehyde production only if consumed heavily (>40 g/day); when an increase is observed it is dose dependent and it increases salivary acetaldehyde levels in average by about 50%. Smoking and alcohol together further increased the salivary acetaldehyde production by about 100% as compared to nonsmokers and moderate alcohol consumers (48).

The microbial background of salivary acetaldehyde production from ethanol has also been investigated. Studies on humans showed that mainly aerobic bacteria (*Streptococcus salivarius, Hemolytic viridans* group, streptococci, *Corynebacterium* sp., *Stomatococcus* sp., and yeasts) are associated with an increased acetaldehyde production (48).

Studies focusing on yeasts in saliva clearly showed that yeast colonization was found significantly higher in high acetaldehyde-producing salivas than in the acetaldehyde-producing salivas (49). Among carriers, the density of yeasts was higher in the high than in low acetaldehyde producers. Moreover, *Candida albicans* strains isolated from the high-acetaldehyde-producing salivas formed significantly higher acetaldehyde levels from ethanol than *C. albicans* strains from low-acetaldehyde-producing salivas.

Recent experience from intestinal bacteria indicated that there are some strains that have much higher acetaldehyde production capacity and ADH activity than others. Also, the ALDH activity differs significantly. Thus, the observed interindividual differences in microbial acetaldehyde production in the upper GI tract and in the intestine of humans may be due to different microbial colonization.

VI. POSSIBLE ROLE OF MICROBIAL ACETALDEHYDE PRODUCTION IN ETHANOL-ASSOCIATED CARCINOGENESIS: LINKS FROM EPIDEMIOLOGICAL TO EXPERIMENTAL EVIDENCE

In addition to the hypothetical possibility, there is epidemiological and experimental evidence that the microbial acetaldehyde production from ethanol might be a major factor of ethanol-associated carcinogenesis.

The upper GI tract is the major target organ of ethanol-associated carcinogenesis. Its epithelial lining is exposed to alcohol during swallowing and, accordingly, very high ethanol concentrations in the mucosa can be achieved. In addition to the systemic mechanisms, the tumor-promoting effect on the mucosa of the upper GI tract may, as pointed out, largely be local.

One common observation is an accelerated cell division locally in the upper and lower GI tract after alcohol intake. Abnormal proliferation is a characteristic sign of cancer and increased cell division can be observed at early stages of carcinogenesis in the upper GI tract (19,20). Hyperproliferation is one consistent

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finding in the esophagus of residents in high-risk regions for esophageal cancer and in patients with Barrett's esophagus. Hyperplastic lesions in the oral cavity show increased cell division and a sequentially enhanced cell proliferation is observed in different stages of preneoplastic lesions during head and neck carcinogenesis (50). Increased proliferation itself can trigger cancer development by accumulation of replication errors during DNA synthesis and by higher susceptibility to ingested carcinogens.

Accelerated cell division after ethanol intake has been observed in the oral cavity, the esophagus, and the rectum (50–52). Accordingly, this is assumed to be one of the effects of local ethanol-associated carcinogenesis. Direct mucosal damage may be an explanation for the hyperproliferating effects of ethanol but this remains speculative, as ethanol up to a concentration of 20% seems to be rather harmless (18).

Interestingly, the two major anatomical sites, the upper GI tract and the rectum, where ethanol-associated hyperproliferation has been demonstrated are regions with very high concentrations of microbially produced acetaldehyde (24–26). Moreover, it has been shown that normal salivary function is essential for this increased cell proliferation in the esophagus. In animals that did not produce saliva owing to the removal of salivary glands, a normal proliferation pattern was observed after ethanol ingestion (51).

In animal studies with rats, we have demonstrated that acetaldehyde administered in drinking water, and thus mimicking salivary acetaldehyde levels without concomitant ethanol intake, causes hyperplastic and hyperproliferating changes in the tongue, epiglottis, and forestomach (53).

Thus, there is experimental evidence that the ethanol-associated hyperproliferation may mainly be induced by its metabolite acetaldehyde and that its local tumor-promoting effect is caused via the salivary redistribution of ethanol. Consequently, microbially produced acetaldehyde in saliva may be responsible for the tumorigenic effect of ethanol on upper-GI-tract cell regeneration.

Strong evidence for acetaldehyde as the major factor behind ethanol-associated carcinogenesis is derived from studies linking the genotypes of ethanol-metabolizing enzymes to tumor risk. Rapidly metabolizing ADHs (ADH3), leading to higher and quicker production of cellular acetaldehyde, and the lack of low-k_m ALDHs (ALDH2), leading to a longer and delayed exposure to acetaldehyde, have recently been shown to be associated with an increased cancer risk of the upper GI tract (14,23). In a recent study in Orientals, the possible correlation between ALDH2 genotype mutation and cancer risk in alcoholics has been expanded to all possible alcohol-related cancers. In this study, the frequencies of mutant ALDH2-2 allele were significantly higher in alcoholics with oropharyngeal, laryngeal, esophageal, stomach, colon, and lung cancer but not in those with liver or other cancers (14). This is very interesting, as all these organs are covered by microbes and the microbial production of acetaldehyde from ethanol

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is described. Thus, it is possible that the hampered detoxification of acetaldehyde from ethanol in ALDH2-deficient subjects might become clinically relevant only in case of exceeding acetaldehyde production by microbes. Hence, there is additional support of microbial acetaldehyde production from ethanol as a possible factor in alcohol-associated carcinogenesis. Moreover, it is possible that the salivary gland itself may produce acetaldehyde, which might be of relevance only in Orientals. Studies to examine this effect are ongoing.

One interesting large epidemiological study showed that alcohol together with a low-folate, low-methione diet leads to an increased cancer risk, introducing possible effects of C1-transmethylating agents in ethanol-associated carcinogenesis (37). In addition to a dietary deficiency among alcoholics, high acetaldehyde levels have been shown to catabolize folate via cleavage at the C9–N10 bond (36). Thus, it is possible, that high local acetaldehyde levels could break down folate. This interesting in vitro mechanism by which alcohol could lead to folate deficiency has often been considered nonsignificant for the situation in vivo, as the reported concentrations of acetaldehyde so far have been thought to be much too low. The observation of extremely high acetaldehyde levels in the colon of rats prompted us to investigate this possible acetaldehyde effect. We could show that alcohol treatment led to folate deficiency in the colon of rats, an effect that could be overcome by antibiotic treatment (which abolishes microbial acetaldehyde production without affecting ethanol levels) (54). Accordingly, local microbial acetaldehyde may cleave folate, an important cancer-protective substance, an effect that could also be true for the upper GI tract, as, with respect to microbial acetaldehyde production, conditions comparable to the colorectum can be seen in the oral cavity. It also offers a unique explanation for the combined effect of low-folate and high-ethanol intake with respect to cancer development.

Although alcohol and tobacco smoke are well-known independent and strong risk factors for upper-GI-tract cancer, their combined effect on these epithelia is poorly understood. Studies have strikingly shown that smokers have higher cancer rates in conjunction with high exposure to alcohol than could be expected from the attributable data for each risk factor alone (2). Hence, with regard to upper-GI-tract cancer alcohol and tobacco act together in a more multiplicative rather than in an additive manner and seem to have synergistic tumor-promoting effects (2–5). Enhanced solubility of concomitantly inhaled tobacco carcinogens might be one explanation. However, our observation that, probably owing to tobacco-induced effects on the oral microflora with an overgrowth of yeasts and gram-positive bacterial strains, smokers have an increased salivary acetaldehyde production from ethanol in contrast to nonsmokers could also be a biologically plausible explanation (26,48). Moreover, it would not necessarily require concomitant misuse of alcohol and tobacco, as there are other postulated cancer-promoting effects. In addition, it has been shown that cancer patients have an increased acetaldehyde production in the oral cavity from ethanol (55).

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Most epidemiological studies have shown that indicators of poor dental hygiene such as tooth loss, poor dentition, and infrequent practice of oral hygiene habits are independent but only weak risk factors for oral cavity cancer (56–60). Some studies demonstrated that heavy drinkers and/or smokers have a substantially higher risk if they have poor oral hygiene than would be expected if the traits were considered additively (56,60). Thus, there is evidence that the higher incidence of poor dental status in subjects with high alcohol intake may worsen the carcinogenic effects of alcohol, suggesting a joint and synergistic effect between these risk factors, as is proven for the combined use of alcohol and tobacco products. In addition, one study analyzed the risk factors of oral cancer patients who were nonusers of tobacco and alcohol and found that in this cohort poor dental status was not associated with an increased cancer risk (59).

We have recently been able to show that independent of ethanol and smoking habits, poor dental status, reflecting poor oral hygiene accompanied by bacterial overgrowth, leads to an increased salivary acetaldehyde production from alcohol mediated by microbes (61).

As alcohol is involved synergistically in the attributable risk of both smoking and poor oral hygiene, it is conceivable to suggest a unifying pathogenic mechanism of alcohol drinking behind these epidemiological findings. This could, again, be the local production of carcinogenic acetaldehyde from ethanol by microbes.

One other hypothesis is the unsatisfactory explanation for the association between alcohol intake and laryngeal carcinoma. Alcohol is a strong and independent risk factor for laryngeal cancer, with the highest risk for supraglottic carcinoma, which decreases stepwise from glottic to subglottic carcinoma, without evidence for a link between ethanol intake and cancer in the lower bronchopulmonary tract and the lung. Local ethanol effects may obviously not account for the tumor-promoting effects. Salivary acetaldehyde, owing to its high vapor pressure and votality, is capable of entering the upper bronchopulmonary tract. On the other hand, owing to its high reactivity and toxicity, it is assumed to bind very rapidly to all macromolecules. In accordance with this observation, carcinogenesis studies in animals could only show an increased cancer risk in regions of the upper aerobronchopulmonary tract after inhalation of acetaldehyde (38–40,53). Thus, one might assume that salivary acetaldehyde levels are much more likely to account for laryngeal cancer development than ethanol itself.

From the investigations on the bacteria involved in ethanol metabolism to acetaldehyde some important conclusions can be drawn. They have been shown to obviously lack sufficient ALDH activity, and the microbes involved include mainly aerobic, facultative aerobic, or microaerophilic bacterial strains and yeasts (24,26,46,48,62). As an overgrowth of yeasts is frequently observed in alcoholics and smokers and, of special interest, in patients with oral cavity cancer, the finding of an association between yeast colonization and acetaldehyde production in

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saliva might indeed be of pathogenic importance (48,49,63). The limited evidence we have from this field, however, supports evidence of a causality of microbes in ethanol metabolism to acetaldehyde.

VII. CONCLUSIONS AND IMPLICATIONS

In conclusion, there is increasing evidence that the major factor behind alcohol-associated carcinogenesis is the intermediate acetaldehyde. The recent data on the genetic linkage of alcohol-metabolizing enzymes and an increased cancer risk, if these mutations lead to an intracellular accumulation of acetaldehyde, have shown striking evidence for this hypothesis. Observation of stable, distinct, and chemically defined DNA adducts is a further step to study the underlying mechanisms of the role of acetaldehyde in alcohol-associated carcinogenesis. Future studies focusing on detecting these adducts in target tissues of high-risk subjects will help to evaluate their causal role in the multistep process of malignant transformation. In vitro studies to investigate this adduct and its role in causing a certain mutational pattern in regulatory genes, such as tumor suppressor genes, will be conducted in the future and would possibly lead to identification of specific ''alcohol-related'' gene mutations, as research has found for tobacco smoke.

The new findings of the possible causal role of microbial acetaldehyde production in upper GI tract and colorectal cancer in alcoholics could open a new microbiological approach to the pathogenesis of oral cavity and upper-GI-tract cancer and may influence future prevention strategies.

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Alcohol and Gastrointestinal Cell Regeneration

Ulrich A. Simanowski and Helmut K. Seitz
Salem Medical Center, Heidelberg, Germany

I. INTRODUCTION

Cellular proliferation or regeneration is an important characteristic of the biology of tissues. It is mandatory for maintaining tissue form and function. A great number of variables are involved in cell regeneration of a given tissue: overall cell number, growth fraction, speed of the cell cycle, age distribution of cells, length of cell cycle phases, possible changes over time versus steady-state conditions. Cell regeneration is an important descriptor in tissue injury and in carcinogenesis. Since alcohol increases cancer risk in certain regions of the gastrointestinal (GI) tract, it seems necessary to evaluate proliferative changes after ethanol exposure. It is a general principle that increased epithelial cellular regeneration constitutes a state of increased susceptibility toward carcinogens and carcinogenesis (1). This concept is supported by the observation that hypoproliferative states correlate with decreased cancer risk (2–4). This review will focus on alcohol-related changes of GI cell regeneration especially in relation to the reported increased cancer risks in the upper GI tract and in the colorectum of regularly drinking subjects.

II. MEASUREMENT OF CELL PROLIFERATION

The majority of cell proliferation studies in humans as well as in experimental animals use so-called state measures, which are one-time-point observations, recording the number of cells in certain phases of the cell cycle. The mitotic index

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(MI) can be tested in routine histological sections; however, the variation of the MI is large and this method is tedious, because the duration of mitoses is very short compared to the length of the cell cycle (5). Therefore, most studies use markers of the longer S phase or markers that are present in cycling cells. Currently the S-phase index is estimated after immunostaining for the proliferating cell nuclear antigen (PCNA), which seems to belong to the cyclins that are involved in regulation of the cell cycle. They are expressed in the late G1, S, and early G2 phases (6). The relatively new method of in situ hybridization for histone H3 produces reliable staining of S-phase cells (6). Previously used markers of S-phase cells include labeling with tritiated thymidine and autoradiography for its detection and bromodesoxyuridin with immunocytochemistry. For estimation of the growth fraction Ki-67 immunostaining is a widely used method, since Ki-67 is expressed almost during the whole cell cycle, except in early G1 (6). To evaluate the actual speed or rate of cell proliferation, which is cells produced per 100 cells per minute, stathmokinetic methods can be used in experimental animals. A stathmokinetic agent, e.g., vincristine, is given, which leads to disruption of the cell cycle by preventing the formation of the spindle apparatus in mitoses. Consequently, metaphases accumulate with time, which can be recorded histologically (5). Other methods of cell proliferation studies utilize determination of the DNA content of cells with flow cytometry to estimate the number of cells in each phase of the cell cycle. However, all or almost all histological information of the tissue under investigation is lost.

III. UPPER GI TRACT

Chronic alcohol consumption is a major risk factor for the development of cancer of the oral cavity, pharynx, larynx, and esophagus (7–13). The effects of alcohol and smoking seem to potentiate each other. The upper GI tract as far as the upper jejunum is directly exposed to ethanol during drinking. From there downward intestinal mucosal ethanol levels are identical to blood levels. The hypothesis evolved that alcohol induces a toxic injury to the upper GI mucosa, resulting in compensatory hyperregeneration, which renders the mucosa more susceptible to the action of carcinogens, while smoking provides those carcinogens. Indeed it has been shown that alcohol produces direct mucosal damage of the oral cavity (14) as well as in other parts of the upper GI tract (1,15–18). This toxic event would in turn lead to reparative mucosal growth and therefore increased cell regeneration of the exposed mucosa. This could be shown in most, but not all, studies of cell proliferation. It may be due to different alcohol concentrations used, different susceptibility of experimental animals, and, last but not least, different study design, since acute exposure to highly concentrated ethanol leads to

initial inhibition of cell proliferation, and then after a time leads to a burst of cell renewal (19–25).

What are the possible mechanisms of ethanol-related mucosal hyperregeneration and propable links to carcinogenesis in the upper aerodigestive tract? In contrast to breast cancer and cancer of the colon and rectum, there is a clear-cut dose-response relationship: The more you drink, the higher the risk. It is from this observation that ethanol-induced mucosal damage seems to be important. It is obvious that highly concentrated spirits have a direct damaging effect. However, beverages with low alcohol content such as beer and wine exhibit the same dose-response effect of cancer risk. So what is the possible mediator to bring about the mucosal damage? The first metabolite of ethanol is acetaldehyde (AA), which is a highly toxic and reactive compound that binds rapidly to protein and even DNA, resulting in cell injury (26–28). In a recent study it was demonstrated that AA feeding results in stimulation of epithelial cell proliferation in the upper GI tract (29). AA can be produced via the mucosal alcohol dehydrogenase (ADH) or by oral bacteria. The bacterial pathway seems to be quantitativly much more important than the ADH pathway (30,31). At present AA seems to be the toxic compound that is responsible for mucosal damage after ethanol exposure, which in turn leads to epithelial hyperregeneration. This does not imply that cellular hyperregeneration is the only mechanism by which alcohol stimulates carcinogenesis (see related chapters of this book). There are also other variables that influence ethanol-related stimulation of cell proliferation. In one study we demonstrated that age influences the magnitude of the stimulatory effect. The same study yielded the observation that the esophageal epithelium depends on trophic factors from saliva to respond to proliferative stimuli (24).

IV. COLON AND RECTUM

The majority of epidemiological studies, especially the more reliable prospective ones, dealing with alcohol consumption and colorectal cancer risk indicate an approximately twofold relative rectal and left-sided colon cancer risk for individuals consuming about 40 g of ethanol per day (32–34). In many studies beer was the incriminated beverage. Furthermore, it has been shown that chronic alcohol consumption increases the risks for the development of adenomatous (35) as well as hyperplastic colorectal polyps (36), both of which indicate an increased cancer risk (37–39), since hyperplastic polyps are probably also of monoclonal origin (40).

There seems to be biological link between the proliferative activity of epithelia and their susceptibility to carcinogens and carcinogenesis. It could be shown that this susceptibility was predictable from the baseline intestinal cell

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proliferation of different strains of experimental animals (41,42). This link holds as well for human conditions like ulcerative colitis (43,44). It is not only the overall rate of cell proliferation that is important. The distribution of proliferative activity inside colorectal crypts, especially the size of the proliferative compartment (PC), seems to be important as well. The PC comprises the lower part of intestinal crypts and hosts most of the proliferating cells. Lipkin (45,46) used the PC to predict individual colorectal cancer risks.

We therefore investigated colorectal cell proliferation under various conditions in chronically ethanol-fed experimental animals (47–49). These experiments yielded the unanimous results that chronic ethanol feeding stimulates epithelial cell proliferation in the left-sided colon and rectum, but not in the right-sided colon. This induced hyperproliferation was often associated with an enlargement of the PC. Ornithine decarboxylase activity (ODC), another marker for proliferative activity, was also increased in the left-sided colon only. This ODC induction after ethanol had been reported earlier (50).

What underlying mechanisms may be responsible for this stimulatory effect of chronic alcohol ingestion? Since the entire colon is exposed to alcohol levels identical to blood levels, but the stimulation of cell proliferation is confined to the left-sided colon and rectum, we concluded that the underlying mechanism must be a local one. When we measured AA levels of the mucosa, we saw surprisingly high levels in the rectal mucosa (47), which were twice as high as in liver tissue. This significant alcohol metabolism in the rectum cannot be explained by mucosal ADH activity, since it is much too low. Microbial metabolism by colonic bacteria is an obvious explanation, since their concentration is much higher in the rectum compared to the right-sided colon, and we could show that AA levels depend on the number of fecal bacteria and that in germfree animals AA production is almost abolished (47). This association of bacterial AA generation and stimulation of cell proliferation by AA was confirmed in vitro as well as in vivo by the work of the group of Salaspuro (29–31,51–53). Some time ago it had been speculated that this pathway via the portal circulation may also be responsible for some of the alcohol-related liver injury (54). Another factor influencing colonic cell proliferation and concomitant cancer risk is propably age. Advanced age is a risk factor for colorectal cancer, as well as adenomatous polyps (35). The aging colorectal mucosa is propably more susceptible to induction of cell proliferation (55). Accordingly, we demonstrated that in aging animals the stimulatory effects of ethanol on cell proliferation in the rectum are more pronounced compared to younger animals (49). Since this chapter deals with ethanol and GI cell proliferation, we will not discuss further the possible alcohol-related effects on the chain of cancer induction and development, which will be dealt with elsewhere.

Recently we investigated rectal cell proliferation and differentiation in human alcoholics and controls (56). In this respect, humans behaved like experimental animals. They exhibited an increased rectal cell proliferation with en-

largenment of the PC. This was demonstrated with staining of rectal biopsies for PCNA, Ki-67, and by in situ hybridization for histone H3. As markers for cell differentiation we performed immunohistological staining for different cytokeratins, which were unchanged compared to controls. On the background of observations of mutagenic potential of AA (57), we also performed immunohistological staining for the gene products of the protooncogenes p53, bcl-2, and Rb1, of which p53 may be especially important for colorectal carcinogenesis (58). We could not detect any differences in bcl-2 and Rb1 expression, as well as no overexpression of p53. The latter finding is not surprising, since p53 mutation and related overexpression is a late event in colorectal tumorigenesis, wheras mucosal hyperregeneration is an early one.

V. CONCLUSION

Chronic ethanol ingestion leads to epithelial hyperregeneration in regions of the GI tract susceptible to alcohol-related carcinogenesis. This increased cellular regeneration is probably caused, at least in part, by the toxic effects of AA, the first metabolite of ethanol, mainly generated by GI bacteria.

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Alcohol and Cancer of the Aerodigestive Tract

Hiromasa Ishii and Shinzo Kato
Keio University, Tokyo, Japan

Akira Yokoyama and Katsuya Maruyama
National Institute on Alcoholism, Kurihama National Hospital, Kanagawa, Japan

I. INTRODUCTION

Epidemiology has provided ample evidence that alcohol intake is causally related to oropharyngolaryngeal, esophageal, and liver cancer. Among 2000 Japanese male alcoholics screened by endoscopy combined with esophageal iodine staining, 5.4% had cancer (1.0%, oropharyngolaryngeal; 3.6%, esophageal; 1.4%, stomach; 0.5%, multiple cancers). Consumption of strong alcoholic beverages and heavy smoking were risk factors for the oropharyngolaryngeal, esophageal, and multiple cancers in this population. The inactive form of aldehyde dehydrogenase-2 (ALDH2), encoded by the gene *ALDH2∗1/2∗2*, which is prevalent in Orientals, exposes them to higher levels of acetaldehyde after drinking and is another strong risk factor for Japanese drinkers. Inactive ALDH2 was associated with oropharyngolaryngeal, esophageal, stomach, colon, and synchronous and metachronous multiple esophageal cancers in Japanese alcoholics, suggesting a general role of acetaldehyde, a recognized animal carcinogen, in carcinogenesis of the human alimentary tract. The responses to a simple questionnaire about facial flushing after drinking can indicate an individual's ALDH2 phenotype fairly well. Use of this questionnaire to obtain information on ALDH2-associated cancer susceptibility could contribute to the prevention of alcohol-related cancer in Orientals.

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II. EPIDEMIOLOGY

Excessive alcohol consumption is associated with a number of health problems, ranging from liver disease, pancreatitis, and neurological disorders to psychosocial problems. An association between drinking alcohol and cancer has long been observed. In 1910, Lamy (1) reported that approximately 80% of patients with cancers of the esophagus and gastric cardia were alcoholics who drank mainly absinthe. Since then, evidence that alcohol is causally related to certain kinds of cancers has come from many epidemiological studies. Geographic (2) and temporal (3) correlations have consistently been reported between per capita alcohol consumption and cancer mortality. Studies of traditional abstainers, such as Seventh Day Adventists (4) and Mormons (5), have shown a low incidence of cancer. Studies of groups of people who consume great quantities of alcoholic beverages (for example, brewery workers who get drinks free of charge) have shown increased risk for cancer (6). Studies of patients with cancer have shown an associated history of drinking. In 1988, the International Agency for Research on Cancer (IARC) of the World Health Organization concluded that there is sufficient evidence that alcoholic beverages are carcinogenic to humans (7), and judged to be causally related to cancer of the oral cavity, pharynx, larynx, esophagus, and liver. Epidemiological studies have also provided data suggesting a positive association between drinking alcohol and both colon cancer and female breast cancer (8). There is little evidence, however, that alcohol consumption plays a causal role in stomach cancer.

Epidemiological studies have provided sufficient evidence that heavy alcohol consumption and cigarette smoking, which often coexist, are important risk factors for cancer in the oral cavity, pharynx, larynx, and esophagus (7). The endolarynx is exposed to tobacco, while the hypopharynx and epilarynx are junctional areas that are exposed to both alcohol and tobacco. An IARC case-control study of more than 1000 cases in Italy, Spain, Switzerland, and France showed that endolaryngeal cancer was mainly induced by tobacco in the presence of alcohol, whereas the development of cancer of the hypopharynx/epilarynx was more influenced by alcohol than by tobacco (9). The IARC study clearly demonstrated the synergistic effects between alcohol and tobacco in both the endolarynx and the hypopharynx/epilarynx. The combination of alcohol and tobacco has also been demonstrated as having a striking effect that is intermediate between additive and multiplicative in the development of esophageal cancer (10). Although alcohol has often been considered to be a solvent or cofactor for tobacco rather than a carcinogen per se, alcohol posed a strong risk for esophageal cancer even among nonsmoking drinkers (11).

Epidemiological evidence of a causal role for alcoholic beverages in the development of rectal cancer is suggestive but inconsistent. A recent review (8) showed that seven of 14 cohort studies reported that the risk of rectal cancer is

positively associated with alcohol consumption, with six of these studies showing a statistically significant increase in risk. The increased risk was associated with beer consumption in four of these studies. Drinking was positively associated with rectal cancer in 19 of 24 case-control studies; the associations were significant in 13 of the 19 studies; and beer consumption was significantly associated with rectal cancer in eight of these 13 studies.

Although little is known about the effect of drinking cessation on the risk for alcohol-related cancer, a large case-control study on esophageal cancer in Hong Kong showed that the risk for esophageal cancer falls fairly rapidly for persons who have quit drinking (12).

III. ETIOLOGICAL MECHANISMS

Several possible mechanistic pathways through which alcohol drinking may cause cancer have been discussed (13): (a) alcohol's contact-related local effects on the upper gastrointestinal tract; (b) alcohol's solvent effects on tobacco and other carcinogens; (c) the induction of microsomal enzymes involved in carcinogen metabolism; (d) the generation of oxygen radicals and lipid peroxidation products; (e) nutritional deficiency, especially vitamin and mineral deficiencies; and (f) suppressed immune function. Nevertheless, understanding of the carcinogenic action of ethanol is poor.

Acetaldehyde has been suspected to be a key substance in alcohol-related cancer, although until recently evidence for its carcinogenicity in humans has been inadequate (14). There is sufficient evidence of the carcinogenicity of this first metabolites formed during the breakdown of the ethanol in experimental animals (14). Inhalation of acetaldehyde has produced tumors of the respiratory tract, specifically adenocarcinomas and squamous cell carcinomas of the nasal mucosa in rats (15) and laryngeal carcinomas in hamsters (16), in which the metabolite served as a promoter in carcinogenesis attributable to benzo(a)pyrene (16). Acetaldehyde induces chromosomal aberrations, micronuclei, and sister chromatid exchanges in cultured mammalian cells (14,17). It can also interact covalently with DNA to form DNA adducts, which may be a critical initiating event in the multistage process of chemical carcinogenesis (18). The formation of N^2-ethyl-2′-deoxyguanosine, one major stable acetaldehyde-DNA adduct (18), was detected in liver DNA from ethanol-treated mice (19). In humans, the levels of acetaldehyde adduct in lymphocyte and granulocyte DNA from alcoholic patients were sevenfold and 13-fold higher, respectively, than the corresponding levels in healthy control individuals (20). Lymphocytes from habitual drinkers with the inactive aldehyde dehydrogenase-2, which cannot efficiently detoxify acetaldehyde, had higher frequencies of sister chromotid exchanges than those from individuals with the normal enzyme (21).

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IV. CANCER SCREENING IN JAPANESE ALCOHOLICS

The main purpose of epidemiology in the field of cancer should be primary prevention, early detection, and noninvasive treatment of substance-induced cancers. The use of diagnostic and therapeutic technologies, such as endoscopy, vital staining, and endoscopic mucosal resection for early digestive tract cancer, has become widespread in Japan. Therefore, the development of prevention efforts and screening programs based on these technologies is appropriate for use with high-risk populations such as heavy drinkers.

At the National Institute on Alcoholism, Kurihama National Hospital, Japan, we routinely apply a regimented cancer screening program, using endoscopy combined with oropharyngolaryngeal inspection and esophageal iodine staining in male alcoholic patients over 40 years old (22,23). The procedure includes final rinsing of the esophageal mucosa with thiosulfate solution to lessen the irritation caused by iodine. From 1993 to 1998, systematic screening of 2000 consecutive male alcoholic patients who had any subjective symptoms referrable to cancer showed cancer in 108 patients, an extremely high rate of 5.4%. Oropharyngolaryngeal cancer was diagnosed in 19 of these patients (1.0%), esophageal cancer in 72 (3.6%), and stomach cancer in 28 (1.4%). Ten of the 72 with esophageal cancer were multiple-cancer patients who also had either oropharyngolaryngeal or stomach cancer or both (Table 1). Prior to our findings, the rates of cancer detection via mass screening by endoscopy in Japanese general populations had been reported as only 0.04% for esophageal cancer (24) and 0.15% for gastric cancer (25).

The high rate of esophageal cancer found in our study was influenced by the improved method of screening. A large majority of the detected esophageal cancers were superficial, and two-thirds of the detected lesions would have been

Table 1 Cancer Screening in Japanese Male Alcoholics Using Endoscopy Combined with Esophageal Iodine Staining

Alcoholic males (≥40 years old)	$n = 2000$
(1) Oropharyngolaryngeal cancer	19 (1.0%)
(2) Esophageal cancer	72 (3.6%)
(3) Stomach cancer	28 (1.4%)
(4) Multiple cancer	10 (0.5%)
(1) + (2)	5
(2) + (3)	3
(1) + (2) + (3)	2
All cancers combined	108 (5.4%)

Source: National Institute on Alcoholism, Japan, 1993–1998.

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missed by conventional endoscopic inspection (22). The detection rate and invasion depth of the esophageal cancers revealed by iodine staining are in sharp contrast to what has been shown by endoscopy only. In fact, when other investigators screened 1513 male Japanese alcoholics over 40 years old by conventional endoscopy only, they detected only 10 patients with esophageal cancer (0.7%) (26), and most of these cancers were at more advanced stages.

Because of the treatment technology of endoscopic mucosal resection in Japan, we no longer perform extensive removal of the esophagus for early cancerous lesions (22,27). Endoscopic mucosal resection achieves a therapeutic goal because cancerous involvement of lymph nodes, lymphatics, and vessels is extremely rare in such mucosal carcinomas (28). Patients receiving prophylactic antibiotic treatment recovered quickly within a few days and enjoyed the same quality of life as before the cancer treatment. On the other hand, when the cancerous growth had invaded the submucosa, metastasis to vessels or lymph nodes was frequently encountered, and the result was a poor 5-year survival rate (50%) (28). Most patients with symptomatic cancer have a much poorer prognosis when a cancer is invasive. Thus, early detection is extremely important in these high-mortality cancers.

Although overall trends suggest that all types of alcoholic beverages increase the risk for cancers of the oral cavity, pharynx, larynx, and esophagus, some epidemiological studies have shown stronger associations between those cancers and alcoholic beverages high in ethanol (7). Until recently, it has not been possible to distinguish the effects of different type of beverages in studies of alcoholics. However, a study in which alcoholic cancer patients identified their alcoholic beverage preferences has shown that the higher the ethanol concentration consumed, the higher the rates of oropharyngolaryngeal and esophageal and multiple cancer, but not stomach cancer (23). After adjusting for patients' age and daily alcohol consumption, the investigators found that the stronger beverages (whiskey or shochu), in contrast with lighter beverage choices (sake or beer), increased the risks for oropharyngolaryngeal, esophageal, and multiple cancers (odds ratios 4.8, 3.2, and 10.5, respectively). Of the alcoholic men screened, 85% were current cigarette smokers and 20% had smoked 50 pack-years of cigarettes (23). Heavy smoking of 50 pack-years or more also increased the risk for those cancers (odds ratios 5.1, 2.8, and 11.8, respectively). These results emphasize the importance of direct exposure of the mucosa to strong alcohol with its solvent effect on tobacco.

V. ETHANOL METABOLISM AND ALDH2 GENOTYPE

Ethanol is eliminated from the body by its oxidation to acetaldehyde and then to acetate, reactions that are mainly catalyzed by alcohol dehydrogenase and aldehyde dehydrogenase, respectively. Most of the acetaldehyde generated during

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alcohol metabolism is promptly eliminated by aldehyde dehydrogenase-2 (ALDH2) and aldehyde dehydrogenase-1 in the liver (29).

Approximately half of all Japanese and Chinese have inactive forms of ALDH2 (30,31), in which the mutant allele ALDH2∗2 encodes an inactive subunit. The distribution of the mutant ALDH2∗2 allele varies by race (32): it is prevalent in Asians, but has not been found in Caucasians or Africans. When this enzyme is inactive, the body fails to metabolize acetaldehyde rapidly, leading to excessive accumulation of acetaldehyde. Blood acetaldehyde concentrations are approximately 19 and 6 times higher in ALDH2∗2/2∗2 homozygous and ALDH2∗1/2∗2 heterozygous individuals, respectively, than in normal ALDH2∗1/2∗1 homozygotes (33).

After drinking alcohol, persons with inactive ALDH2 exhibit the so-called flushing response, which includes facial flushing, palpitations, drowsiness, and other unpleasant symptoms (34). By causing acetaldehydemia with the flushing response, the inactive forms of ALDH2 act to prevent many Japanese from drinking heavily and developing alcoholism (35). The preventive effect of heterozygous inactive ALDH2 is incomplete, however; individuals with the inactive enzyme often become alcoholics. In fact, as many as 10% of Japanese and Chinese alcoholics have the heterozygous form of inactive ALDH2 (30,31).

VI. ALCOHOL-RELATED CANCER AND ALDH2 GENOTYPE

Recently, researchers at the National Institute on Alcoholism, Japan, discovered that, among Japanese, the inactive form of ALDH2 encoded by the ALDH2∗1/2∗2 gene is a strong risk factor for esophageal cancer in both alcoholics and nonalcoholic everyday drinkers (36). Comparison of the ALDH2 genotype frequencies among alcoholics and nonalcoholic everyday drinkers with and without esophageal cancer showed a much higher frequency of heterozygous ALDH2∗1/2∗2 among the esophageal cancer patients, both alcoholics (52.5%) and everyday drinkers (72.4%), than among cancer-free controls (alcoholics, 12.7%; nonalcoholic, 17.9%). Whereas acetaldehyde has been established as a carcinogen to experimental animals (14), this link between the results of epidemiological and animal studies suggests the role of acetaldehyde in esophageal cancer. Moreover, in confirmation of our findings, other investigators have shown that 75.3% of Japanese patients with esophageal cancer treated in a general hospital were ALDH2∗1/2∗2 heterozygotes (37).

Multiple intraesophageal cancers and upper aerodigestive tract cancers associated with esophageal cancer are common, especially in heavy drinkers (38,39). This multiplicity of cancer is often explained by the concept of field cancerization, which suggests a common etiology (40). Inactive ALDH2 is a risk

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factor for synchronous multiple cancers of the esophagus in alcoholics. Of 17 patients with inactive ALDH2 in one study, 13 (76.5%) had multiple intraesophageal cancers, compared with only five of 16 (31.3%) patients with active ALDH2 who had multiple cancers. The incidence of concurrent upper aerodigestive tract cancer was 29.4% among those with inactive ALDH2, compared with 6.3% among those patients with active ALDH2 (38).

The frequent occurrence of a second primary cancer after the first treatment of oropharyngolaryngeal cancer has long been recognized (41). Both alcohol drinking and tobacco smoking have been reported to be significant predictors of the likelihood of developing second cancers (39,41). Follow-up of 126 cases of superficial esophageal cancer treated by endoscopic resection in a Japanese general hospital showed that the second esophageal cancers developed after a median of 26 months in only six patients (4.8%) (42). However, short-term follow-up of Japanese alcoholics showed that additional primary esophageal cancers developed much more frequently (42.1%) among those with heterozygous inactive ALDH2 (43). This high incidence of simultaneous and metachronous development of multiple esophageal cancers among alcoholics with inactive ALDH2 suggests that systemic acetaldehydemia plays a critical role in multicentric or field cancerization throughout the entire mucosal surface of the esophageal and oropharyngolaryngeal areas.

A comprehensive study of the ALDH2 genotype and cancers prevalent in Japanese alcoholics showed that the frequency of inactive ALDH2 increased

Table 2 ALDH2 Genotype and Risk for Cancer in Japanese Male Alcoholics

Subjects, by cancer status	*n*	ALDH2 genotype		OR for inactive ALDH2 (95%CI)
		2∗1/2∗1	2∗1/2∗2	
Cancer-free	487	91.0%	9.0%	—
Cancer				
Oropharyngolaryngeal	34	47.1%	52.9%‡	11.1 (5.1–24.4)
Oral/Oropharyngeal	16	50.0%	50.0%‡	11.1 (3.8–32.7)
Hypopharyngeal	10	30.0%	70.0%‡	23.7 (5.2–107.6)
Laryngeal	10	50.0%	50.0%†	13.0 (3.3–51.3)
Esophageal	87	47.1%	52.9%‡	12.5 (7.2–21.6)
Stomach	58	77.6%	22.4%†	3.5 (1.6–7.4)
Colon	46	78.3%	21.7%*	3.4 (1.5–7.5)
Liver	18	94.4%	5.6%	0.7 (0.1–5.6)
Lung	7	71.4%	28.6%	8.2 (1.3–53.2)
Multiple esophageal	14	21.3%	78.6%‡	54.2 (11.5–255.2)

ALDH2, aldehyde dehydrogenase-2; ALDH2∗1, normal allele; ALDH2∗2, mutant allele. *$p < 0.05$; †$p < 0.01$; ‡$p < 0.001$ vs. the cancer-free alcoholics
Source: Ref. 44.

markedly among alcoholics with cancers of the oral cavity/oropharynx, hypopharynx, larynx, and esophagus. Adjustment for age and for alcohol and cigarette consumption showed the odds of those cancers occurring in patients with inactive ALDH2 were more than 10-fold the odds for patients with active ALDH2 (44) (Table 2). For colon and stomach cancers, epidemiological evidence of the association with alcohol drinking has been controversial, but significantly (threefold) higher odds ratios for these cancers in alcoholics with inactive ALDH2 suggests that some of these cancers are also associated with severe acetaldehydemia (44). Screening Japanese alcoholics for colon cancer using immunofecal occult blood tests yielded a detection rate of 1.5% (45), far exceeding the 0.15% rate obtained in a nationwide mass-screening survey in Japan (25).

The ALDH2 effect was not observed with liver cancer (44,46). The confounding effects of other risk factors, including the development of liver cirrhosis and concomitant hepatitis B or C virus infection, may more strongly predict carcinogenicity in the liver.

VII. TOPICAL ETHANOL OXIDATION IN THE AERODIGESTIVE TRACT

Poor dentition, inadequate oral hygiene, and the use of mouthwash are other suspected risk factors for oral cancer. The largest investigation into the role of mouthwash was carried out in the United States, where mouthwash has been used regularly by over 40% of adults. The study revealed that the risk for oral or pharyngeal cancer was elevated with the duration and frequency of mouthwash use, but that the significant association was limited to mouthwashes having an alcohol content of 25% or higher (47). Because few subjects reported swallowing mouthwash, the results indicate that the exposure of topical mucosal tissue to alcohol may be involved in oral carcinogenesis.

New evidence of a general role for acetaldehyde in the development of alcohol-related cancer (44) highlights the possibility of topical ethanol oxidation by mucosal enzymes or bacteria in the lumen of the alimentary tract. The mucosal expression patterns of alcohol dehydrogenase (ADH) and ALDH and the ethanol- and acetaldehyde-oxidizing abilities differ considerably along the alimentary tract. The esophagus and oral cavity are high in ADH activity and have strong expression of ADH7 (48,49), which has high K_m and is very active at high ethanol concentrations. In contrast, the esophagus, tongue, and gingiva are low in ALDH activity and completely lack ALDH2 activity (49). Thus, in the esophagus and oral cavity, inefficient degradation of the acetaldehyde derived from the oxidation of systemic and mucosal ethanol may enhance the chances for acetaldehyde-associated carcinogenesis.

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In human stomach the documented mucosal metabolism of first-pass alcohol (50,51), ADH3, ADH7, ALDH1, and ALDH2 have been shown to be the main enzymes (49). The expression of ADH7 in the stomach displays ethnic variability, being absent or markedly decreased in 70–80% of Japanese (52) and Chinese (49). ADH3 and ALDH2 have genetic polymorphisms, and their ethanol- and acetaldehyde-oxidizing abilities in the stomach correlate with their different expression patterns (49). In the stomach the ALDH activity is three and four times the level of this activity in the colon and esophagus, respectively, suggesting the stomach's greater acetaldehyde-degrading ability (49).

Ingested alcohol is distributed to water in the colon via blood circulation and diffusion to achieve an ethanol concentration equal to that in the blood (53). A significant amount of ADH3 activity occurs in the mucosa of the colon (54). In addition to mucosal enzyme, the existence of metabolism of alcohol, a bacteriocolonic pathway of ethanol oxidation was recently proposed after concomitant administration of ethanol and a potent aldehyde dehydrogenase inhibitor accelerated chemically induced carcinogenesis in the rat rectum (55). The study showed a high mucosal concentration of acetaldehyde in the distal colon and its association with a number of rectal bacteria. A positive correlation between bacterial alcohol dehydrogenase activity in the human colonic flora and the capacity of these bacteria to produce acetaldehyde from ethanol also has been reported (56). However, given the colon's relatively low level of ALDH activity and its weak expression of ALDH2 (54), the colon may not be efficient in metabolizing acetaldehyde derived from bacterial fermentation as well as systemic and mucosal ethanol oxidation.

VIII. ESOPHAGEAL CANCER AND GENETIC POLYMORPHISMS OF ADH2 AND P4502E1

Like ALDH2, alcohol dehydrogenase-2 (ADH2) is polymorphic. More than 90% of Japanese and Chinese have atypical ADH2 encoded by homozygous ADH2*2/2*2 or heterozygous ADH2*1/2*2 (57). In vitro studies showed that these mutant isozymes have higher catalytic activity than the typical ADH2*1/2*1 form of ADH2 found in most Caucasians and Africans (58,59).

Striking differences have been reported between the frequencies of ADH2 genotypes in alcoholics and normal controls in both Japanese and Chinese populations (30,31). Increased frequency of the typical ADH2 and decreased frequency of the atypical ADH2 were found in alcoholics, compared with normal controls. A similar tendency was reported in Japanese and Chinese patients with alcoholic liver disease, compared with normal controls (60,61). In a study of Japanese patients with esophageal cancer treated in a general hospital, the fre-

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quency of typical ADH2 was increased and that of atypical ADH2 homozygote was decreased (37). The majority of those patients were drinkers. The ADH2 effects on alcohol-related diseases (alcoholism, alcoholic liver disease, and esophageal cancer) suggest that, in contrast to Caucasians and Africans, Asians are genetically protected against alcohol-related harm by the mutant ADH2∗2 allele. In fact, the odds of esophageal cancer decreased to 0.6 for ADH2∗1/2∗2 heterozygote and to 0.2 for ADH2∗2/2∗2 homozygote (37).

The role of ADH2 remains puzzling. It have been demonstrated that the polymorphism of ADH2 has little effect on an individual's blood acetaldehyde concentration and ethanol elimination rate in vivo (33), and the mechanisms of ADH2 action are not clear.

Recently, many investigators have reported associations between cancer susceptibility and the genetic polymorphism of drug-metabolizing enzymes such as cytochromes P-4501A1, P-4502E1, and P-4502D6 (62). The cytochrome P-450s are involved in the metabolic activation of procarcinogens. Although cytochrome P-4502E1 is an ethanol-inducible form of cytochrome P-450 (63) and presents in the esophageal mucosa (64), several studies have reported that there is no association between esophageal cancer and the genetic polymorphism of P-4502E1 (37,65).

IX. SCREENING FOR INACTIVE ALDH2

New evidence concerning the pathogenesis of alcohol-related cancers could renew interest in acetaldehyde as an important subject for future cancer research and it has special public health implications for Asian populations. Because almost half of Japanese and Chinese people have inactive ALDH2, screening for this enzyme could serve as a powerful tool in cancer prevention.

A. Flushing Questionnaire

The flushing questionnaire we have developed to identify the presence of inactive ALDH2 ask two simple questions: (a) Do you flush in the face immediately after drinking a glass of beer: always, sometimes, or never? (b) Did you flush in the face immediately after drinking a glass of beer during the first to second year after you started drinking: always, sometimes, or never? (66) In tests of the reliability of this questionnaire in detecting inactive ALDH2, blinded genotyping of 266 elderly Japanese male respondents over age 50 showed inactive ALDH2 for 94% of those who reported currently or formerly always flushing and for 48% of those who reported sometimes flushing, whereas 96% of the subjects who reported never flushing had active ALDH2. When all three categories of flushing (current always, formerly always, and sometimes) were considered to have inac-

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tive ALDH2, 96% of those with inactive ALDH2 and 87% of the total subjects were discriminated correctly. These results suggest the utility of this simple flushing questionnaire in daily practice as well as in large-scale cohort studies and in activities aimed at preventing alcohol-related cancer in Asians.

B. Ethanol Patch Test

Another reliable indicator of inactive ALDH2 is the ethanol patch test. This cutaneous model for the flushing response is especially useful in health education for youth because it can reveal the phenotype in persons who have never consumed alcohol (34). We have found the reliability of the ethanol patch test to be unsatisfactory for use in men over age 50 because many of them have a long history of drinking (66). This test is less reliable than the flushing questionnaire in alcoholics, probably because of acquired tolerance to acetaldehyde after long-term exposure to alcohol. However, the majority of ALDH2∗1/2∗2 heterozygous alcoholics are able to recall their former always-flushing status suggesting the merit of using the flushing questionnaire in the identification of high-risk drinkers.

X. CONCLUSION

In addition to alcohol consumption, choosing stronger alcoholic beverages, heavy smoking, and having the heterozygous ALDH2∗1/2∗2 genotype are important risk factors for alcohol-related aerodigestive tract cancer. Atypical ADH2 protects against esophageal cancer probably by preventing heavy drinking. Public dissemination of information on ALDH2-associated cancer susceptibility and the use of a simple questionnaire about alcohol flushing could contribute to the prevention of alcohol-related cancer in Asians. High-risk drinkers should be advised to undergo cancer screening as a means of immediately reducing their risk for cancer and to reduce their alcohol intake or quit drinking.

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Alcohol and Cardiovascular Diseases

Arthur L. Klatsky
Kaiser Permanente Medical Center, Oakland, California

I. INTRODUCTION

Disparities in the relationships between alcohol consumption and various cardiovascular conditions are now evident, with complex interrelationships between conditions. Thus, it is best to consider separately the relationships of alcohol to several disorders, as follows: (1) The evidence continues to mount that susceptible persons may suffer heart muscle damage from chronic use of large amounts of alcohol, leading to *alcoholic cardiomyopathy*. (2) Strong, consistent epidemiological data support a relationship of heavier drinking to higher blood pressure (*hypertension*). Clinical experiments confirm a hypertensive effect of alcohol, which appears and regresses within several days, but a mechanism has not yet been established. (3) Heavier, and possibly lighter, drinking is related to higher risk of *hemorrhagic stroke* (due to ruptured blood vessels), but lighter drinking is associated with lower risk of *ischemic stroke* (due to blocked blood vessels). (4) Heavier drinking, especially binge drinking, is associated with certain *heart rhythm disturbances*. (5) An inverse relationship of alcohol use to *coronary heart disease* is consistently supported by many population studies. Interpretation of these data as a protective effect of alcohol against coronary disease is strengthened by plausible mechanisms, including increased high-density-lipoprotein (HDL) cholesterol in alcohol drinkers, and anticlotting actions of alcohol. The high prevalence of coronary disease (about 60% of all cardiovascular deaths and about 25% of all deaths) results in an impact on total mortality statistics, such that lighter drinkers are at slightly lower risk than abstainers of death within a given time period.

International comparisons suggest that wine may be more protective against

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coronary disease than liquor or beer. Reports of antioxidants, endothelial relaxant activity, and antithrombotic activity in wine (especially red) support the hypothesis of possible nonalcohol beneficial components in wine. However, prospective population studies show no consensus; apparent protection has been found for beer, wine, or liquor, and it has been suggested that favorable traits or drinking patterns of wine drinkers might explain the international study findings. Furthermore, observational data and limited data from randomized clinical trials are inconclusive, at best, with respect to benefits from antioxidant supplements. Whatever the facts, it is worth noting that prominent lay media dissemination has probably led to widespread public acceptance of specific benefits of red wine.

As with most aspects of alcohol and health effects, the body of evidence does not suggest monotonic relationships of alcohol with any cardiovascular condition. Thus, amount of alcohol taken is a crucial consideration. Advice to concerned persons needs to take into account individual risk/benefit factors in drinkers or potential drinkers. For drinkers, there are no compelling health-related data that preclude personal preference as the best guide to choice of beverage.

Disparities in relationships between drinking alcoholic beverages and various cardiovascular (CV) conditions (1–4) make it desirable to consider several disorders separately. Because of past diversions in understanding alcohol-CV relationships, it is relevant to include with some emphasis historical review in this presentation. (1) Although perceived 150 years ago, understanding of alcoholic cardiomyopathy was clouded by recognition of beriberi and of synergistic toxicity from alcohol with arsenic or cobalt. (2) A report of a link between heavy drinking and hypertension (HTN) in World War I French soldiers was apparently ignored for $>$50 years. Epidemiological and intervention studies have now firmly established this association, but a mechanism remains elusive. (3) The ''holiday heart syndrome,'' an increased risk of supraventricular rhythm disturbances in binge drinkers, has been widely known to clinicians for 25 years. Data remain sparse about the total role of heavier drinking in cardiac rhythm disturbances. (4) Failure of earlier studies to distinguish types of stroke impeded understanding; it now seems probable that alcohol drinking increases risk of hemorrhagic stroke but lowers risk of ischemic stroke. (5) In 1786 William Heberden reported angina pectoris relief by alcohol, and pathologists observed an inverse alcohol-atherosclerosis association in the early 1900s. Recent population studies and plausible mechanisms support a protective effect of alcohol drinking against coronary heart disease (CHD). International comparisons dating back to 1819 suggest beverage choice as a factor, but this issue remains unresolved.

II. DEFINITIONS OF MODERATE AND HEAVY DRINKING

Any definition of moderate drinking is arbitrary. The operational definition used here is based upon the level of drinking in epidemiological studies above which

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net harm is usually seen. Thus, less than three drinks per day is called ''lighter'' or ''moderate'' drinking, and three or more drinks per day ''heavy'' drinking. Sex, age, and individual factors lower the upper limit for some persons and raise it for others. In data based upon surveys, systematic ''underestimation'' (lying) probably tends to lower the *apparent* threshold for harmful alcohol effects.

Fortunately, the amount of alcohol in a standard-sized drink of wine, liquor, or beer is approximately the same. Since people think in terms of ''drinks,'' not milliliters or grams of alcohol, it seems best to describe alcohol relations in terms of drinks per day or week. When talking with patients, health professionals should always remember the importance of defining the size of drinks.

III. ALCOHOLIC CARDIOMYOPATHY

A. Definition of Cardiomyopathy

The word ''cardiomyopathy'' (CM) is used by some to mean heart muscle disease, regardless of the cause. Others use it to refer to heart muscle disease only of unknown cause (''idiopathic''). Many, including this author, use the term to mean heart muscle disease independent of the valves, coronary arteries, pericardium, and congenital malformations. Some cases have known causes; many do not. The cardiomyopathies include anatomical subtypes, depending upon whether the heart chambers are thickened but not dilated (''hypertrophic'' CM), infiltrated by abnormal tissue (''restrictive'' CM), or enlarged disproportionately to thickening with weakened contraction (''dilated'' CM). Sustained heavy alcohol drinking is believed to be one of the causes of dilated CM (5). The clinical picture of dilated CM ranges from abnormalities detectable only by testing (''subclinical''), to severe illness with heart failure and high mortality rate.

B. Alcohol's Role in Dilated Cardiomyopathy

A number of famous nineteenth-century physicians commented on an apparent relationship between chronic intake of large amounts of alcohol and heart disease (6–10). A German pathologist (11) described cardiac dilatation and hypertrophy among Bavarian beer drinkers, who averaged 432 L/year; this became known as the ''*Munchener bierherz*.''

In 1900, an epidemic of heart disease due to arsenic-contaminated beer occurred in Manchester, England. Before this event, Graham Steell (9) stated, ''Not only do I recognize alcoholism as one of the causes of muscle failure of the heart but I find it a comparatively common one.'' Following the arsenic-beer episode, Steell (12) wrote, ''In the production of the combined affection of the peripheral nerves and the heart met with in beer drinkers, arsenic has been shown to play a conspicuous part.'' In his textbook, *The Study of the Pulse*, William MacKenzie (13) described cases of heart failure attributed to alcohol and first

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used the term ''alcoholic heart disease.'' Early in the twentieth century, there was general doubt that alcohol had a direct role in producing heart muscle disease, although some (14) took a strong view in favor of such a relationship. After the detailed descriptions of cardiovascular beriberi (15,16), the concept of ''beriberi heart disease'' dominated thinking about the effects of alcohol upon the heart for several decades.

In recent decades increasing interest has been evident in possible direct toxicity of alcohol upon the myocardial cells and the existence of alcoholic CM (ACM) has now become solidly established (17,18). Many series of cases in various types of practice have been reported (19–26). Varying proportions of chronic heavy alcohol users have been reported in these series, probably dependent mostly upon the drinking habits of the study population. The absence of diagnostic tests has been a major impediment to epidemiological study, since the entity has been indistinguishable from other forms of dilated cardiomyopathy. Most cases of dilated cardiomyopathy remain of unknown cause, with a postviral autoimmune process the leading etiological hypothesis. The proportion of heavy drinkers who develop cardiomyopathy is not known, but is smaller than the proportion who develop liver cirrhosis. Also not known is the proportion who demonstrate regression or partial regression with abstinence but data showing that such regression has been present for decades (27). The most convincing evidence that alcohol can cause cardiomyopathy consists of extensive data, in animals and humans, of nonspecific functional and structural abnormalities related to alcohol (21,22,28–38). These data include autopsy studies, cardiac biopsies in some, and noninvasive measures of heart function, such as nucleide and echocardiographic studies, in several. Subclinical abnormalities of function and structure may precede evident illness for years.

A landmark study (25) showed a clear relation in alcoholics of lifetime alcohol consumption to structural and functional myocardial and skeletal muscle abnormalities. The large amounts of alcohol needed—equivalent to 120 g alcohol per day for 20 years—make the term ''cirrhosis of the heart'' (7) appropriate.

Another study from Spain (39) investigated alcohol-related active myocardial damage by use of indium 111–labeled monoclonal antimyosin antibodies. The major group studied was 56 ACM patients referred for heart transplant, all of whom had taken 100+ g of alcohol per day for at least 10 years. If actively drinking, patients had much higher indium 111 uptake. This decreased in most with 3 months of abstinence, associated with increased left ventricular function, as measured by ejection fractions. The indium 111 uptake was stable if the ACM patients had taken no alcohol for 3+ months. In 15 detoxification patients without ACM and six healthy subjects, there was essentially no indium 111 uptake. These data confirm prior important clinical observations that have suggested improvement in myocardial damage with abstinence and variability in myocardial susceptibility to alcohol.

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A possible nonoxidative metabolic pathway for alcohol has been reported (40) in the heart, muscle, pancreas, and brain, related to fatty acid metabolism. Fatty acid ethyl ester accumulation was related to blood alcohol levels and mitochondrial metabolism. Other increased enzymatic activity in myocardial cells has also been reported, including apha-hydroxybutyric dehydrogenase, creatine kinase, lactic dehydrogenase, and malic dehydrogenase (18). It is not clear whether the reported enzymatic activity reflects causative processes or an adaptive reaction. The histological findings include evidence of inflammation, lipid deposits, focal or diffuse fibrosis, and mitochondrial damage (21,25,30,41). Some (18) believe that hypertrophy, fibrosis, and cell nuclear disruption are greater in alcoholic than in other forms of dilated CM, but the histology has not been generally considered sufficiently characteristic for specific diagnosis. Data showing a relationship between alcohol drinking and left ventricular hypertrophy or mass have been reported (42,43) but it is not clear that this is independent of alcohol-related hypertension (see below).

C. Diagnosis and Clinical Picture

The diagnosis depends upon the combination of a compatible alcohol drinking history and the presence of heart muscle disease without other evident cause. When clinical evidence appears, early manifestations are nonspecific electrocardiographic findings and, possibly, rhythm disturbances (44). Evans (45) described electrocardiographic T-wave variations that he considered characteristic, but these have not been widely reported. The late picture includes (congestive) heart failure, chronic rhythm disturbances, conduction abnormalities, systemic emboli, and death (28,36). The onset may be insidious, but sometimes seems subacute.

D. Possible Cofactors with Alcohol in Cardiomyopathy

Because the diagnosis of ACM is based on excluding other causes of CM and other types of heart disease, the role of alcohol as a contributing factor remains unknown. It seems plausible that amounts of drinking substantially less than needed to produce CM might act in concert with other conditions or cofactors to cause heart muscle dysfunction. In this connection, it seems appropriate to consider further the arsenic and cobalt beer drinker episodes and thiamine (cocarboxylase or vitamin B_1) deficiency—or beriberi heart disease. *Arsenic-beer drinkers' disease* refers to a 1900 epidemic (6000+ cases with 70+ deaths) in Manchester, England, which proved to be due to contamination of beer by arsenic with prominent cardiovascular manifestations, especially heart failure (46). It was determined that the affected beer had 2–4 parts per million of arsenic, not—in itself—an amount likely to cause serious toxicity (47), and that some persons seemed to have a ''peculiar idiosyncrasy'' (46). An appointed committee report

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(48) suggested that ''alcohol predisposed people to arsenic poisoning'' but, apparently, no one suggested the converse. *Cobalt-beer drinkers' disease*, recognized 65 years after the arsenic-beer episode, was similar in some respects. In the mid-1960s reports appeared of heart failure epidemics among beer drinkers in Omaha and Minneapolis in the United States, Quebec in Canada, and Leuven, Belgium, with, generally, abrupt onset in chronic heavy beer drinkers. The explanation proved to be the addition of small amounts of cobalt chloride by certain breweries to improve the foaming qualities of beer. This etiology was tracked down largely by Quebec investigators (49), and the condition became justly known as Quebec beer-drinkers' cardiomyopathy. Removal of the cobalt additive ended the epidemic in all locations. Even in Quebec, where cobalt doses were greatest, 12 L of contaminated beer provided only about 8 mg of cobalt, less than 20% of the dose sometimes used as a hematinic and not implicated as a cause of heart disease. Most exposed persons did not develop the condition. Thus, it was established that both cobalt and substantial amounts of alcohol seemed needed to produce this condition. Despite much speculation, biochemical mechanisms were not established. One observer (50) summed up the arsenic and cobalt episodes thus: ''This is the second known metal induced cardiotoxic syndrome produced by contaminated beer.''

The arsenic and cobalt episodes raise the possibility of other cofactors in alcoholic cardiomyopathy, such as cardiotropic viruses, drugs, selenium, copper, and iron. Deficiencies of zinc, magnesium, protein, and various vitamins have also been suggested as cofactors, but deficiency of thiamine is probably the only one with solid proof of cardiac malfunction.

Cardiovascular beriberi dominated thinking about alcohol and cardiovascular disease for many years. The classic description (15) defined high-output heart failure in Javanese polished-rice eaters, with decreased peripheral vascular resistance as the physiological basis. It became assumed that heart failure in heavy alcohol drinkers in the West was due to associated nutritional deficiency states. Although some heart failure cases in North American and European alcoholics fitted this clinical pattern, most did not (51,52). Many had low-output heart failure, were well nourished, and responded poorly to thiamine. Some felt that these facts were due to the chronicity of the condition, which ultimately might become irreversible. However, Blacket and Palmer (53) stated the following: ''It [beriberi] responds completely to thiamine, but merges imperceptibly into another disease, called alcoholic cardiomyopathy, which doesn't respond to thiamine.'' Modern physiological techniques have established that, in beriberi, there is generalized dilatation of peripheral arterioles, not heart muscle disease, and a few cases of complete recovery with thiamine within 1–2 weeks were documented. Thus, it is evident that many cases earlier called ''cardiovascular beriberi'' would now be called ''alcoholic heart disease.'' Does chronic thiamine deficiency play a role

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in some cases of alcoholic cardiomyopathy? This currently unpopular thesis has not been proved or disproved.

In view of the history just cited, it seems noteworthy that there has been little work so far about possible cofactors or predisposing traits for alcoholic cardiomyopathy.

IV. HYPERTENSION

A. Background

Although an association between heavy drinking and HTN was reported in 1915 in middle-aged French servicemen (54), it was more than 50 years before further attention was paid to this subject (4,55,56). Since the mid-1970s, dozens of cross-sectional and prospective epidemiological studies have solidly established an empirical alcohol-HTN link, and clinical experiments have confirmed this (57–59). So far, a mechanism has not been demonstrated. The evidence is sufficient so that clinicians should consider heavy alcohol drinking to be a probable HTN risk factor.

B. Epidemiological Studies

Almost all of approximately 50 cross-sectional studies show higher mean blood pressures and/or higher HTN prevalence with increasing alcohol drinking. Reviews (57–59) detail this observation in North American, European, Australian, and Japanese populations and show its independence from adiposity, salt intake, education, cigarette smoking, and several other potential confounders. Most studies do *not* show any increase in blood pressure at light-moderate alcohol drinking, and several studies show an unexplained J-shaped curve in women (59–62), with lowest pressures in lighter drinkers. Data from the first Kaiser Permanente study (Fig. 1) show these relationships in the two sexes in each of three racial groups. A later Kaiser Permanente study (63) again showed a J curve in women, but a continuous relationship in men starting at 1–2 drinks per day. The data from this later study suggested that ex-drinkers had similar blood pressures to those of nondrinkers and that elevated blood pressures regressed within a week upon abstinence from alcohol. In both studies, HTN prevalence was approximately doubled among the heaviest ($\geq$6 drinks daily) drinkers, compared to abstainers or light drinkers.

Data from prospective studies (56,62,64–66) show higher risk of HTN development among heavier alcohol drinkers. At least two studies (62,66) were well controlled for multiple nutritional factors.

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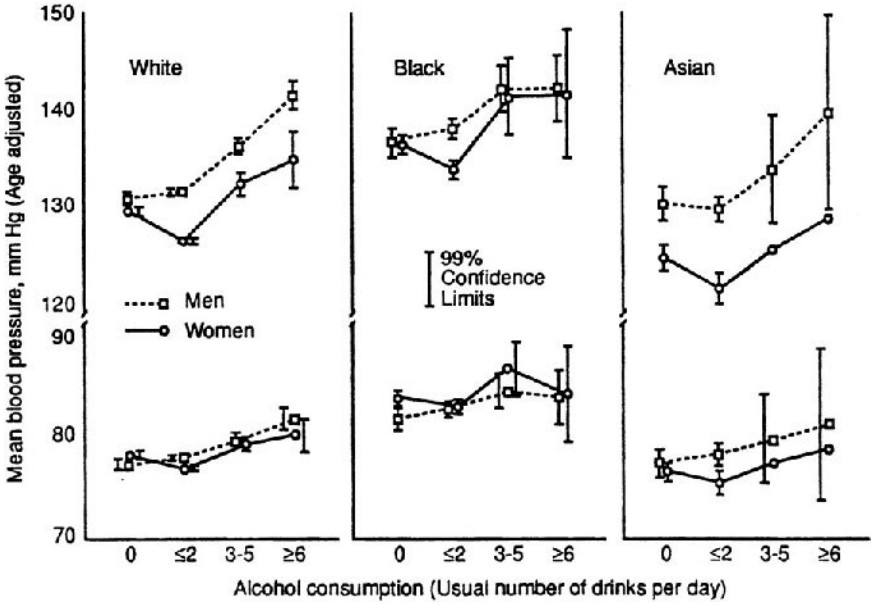

Figure 1 Mean systolic blood pressures (upper half) and mean diastolic blood pressures (lower half) for white, black, or Asian men and women with known drinking habits. Small circles represent data based on fewer than 30 persons. (From ref. 60. Used by permission.)

C. Intervention Studies

A landmark study (67) showed in hospitalized hypertensive men that 3–4 days of intake of 4 pints of beer raised blood pressure and that 3–4 days of abstinence resulted in lower pressures. A 12-week crossover design trial (68) showed similar results in ambulatory normotensives and the observation was later confirmed in hypertensives (69). Other studies show that heavier alcohol intake interferes with drug treatment of HTN (70) and that moderation or avoidance of alcohol supplements or betters other nonpharmacological interventions for blood pressure lowering, such as weight reduction (71), exercise (72), or sodium restriction (73).

D. Possible Mechanisms

The alcohol-HTN relation is a subacute one, developing in days to weeks (67,69–73). Acute human and animal experiments show no consistent increase in blood pressure after alcohol administration (57–59,74). Ambulatory monitoring has shown a depressor effect of a substantial dinnertime alcohol dose, lasting up to 8 hr, with a pressor effect the next morning (75).

Much work has failed to uncover a biological mechanism, including no consistent relationships to plasma renin, aldosterone, cortisol, catecholamines, or insulin (57,58,76–78). Experiments suggest independence from acetaldehyde-induced flushing (75) and raise the possibility of changes in intracellular sodium metabolism (78,79). Other speculations include a direct smooth muscle effect via calcium transport mechanisms, impaired insulin sensitivity, impaired baroreflex activity, magnesium depletion, and a heightened responsiveness of the sympathetic nervous system (79,80). An overactive sympathetic nervous system exists during the alcohol withdrawal state, but this is not the likely explanation for the alcohol-HTN relation.

E. Sequelae of Alcohol-Associated HTN

Complex interactions of alcohol, various cardiovascular conditions, and risk factors make the study of this important subject difficult. Since coronary heart disease (CHD) and strokes of all types are major cardiovascular sequelae of HTN, the lower risk of CHD and occlusive stroke among drinkers confounds study of this aspect. A counterbalancing role of HTN has been observed in two alcohol-CHD studies (81,82). An attempt to study whether alcohol-associated HTN had the same prognosis as HTN not so associated led to the conclusion that alcohol's harmful and beneficial effects so dominated the outcome that the basic question could not be answered (59).

F. Interpretive Problems and Conclusions

A satisfactory long-term clinical trial of alcohol, HTN, and HTN sequelae is unlikely to be performed. Thus, the closest practical alternatives are prospective observational studies and short to intermediate-term clinical trials. The intrinsic problems in studies of alcohol and health effects are well known (59). Underreporting of heavier alcohol intake is one of these, but is incorrectly cited as a factor in the alcohol-HTN relation since the major effect of such underreporting would produce an apparent, but spurious, relationship of HTN to *lighter* drinking. The threshold for the relation could be higher than suggested by the epidemiological data.

Because many traits are related to alcohol drinking or to HTN, it is difficult to rule out all indirect explanations. Psychological or social stress is especially difficult to exclude, but some data show independence from several measures of such stress (76). The intervention studies provide good evidence against most indirect explanations.

Except for the failure, so far, to demonstrate a biological mechanism, other criteria for causality are satisfactorily fulfilled. It is the author's opinion that the relationship between heavier drinking and higher risk of HTN is causal and that

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alcohol-related HTN is the commonest reversible form of HTN. Alcoholic beverage type (wine, liquor, or beer) seems to be a minor factor. Estimates of the proportion of HTN due to heavy drinking vary with the population involved; the contribution of alcohol depends substantially upon the drinking habits of the group under study. Among the lowest estimates are 5% (83) or 7% (57) of hypertension, considering both sexes together. This translates into 1–2 million people with alcohol-associated HTN in the United States, using 20–40 million hypertensives as the denominator. It is probable that alcohol restriction plays a major role in HTN management and prevention (84).

V. CORONARY ARTERY DISEASE

A. Background

Although incidence is decreasing in developed countries, CHD remains the leading cause of death in men and women. Since it causes a majority of all cardiovascular deaths, CHD dominates statistics for cardiovascular mortality and has impact upon total mortality. Epidemiological studies have uncovered several, probably causal, CHD risk factors, including cigarette smoking, HTN, diabetes mellitus, high low-density lipoprotein (LDL) cholesterol, and low high-density lipoprotein (HDL) cholesterol. Atherosclerotic narrowing of major epicardial vessels is the usual basis, with thrombosis in narrowed vessels playing a critical role in major events, such as acute myocardial infarction or sudden death. Another major clinical expression of CHD is angina pectoris. Since Heberden's description of angina relief by alcohol in 1786 (85), many have assumed that alcohol is a coronary vasodilator, but exercise electrocardiographic data (86) suggest that alcohol's effect is purely subjective. Thus, it is probably dangerous for CHD patients to drink before exercise.

In the early 1900s, reports appeared of an inverse relation between heavy alcohol drinking and atherosclerotic disease (87,88), but others (89,90) explained this as an artifact due to premature deaths of many heavy drinkers. Early studies of alcoholics and problem drinkers suggested a high CHD rate (91–94), but some of these studies did not allow for the role of traits associated with alcoholism, such as cigarette smoking. Such studies can tell nothing about the role of light-moderate drinking.

B. Epidemiological Studies

Epidemiological studies consistently show reduced risk of acute myocardial infarction and CHD death in light-moderate drinkers (95–100). Angina pectoris is subjective and difficult to quantify and thus has been relatively little studied epidemiologically in relation to alcohol. Studies showing more CHD events in

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alcohol abstainers than in drinkers include international comparisons (101–103), time-trend analyses of CHD over many years (104), case-control studies (105–111), prospective population studies (2,3,112–133), and studies of coronary arteriograms (134,135). Data from prospective population studies in which alcohol use is determined before CHD events are the most convincing type. Reduced CHD risk among light-moderate drinkers is present in various studies in both sexes, in whites, blacks, and Asians, and at various ages. The impact upon total mortality risk may be strongest among persons $\geq$ 50 years of age (115,124).

Most population studies of nonfatal infarction show that both lighter and heavier drinkers are at lower risk, but studies of CHD mortality tend to show a U-curve relation to alcohol, with abstainers and heavier drinkers at higher risk than light-moderate drinkers (95–99). The reasons for the upper limb of the U may include the effects of spree drinking, alcohol-associated HTN or arrhythmias, misdiagnosis of other conditions (e.g., dilated cardiomyopathy) as CHD, or a truly different effect of heavier drinking upon CHD.

Some population studies did not separate lifelong abstainers from ex-drinkers, or did not adequately control for baseline CHD risk. This led to the ''sick quitter'' hypothesis, which stated that the nondrinking referent groups in these studies were at higher risk for reasons other than abstinence (136). This hypothesis is refuted by prospective studies that separate ex-drinkers from lifelong abstainers and that also control well for baseline CHD (95–99). A few examples will suffice:

1. Data from Kaiser Permanente studies are summarized in Tables 1–3. Analysis of alcohol habits in relation to CHD hospitalizations (113) showed that ex-drinkers and infrequent ($<$1/month) drinkers were at risk similar to that of lifelong abstainers (Table 1). A lower CHD risk was present among all other drinkers, independent of a number of potential confounders, baseline CHD risk at examination (Table 2), and beverage choice. In a study of total cardiovascular (CV) mortality (114), ex-drinkers had higher age-adjusted CHD and overall CV mortality risk than lifelong abstainers, but the difference disappeared when adjusted for other traits. Among drinkers, there were U-shaped mortality curves, independent of baseline risk, relating amounts of alcohol to CV and CAD deaths, with a nadir at 1–2 and 3–5 drinks/day. The study demonstrated the expected disparities between alcohol and various CV conditions (Table 3).
2. A large prospective study among women free of CHD at examination (119) showed a progressive inverse relation of alcohol use to CHD events, independent of prior reduction in alcohol intake and detailed analysis of nutrient intake. Further analysis of these data in women (124) demonstrated that net beneficial effects of moderate alcohol use

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Table 1 Relative Risk of Coronary Artery Disease Hospitalization[a] According to Alcohol Use

Alcohol use	RR[b]	95% CI	*p* value
Nondrinkers			
Abstainer	1.0 (ref)	—	—
Ex-drinker	1.0	(0.7, 1.4)	0.9
Drinkers			
<1/month	0.9	(0.7, 1.2)	0.6
<1/day, >1/month	0.7	(0.5, 0.8)	<0.001
1–2/day	0.6	(0.5, 0.7)	<0.0001
3–5/day	0.5	(0.4, 0.8)	<0.001
6–8/day	0.5	(0.3, 1.1)	0.1
≥9/day	0.5	(0.2, 1.5)	0.2

[a] First for any CAD diagnosis ($n = 756$).
[b] Computed from coefficients estimated by Cox proportional hazards model; covariates include sex, age, race, smoking, education, coffee. RR = relative risk; CI = confidence interval.
Source: Adapted from ref. 113.

Table 2 Relative Risk of Coronary Artery Disease Hospitalization According to Alcohol Use Among Persons Free of Coronary Risk/Symptoms or Recent Major Illness[a]

Alcohol use	RR[b]	95% CI	*p* value
Nondrinkers			
Abstainer	1.0 (ref)	—	—
Ex-drinker	0.9	(0.6, 1.6)	0.8
Drinkers			
<1/month	0.9	(0.6, 1.3)	0.8
<1/day, >1/month	0.6	(0.4, 0.9)	<0.01
1–2/day	0.5	(0.3, 0.7)	<0.0001
3–5/day	0.5	(0.3, 0.8)	<0.01
6–8/day	0.7	(0.2, 1.8)	0.4
≥9/day	0.5	(0.1, 3.8)	0.5

[a] First for any CAD diagnosis ($n = 336$) among persons with no CHD risk/symptoms (12 items) or other major illness in the past year.
[b] Computed from coefficients estimated by Cox proportional hazards model; covariates include sex, age, race, smoking, education, coffee. RR = relative risk; CI = confidence interval.
Source: Adapted from ref. 113.

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Table 3 Relative Risk[a] of Death of Various Cardiovascular Conditions and Cirrhosis by Alcohol Use

	RR for each drinking category vs. lifelong abstainers					
Condition (*n* deaths)	Ex-drinkers	<1/mo	>1/day; <1/mo	1–2/day	3–5/day	6+/day
All CAD (600)	1.0	0.9	0.8*	0.7**	0.7**	0.8
AMI (284)	1.0	0.7	0.8	0.6**	0.5**	0.6
Other CAD (316)	0.9	1.0	0.7	0.8	0.7	1.0
Stroke (138)	1.0	0.8	0.8	0.8	0.7	1.4
Hemorrhagic (41)	1.4	1.5	1.6	1.8	1.3	4.7
Ischemic (34)	0.9	0.5	0.5	0.3	0.4	****
Nonspecific (63)	1.1	0.7	0.9	1.0	1.0	1.2
Hypertension (64)	2.8	2.4	1.9	1.3	2.2	2.1
Cardiomyopathy (24)	3.4	8.5*	4.0	5.6	2.4	8.0
Syndromes (82)[b]	0.6	0.6	0.5	0.4*	0.6	1.0
Arterial (41)[c]	—(d)	1.1	1.6	0.4*	1.7	****
Cirrhosis (42)	10.8(c)	1.4	1.0	4.3	8.1(b)	22.0(c)

[a] Computed from coefficients estimated by Cox proportional hazards model; covariates include sex, age, race, smoking, education, coffee; reference group is lifelong abstainers.

[b] Includes ''symptomatic heart disease'' ($n = 32$); disorders of heart rhythm ($n = 22$); and ill-defined heart disease ($n = 28$).

[c] Includes arteriosclerosis ($n = 15$); aneurysms ($n = 23$); peripheral vascular disease ($n = 2$); and arterial embolism and thrombosis ($n = 1$).

* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** insufficient cases for estimate. RR = relative risk; CAD = coronary artery disease; AMI = acute myocardial infarction.

Source: Adapted from ref. 114.

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in women were limited by adverse effects to persons clearly at above-average CHD risk (i.e., those above 50 years of age).

3. The 12-year prospective American Cancer Society Study (120) of 276,802 men showed a U curve for CHD mortality, with a relative risk of 0.8 (vs. abstainers) at 1–2 drinks/day. In the Health Professional Followup Study of 51,529 men (121), well controlled for dietary habits, newly diagnosed CHD was inversely related to increasing alcohol intake. A study in both sexes, the Auckland Heart Study (109), designed to study the hypothesis that persons at high CHD risk are likely to become nondrinkers, showed that moderate drinkers had lower CHD risk than both lifelong abstainers and ex-drinkers.

C. Possible Mechanisms for CAD Protection by Alcohol

1. Via Blood Lipid Factors

The most studied mechanism, and the most plausible for overall protection by alcohol against atherosclerotic disease, is a link via blood lipid factors. These play a central role in development of this condition (137), with a positive relationship between CHD and higher levels of LDL cholesterol, the so-called ''bad cholesterol,'' and an inverse relationship between higher levels of HDL cholesterol, the so-called ''good cholesterol.'' Triglycerides may play an independent role and some feel that the ratio between total cholesterol and HDL cholesterol, which indirectly incorporates data about LDL, HDL, and triglycerides, may be the best single CHD risk indicator (137). A subset of heavier drinkers has a substantial increase in triglyceride levels, but this is infrequently seen with lighter/moderate drinking. Alcohol may be associated with lower LDL levels (125), but it is unclear that this is independent of other dietary factors. The case for a lipid link for alcohol's protection against CHD rests primarily upon HDL effects.

HDL levels are inversely related to CHD risk (98,137,138), possibly acting by abetting removal of lipid deposits in large blood vessels. HDL binds with cholesterol in the tissues and may aid in preventing tissue oxidation of LDL cholesterol; it then carries it back to the liver for elimination or reprocessing. The net effect is reduction of cholesterol buildup in the walls of large blood vessels, such as the coronary arteries. In the absence of severe liver impairment, alcohol ingestion raises HDL levels (98,121,122,137,139). Among HDL subspecies, some data suggest that HDL_2 may be more protective (140), but several recent studies suggest that both HDL_2 and HDL_3 are protective (122,141–143). The biochemical pathways for the HDL effect of alcohol are incompletely understood. Also pertinent are data that show elevation by alcohol of apolipoproteins A_1 and A_2, associated with HDL particle formation (144–146).

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The hypothesis that the apparent protective effect of alcohol against CHD is mediated by higher HDL cholesterol levels in drinkers has been examined quantitatively in three separate studies (81,122,147). All three analyses yielded similar findings suggesting that higher HDL levels in drinkers mediated about half of the lower CHD risk. One of these studies (122) suggests that both HDL2 and HDL3 are involved. HDL3 may be more strongly related to lighter alcohol intake, but is probably related as strongly as HDL2 to lower CHD risk.

2. Via Antithrombotic Mechanisms

There are data that support an inhibitory effect of alcohol upon various aspects of clotting (98,99,148), including decreased platelet stickiness (149–152), increased thromboxane-prostacyclin ratio (153), lower fibrinogen levels (154–156), interaction with aspirin in prolonging bleeding time (157), and increased release of plasminogen activator (122,158). Perhaps the evidence about anticlotting effects of alcohol is best for fibrinogen lowering (148). An antithrombotic action of alcohol could partially account for the lower CHD risk at very light drinking levels (e.g., several drinks per week) seen in several of the epidemiological studies.

3. Via Glucose Metabolism

Although alcohol drinking, especially heavier intake, has been associated with higher blood glucose levels (159), lighter drinking has also been associated with possible beneficial changes in insulin and glucose metabolism (160–163). Since glucose intolerance is one of the major CHD risk factors, these effects could hypothetically play a role in protection by alcohol against CHD.

4. Via Stress Reduction

Hypothetical considerations about possible benefit from antianxiety or stress-reducing effects of alcohol have no good supporting data.

D. Role of Beverage Choice (Wine, Liquor, Beer)

In 1819 Dr. Samuel Black, a perceptive Irish physician with a great interest in angina pectoris, wrote probably the first commentary pertinent to the ''French paradox.'' Noting apparent angina disparity between Ireland and France, he attributed the low prevalence in the latter to ''the French habits and modes of living, coinciding with the benignity of their climate and the peculiar character of their moral affections'' (164). It was to be 160 years before data were presented from the first international comparison study to suggest less CHD in wine-drinking

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countries than in beer or liquor-drinking countries (101), and there are confirmatory international comparison studies (102,103,130). The ''French paradox'' concept has arisen from these data; it refers to the fact that France tends to be an outlier on graphs of mean dietary fat intake versus CHD mortality, unless adjusted for wine alcohol intake (102,103). Reports of nonalcohol antioxidant phenolic compounds (165–167) or antithrombotic substances (168–170) in wine, especially red wine, have appeared. Inhibition of oxidative modification of LDL cholesterol is probably antiatherogenic, although prospective clinical trials of antioxidant supplements, vitamin E possibly excepted, are not yet conclusive (171–173).

A Kaiser Permanente cohort study of 221 persons who died of CHD (174) and who took 80–90% of their beverage alcohol as the preferred beverage showed that, compared to nondrinkers, CHD risk was significantly lower among preferrers of each beverage type. When the CHD risks of the beverage preferrer groups were compared, there was a gradient of apparently increasing protection from liquor to beer to wine. There were substantial differences in traits between the preference groups (175). The wine drinkers had the most favorable CHD risk profiles, leading to the hypothesis that favorable uncontrolled traits (e.g., dietary habits, physical exercise, use of antioxidant supplements) of wine preferrers might explain the findings.

An analysis of the role of beverage choice among 3931 persons hospitalized for coronary disease used a proxy variable for reported frequency of drinking each beverage type, enabling use of all available beverage choice data (116). Adjusted analyses, not controlled for total alcohol intake, showed inverse relationships to CHD risk for each beverage type, weakest for liquor use. In sex-specific data this inverse relation was significant for beer use in men and for wine use in women. When analyses were controlled for total alcohol intake, only beer use in men remained significantly related. There were no significant differences in risk between drinkers of red, white, both red and white, and other types of wine. It was concluded that all beverage types protect against CHD, with additional protection by specific beverages likely to be minor.

Although antioxidants and other substances in wine are an attractive hypothetical explanation for CHD protection, the prospective population studies provide no consensus that wine has additional benefits, and various studies show benefit for wine, beer, liquor, or all three major beverage types (98,99,116,125, 176). The beverages differ in user traits, with wine drinkers having the most favorable CHD risk profile (175), and drinking pattern differences among the beverage types are another hypothetical factor. The wine/liquor/beer issue is unresolved at this time (116,127,176), but it seems likely that ethyl alcohol is the major factor with respect to lower CHD risk. There seem to be no compelling health-related data that preclude personal preference as the best guide to choice of beverage.

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E. A Causal Relation?

It remains theoretically possible that lifelong abstainers could differ from drinkers in psychological traits, dietary habits, physical exercise habits, or some other way that could be related to CHD risk, but there is no good evidence for such a trait. The various studies indicate that such a correlate would need to be present in persons of both sexes, various countries, and multiple racial groups. While it remains possible that other factors play a role, a causal, protective effect of alcohol is a simpler and more plausible explanation.

VI. CEREBROVASCULAR DISEASE

Earlier studies of relationships of alcohol drinking to stroke were made difficult by imprecise diagnosis of stroke type before modern imaging techniques improved diagnostic accuracy. Risk factors differ somewhat for the two major stroke types; these are hemorrhagic stroke, due to ruptured blood vessels on the brain surface (subarachnoid hemorrhage) and in the brain substance, and ischemic (occlusive) stroke, due to blockage of blood vessels by thrombosis in the brain blood vessels, emboli to the brain from elsewhere, or blockage of blood vessels outside the brain (most notably the carotid arteries.) All studies of alcohol and stroke are greatly complicated by the disparate relationships of both stroke and alcohol to other cardiovascular conditions. Age and HTN are major risk factors for all stroke types, and most cardiovascular conditions have differing relations to various types of stroke. When we add in the disparities in alcohol-CV relationships and the lighter/heavier/binge drinking differences, we end up with almost Byzantine complexity in alcohol-stroke relationships.

As indicated in a comprehensive review (177), several reports suggest that alcohol use, especially heavier drinking, is associated with higher risk of stroke. Some studies examined only drinking sprees; some others did not differentiate between hemorrhagic and ischemic strokes. The importance of these deficiencies is highlighted by several recent studies suggesting that regular lighter drinkers are at higher risk of hemorrhagic stroke types, but at lower risk of several types of ischemic stroke (177). For example, the Nurse's Health Study (119) showed drinkers to be at higher risk of subarachnoid hemorrhage, but lower risk of occlusive stroke. Another example is a Kaiser Permanente study that looked at the relations between reported alcohol use and the incidence of hospitalization for several types of cerebrovascular disease (178). Daily 3+ drink consumption, but not lighter drinking, was related to higher hospitalization rates for hemorrhagic stroke; higher blood pressure appeared to be a partial mediator of this relation. Alcohol use was associated with lower hospitalization rates for ischemic stroke, an inverse relation present in both sexes, whites and blacks, and extracranial and

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intracerebral occlusive lesions. A much larger Kaiser Permanente study is now underway.

Thus, some data suggest that heavier drinking increases the risk of hemorrhagic cerebrovascular events, but that alcohol use lessens the risk of occlusive lesions. In stroke risk the antithrombotic (or anticlotting) actions of alcohol may be important, increasing risk of hemorrhagic strokes and decreasing risk of ischemic strokes. At this time there is no consensus about the relations of alcohol drinking to the various types of cerebrovascular disease and agreement only that more study of this important area is needed (96).

VII. CARDIAC ARRHYTHMIAS

An association of heavier alcohol consumption with atrial arrhythmias has been suspected for decades, with typical occurrence after a large meal accompanied by much alcohol. The concept of the ''holiday heart phenomenon'' has become widely known. The basis of this term was the observation (179) that supraventricular arrhythmias in alcoholics without overt cardiomyopathy were most likely to occur on Mondays or between Christmas and New Year's Day. Various atrial arrhythmias have been reported to be associated with spree drinking with atrial fibrillation the commonest manifestation. The arrhythmia typically resolves with abstinence, with or without other specific treatment. A Kaiser Permanente study

Table 4 Relative Risk[a] of Supraventricular Arrhythmia in Persons with High Versus Low Daily Alcohol Intake

	Persons with arrhythmia					
	6+ drinks day (n = 1,332)		<1 drink day (n = 2,664)			
Rhythm	No.	%	No.	%	RR (6+/<1)	p value
Atrial fibrillation	15	1.1	13	0.5	2.3	0.02
Atrial flutter	8	0.6	6	0.2	3.0	0.05
SVT	5	0.4	2	0.1	5.0	0.03
APBs	43	3.3	32	1.3	3.0	<0.01
Fibrillation, flutter, or SVT	21	1.6	19	0.7	2.3	<0.01

[a] Relative risks and p values estimated using McNemar's method for matched pairs. RR = relative risk; SVT = supraventricular tachycardia; APBs = atrial premature beats.
Source: Adapted from ref. 180.

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(180) compared atrial arrhythmias in 1,322 persons reporting 6+ drinks per day to arrhythmias in 2644 light drinkers. The relative risk in the heavier drinkers was at least doubled for atrial fibrillation (AF) atrial flutter, supraventricular tachycardia, and atrial premature complexes (Table 4).

Increased ventricular ectopic activity has been documented after ingestion of substantial amounts of alcohol, although epidemiological studies have not shown a higher risk of sudden death in drinkers (108).

Speculation about mechanisms for the relationship between heavier drinking and arrhythmias has included myocardial damage, electrolyte/metabolic effects, vagal reflexes, effects upon conduction/refractory times, and possible roles for catecholamines or acetaldehyde. A recent report from Finland (181) studied these in men with recurrent alcohol-associated AF. In controlled analyses of a number of tests and measurements, there was some evidence for exaggerated sympathetic nervous system reaction in these persons.

VIII. CONCLUSION

This survey documents the evidence for disparity in the relations of alcohol and CV disorders. Table 5 summarizes the relations, with emphasis on the disparity

Table 5 Relationship of Alcohol Drinking to Cardiovascular Conditions

	Amount of alcohol drinking		
Condition	Small	Large	Comment
Dilated cardiomyopathy	No relationship	Probably causal	? Unknown cofactors
Beriberi	No relationship	No relationship	Thiamine deficiency
(Arsenic) Cobalt—beer disease	No relationship	Synergistic	Examples of cofactors
Hypertension	Little or no relationship	Probably causal	Mechanism unknown
Coronary disease	Protective	? Protective	Via HDL, antithrombotic effects; beverage type minor factor
Arrhythmia	? None	Probably causal	? Susceptibility factors
Hemorrhagic stroke	? Increased risk	Increased risk	Via higher BP, antithrombotic actions
Ischemic stroke	Protective	? Protective	Complex interactions with other conditions

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between the overall favorable relations of lighter drinking and the overall unfavorable relations of heavier drinking. Advice to the general public or concerned persons about the health effects of alcohol drinking needs to be individualized according to the persons' specific medical history and risks (182,183). A few rules seem sensible: (1) The overall health risk of a heavier drinker is likely to be reduced by reduction or abstinence. (2) Because of the unknown risk of progression to heavier drinking, abstainers cannot be indiscriminately advised to drink for CV health benefit. (3) The majority of persons who are light/moderate drinkers need no change in drinking habits except in special circumstances.

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Beverage Alcohol Consumption as a Negative Risk Factor for Coronary Heart Disease

Biochemical Mechanisms

David M. Goldberg
University of Toronto, Toronto, Ontario, Canada

George J. Soleas
Liquor Control Board of Ontario, Toronto, Ontario, Canada

I. INTRODUCTION

In 1991, Marmot and Brunner reviewed 28 epidemiological investigations analyzing the relative risk of coronary heart disease (CHD) in moderate alcohol consumers (1). Eight were case-control studies, of which two showed no risk reduction, while the median risk reduction in the other six ranged from 0.18 to 0.70. Twenty were prospective studies of which one concluded that moderate alcohol consumption *increased sudden cardiac death* by a factor of 1.4, one study showed no benefit, and the other 18 demonstrated median relative risks ranging from 0.4 to 0.9. Since that time, the notion that moderate consumption of beverage alcohol may be a negative risk factor for CHD has gained further support from epidemiological studies involving subjects of both sexes from more than 20 countries and numbering hundreds of thousands when aggregated. These have been extensively reviewed by the present authors (2,3), and have been described and discussed by others (chapters by Klatsky and by Criqui, this volume).

The beneficial effects of beverage alcohol apply to all CHD end-points: death (4,5), hospitalization (6,7), utilization of health services (8), and angina

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pectoris (9). Even after myocardial infarction, the risk of a subsequent coronary event is significantly alleviated by the moderate consumption of beverage alcohol (10). Critics of these epidemiological investigations have asserted that the favorable effects of beverage alcohol are artifactual, and due to the possibility that many of the abstainers comprising the control groups did not drink because of poor health, stopped drinking for the same reasons, or were previous heavy drinkers who quit for therapeutic purposes (11,12). In the last decade, virtually every epidemiological study has employed a control group restricted to healthy abstainers, yet the results have been almost identical (13–15). Thus, population-based studies using questionnaires to assess individual alcohol intake, or government-derived data to calculate average national per capita beverage alcohol consumption, have demonstrated a reduced risk for CHD among moderate alcohol consumers that is consistent, compelling, and convincing within the limitations of the techniques employed in these studies.

It is, therefore, legitimate to pose the question: Does beverage alcohol truly reduce the risk of CHD and its clinical manifestations? On the basis of this epidemiological evidence, most clinicians and scientists would reply in the affirmative, although they might disagree about issues such as threshold effects, dose-response relationships, and optimal daily intake. We do not count ourselves among that number. The nature of the population studies (retrospective or prospective) that form the evidence does not in itself allow us to draw causal relationships, and we cannot conclude on these grounds alone that moderate alcohol consumption prevents CHD. At best, we can say that there is an *inverse relationship* between these two phenomena in almost all populations studied to date, but this could be mere *association* that may have direct or indirect explanations. Although certain investigators have attempted to use homogenous populations (16–18), we cannot attribute a causal relationship to a feature that may merely be an association reflecting other variables (possibly unknown as yet) in the population examined. Epidemiological population-based studies (unless, as with infectious diseases, they fulfill Koch's postulates) can of themselves rarely provide concrete evidence as to causality, but they are helpful in revealing associations that can then be tested by intervention studies in human populations, and by making use of laboratory experiments to determine whether plausible mechanisms exist that can explain a causal relationship or that are consistent with it.

The object of this chapter is to examine whether ethanol (and possibly other constituents of alcoholic beverages) possesses biochemical and biological properties that might explain and establish its putative role as a negative risk factor for CHD. The latter, in the vast majority of cases, is a sequel to chronic degeneration of the arteries encompassed by the term *atherosclerosis*, upon which is superimposed an acute event such as thrombosis or plaque rupture. This review will be devoted to an analysis of the effects of ethanol upon the cellular and biochemical processes that lead to the initiation and progression of atherosclero-

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sis. For the reader unfamiliar with these mechanisms, a number of reviews can be recommended (19–21).

II. LIPID DEPOSITION IN THE ARTERIAL WALL

The earliest event in the genesis of atherosclerosis is the formation of arterial lesions known as *fatty streaks* owing to the deposition of lipids within the proximal layer of the arterial wall, the intima. The major lipid comprises cholesterol esters. Physiologically, cholesterol is synthesized and secreted by the liver as a component of VLDL, which undergo remodeling in the circulation to form the cholesterol-ester-rich LDL. These are taken up by peripheral cells, including those of the arterial wall, by specific LDL receptors (22). Uptake is saturable, tightly regulated, and takes place only when the cell requires cholesterol. Should cholesterol accumulate in excess of cellular requirements, it is removed by HDL and is eventually returned to the liver for excretion or conversion to bile acids, a process known as *reverse cholesterol transport* (23). The fatty streak is a consequence of two processes: excess uptake of cholesterol by cells in the intimal layer of the blood vessel wall, and/or impaired removal of cholesterol from these cells.

Conditions associated with high blood cholesterol concentrations will tend to promote its cellular uptake by increasing circulating LDL. Native LDL contributes little toward this uptake in view of the limits set upon its endocytosis by the specific LDL receptor. However, these lipoprotein particles are subject to oxidation in the circulation and in the tissues (24). Oxidized LDL (Ox-LDL) lose their affinity for the LDL receptor and instead are taken up by means of *scavenger receptors* (25). These are especially abundant in macrophages and other cells of the reticuloendothelial system. Ox-LDL are toxic to cells and inhibit uptake of cholesterol by HDL (26). The engorgement of macrophages with Ox-LDL eventually leads to their death and disintegration, with release of cholesterol esters into the interstitial space of the vascular intima. Ox-LDL are immunogenic and stimulate the entry of immunocompetent cells into lesions in which they are present extracellularly (27). This sets up an inflammatory reaction leading to architectural distortion of the vessel wall and release of cytokines that induce migration into the intimal layers of monocytes and leukocytes from the peripheral blood, and smooth muscle cells from the adjacent medial layer. As a consequence, the number of cells capable of ingesting Ox-LDL and becoming lipid-laden foam cells greatly increases, collagen formation is stimulated, and arterial thickening becomes evident.

Ethanol cannot prevent oxidation of LDL; if anything, it may enhance this process by virtue of its pro-oxidant potential (28). However, polyphenolic antioxidants present in red wine have been shown to inhibit the oxidation of human LDL in vitro (29,30) and may exercise similar effects in vivo (31) although this

has been recently disputed (32). It has been shown that distilled spirits aged in wood acquire significant concentrations of antioxidants (33,34) that differ from those present in red wine. It is possible, but not yet demonstrated, that these may also inhibit the oxidation of LDL. This finding implies that the biological properties of wines and certain distilled spirits may differ less than was previously thought, and could help to explain the failure of most epidemiological studies to find a lower risk of heart disease among wine drinkers than among those whose preference is distilled spirits (35–37).

With regard to cholesterol removal from the arterial wall, ethanol has the ability to enhance this process by increasing circulating levels of HDL. This was first shown in population-based studies in which a positive and dose-dependent relationship between alcohol consumption and HDL concentration was demonstrated (38,39); the results of one such investigation are illustrated in Figure 1 (40). In themselves, these do not establish a causal role for ethanol in elevating plasma HDL, although they are suggestive. The effect does not seem to be especially dramatic in quantitative terms, but it has major potential biological significance in view of the fact that the estimated risk of CHD is reduced by 10% when HDL concentrations increase by 0.1 mmol/L.

A causal role has, however, been established experimentally by a number of different approaches. Hepatic cells cultured in the presence of ethanol manifest

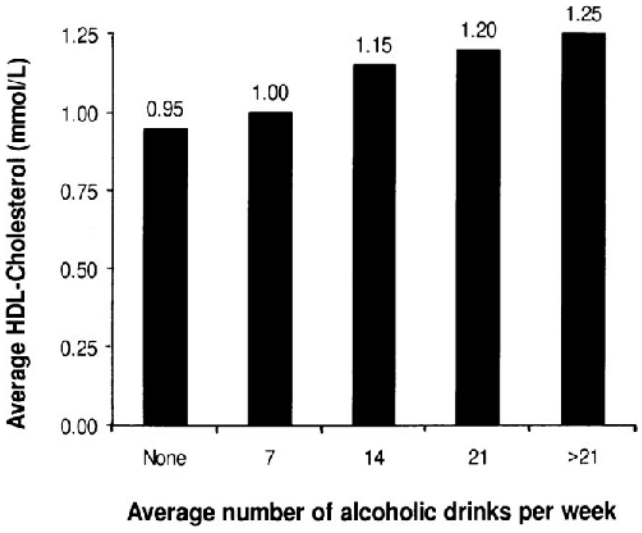

Figure 1 Epidemiological relationship between plasma HDL cholesterol and weekly beverage alcohol consumption. (Adapted from ref. 40.)

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a significant increase in the secretion of Apo-A-I, the main structural protein of HDL and present exclusively in this lipoprotein (41). Human studies have shown an increase in HDL after administration of beverage alcohol (42,43); one set of experiments is illustrated in Figure 2 (44). The latter investigators also demonstrated significant increments in plasma concentrations of Apo-A-I and Apo-A-II after 4 weeks of moderate alcohol consumption (Figs. 3 and 4), there being no difference between red and white wine in these experiments, suggesting that the effects were attributable to their ethanol content and not to polyphenols that predominate in red wine but not white (44).

The fact that plaque regression has been demonstrated angiographically in subjects receiving drugs that lower cholesterol and increase HDL (45,46) implies that reverse cholesterol transport can be enhanced by raising HDL concentrations, thus helping to overcome the inhibitory effects of Ox-LDL upon this process. At one time it was believed that ethanol increased only HDL_3, not HDL_2, which were then thought to be the only HDL particles playing a direct role in the removal of cholesterol from cells and tissues (47). A careful epidemiological study demonstrated increased concentrations of both HDL classes in direct proportion to alcohol consumption (48). Both classes have now been shown to contribute equally and cooperatively to the overall efficiency of reverse cholesterol transport (49). Heterogeneity of HDL particles with respect to their apolipoprotein compo-

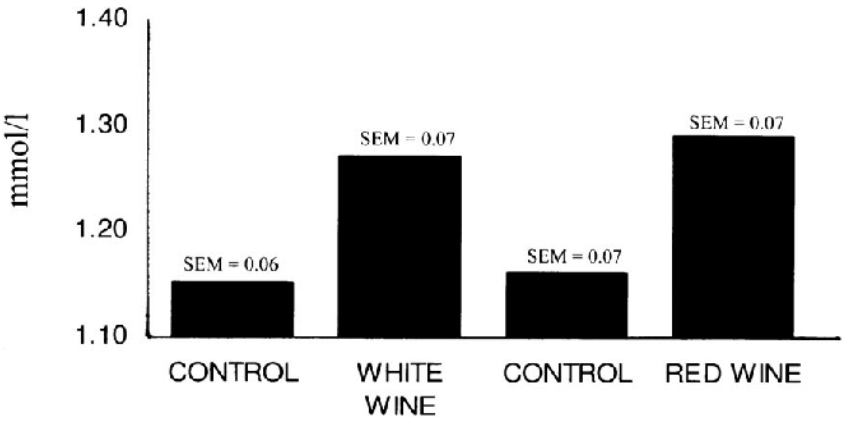

Figure 2 Plasma HDL cholesterol concentrations in 24 healthy men who, in randomly assigned order, drank 375 ml of white or red wine per day for 4-week periods as their sole source of alcohol. During the control periods that randomly preceded or succeeded each wine-consumption period, they avoided alcohol but drank 500 ml/day of commercial grape juice. Bars represent means and SEM is indicated numerically. (Adapted from ref. 44.)

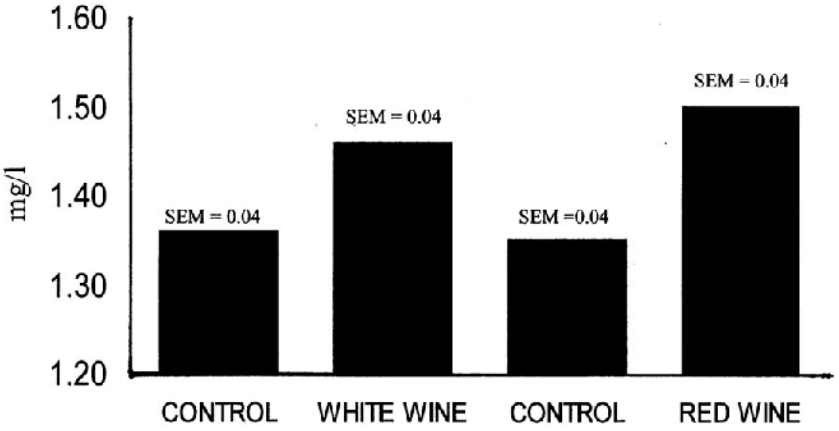

Figure 3 Plasma Apo-AI concentrations in the same subjects as Figure 2. (Adapted from ref. 44.)

sition has also obfuscated the functional relationship between ethanol and cholesterol removal from the tissues. HDL possessing only Apo-A-I is thought to be more effective than particles in which both apo-A-I and apo-A-II are present (50). Beverage alcohol was reported to increase the latter particles more than the former (51), but it was subsequently shown that both are increased (52). The

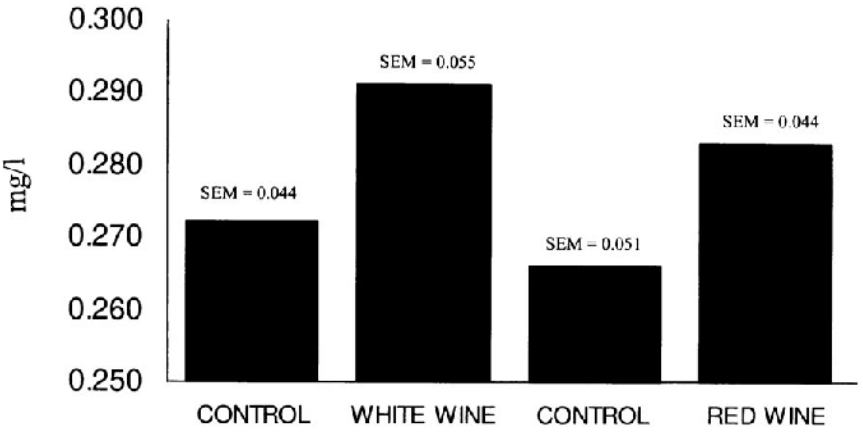

Figure 4 Plasma Apo-A-II concentrations in the same subjects as Figure 2. (Adapted from ref. 44.)

general consensus at present is that the increase in circulating HDL mediated by beverage alcohol is exclusively due to its content of ethanol; this increment enhances reverse cholesterol transport and reduces the risk of CHD, accounting for about 50% of the protection afforded by moderate alcohol consumption against this outcome (53).

Triglyceride, the other major lipid synthesized by the liver and transported to peripheral tissues, is raised by heavy alcohol consumption (54). Slight increases have been noted among moderate drinkers in some investigations (55). For many years, there was considerable debate whether hypertriglyceridemia was an independent risk factor for CHD. It is now accepted that such is the case in a number of clinical situations (56), notably obesity and type II diabetes mellitus (NIDDM). However, it is far from clear how triglycerides contribute to the pathogenesis of atherosclerosis. It does appear, however, that the tendency toward higher plasma triglycerides in moderate drinkers may slightly reduce their overall protection against CHD (55).

III. PLATELET AGGREGATION AND BLOOD CLOTTING

Clot formation within the lumen of the coronary arteries and other blood vessels is an extremely complex process that is subject to exquisite homeostatic control. Three main components are involved: platelets, fibrinogen, and the plasminogen system (57). The aggregation of blood platelets is probably the first event in the overall process. Normally, platelets do not aggregate spontaneously and require a stimulus or agonist to do so (58). Common agonists that probably account for in vivo platelet aggregation include collagen and thrombin; the former is exposed when damage to the vascular endothelium causes gaps uncovering the underlying intima, while the latter is released from prothrombin by an enzymatic cascade that is initiated by inflammatory events and tissue damage.

Aggregating platelets generate a number of metabolically active agents that, operating in an autocrine and apocrine fashion, stimulate aggregation in neighboring platelets and continue the aggregation process once it has begun (59). These initiators of secondary platelet aggregation include ADP and thromboxane A_2, the latter being an eicosanoid synthesized by the cyclo-oxygenase pathway from arachidonic acid that is released from membrane phospholipids by the catalytic action of phospholipase A_2. Ethanol inhibits this step (60), decreasing the production of thromboxane A_2, but also that of other eicosanoids including the prostaglandins that are also synthesized from arachidonic acid (61). Thus, ethanol diminishes the production of an important agonist causing secondary platelet aggregation and will also decrease the production of prostaglandins, most of which cause smooth muscle contraction resulting in reduced organ perfusion and increased blood pressure.

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The likelihood of aggregation taking place depends upon the balance between the concentration of the agonist and the intrinsic sensitivity of the platelets, often thought of as their *stickiness*. This latter property is measured as the concentration of agonist required to induce half-maximal platelet aggregation, referred to as EC_{50}.

Apart from platelets, blood clot contains a matrix of fibers composed of the protein fibrin, together with red blood cells that become passively entrapped as platelet aggregation and fibrin formation proceed locally. Fibrin, formed from fibrinogen through specific proteolytic cleavage carried out by thrombin, is degraded by another proteolytic enzyme called plasmin derived in a similar manner by the cleavage of plasminogen (62). This process is initiated by a family of proteolytic enzymes described as *plasminogen activators*, of which the tissue-type (t-PA) are the best characterized (63). Opposing this process is *plasminogen activator inhibitor* (PA-in). The overall balance between these competing reactions will be reflected in the total fibrinolytic activity of the plasma, measured as its ability to convert insoluble fibrin to soluble degradation products (FDP). Largely through the work of Renaud and associates (64,65), it has been shown that the consumption of beverage alcohol by free-living human populations is accompanied by a dose-related decrease in the potential coagulability of blood withdrawn from the subjects, and measured by a number of ex vivo tests, as illustrated in Figure 5. Many experiments using in vitro and ex vivo techniques

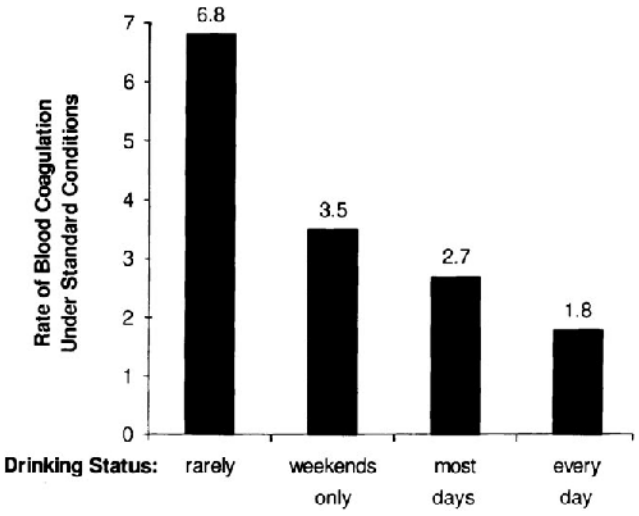

Figure 5 The epidemiological relationship between frequency of beverage alcohol consumption and inhibition of blood coagulation. (Adapted from ref. 65.)

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have shown that ethanol can prevent coagulation when added to the blood of experimental animals and human subjects (see ref. 66 for review), but the ethanol concentrations employed in these investigations were generally much greater than those that can be achieved in vivo by even high levels of alcohol intake.

We have examined this relationship in a cohort of 24 healthy males who were given 375 ml of red wine or white wine, each for a period of 4 weeks in random sequence preceded by a controlled 4-week period during which an isocaloric amount of grape juice was consumed. Each control and wine consumption period was separated by 2 weeks. Throughout the entire period the subjects were asked to adhere to a constant exercise and dietary regime, to avoid all medication, and to consume no alcohol other than the scheduled wines.

Figure 6 shows that the EC_{50} for thrombin-induced activation of washed platelets in suspension was significantly increased after 4 weeks of consuming white or red wine, demonstrating that during this period platelet stickiness was decreased together with their susceptibility to primary aggregation (67). Similar increases in the EC_{50} for ADP-induced aggregation were consistent with a reduced susceptibility to secondary aggregation. Thromboxane A_2 is very unstable and is rapidly converted to its stable metabolite, thromboxane B_2, which provides a measure of cumulative endogenous thromboxane A_2 production. This was decreased during both wine consumption periods relative to the control periods (Fig. 7). The ex vivo reduction of thromboxane A_2 synthesis from the washed

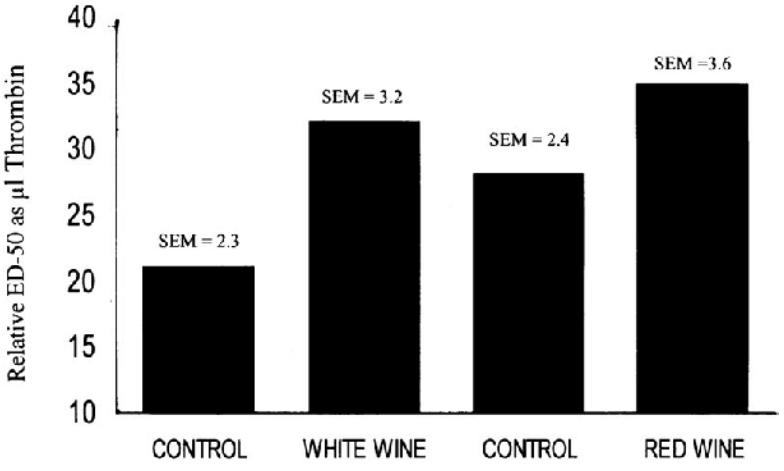

Figure 6 ED-50 (EC_{50}) values for thrombin-induced activation of washed platelets from 24 healthy men who went through the beverage schedules described in the legend to Figure 2. Bars represent means and SEM is indicated numerically. (Adapted from ref. 67.)

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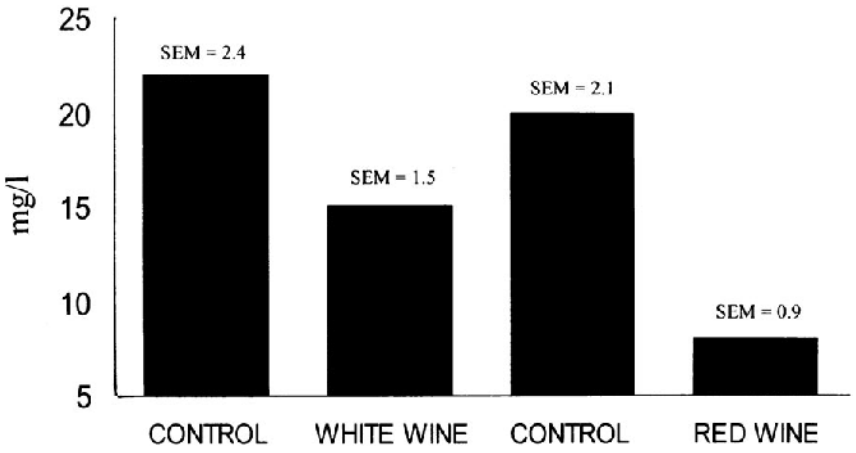

Figure 7 Plasma thromboxane B_2 concentrations in the same subjects as Figure 6. (Adapted from ref. 67.)

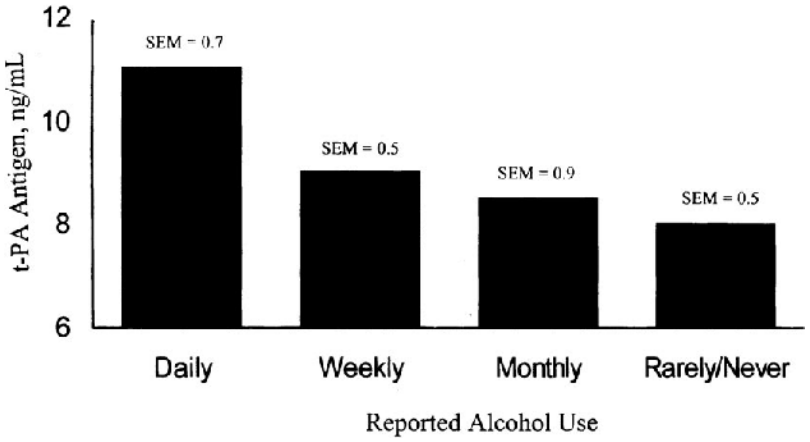

Figure 8 Epidemiological relationship between tissue-type plasminogen activator (t-PA) antigen concentrations and frequency of beverage alcohol consumption. Bars represent means and SEM is indicated numerically. (Adapted from ref. 70.)

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platelets of these subjects, measured as the rate of formation of [^{14}C]-thromboxane B_2 from [^{14}C]-arachidonate, was also lowered by consumption of both wines. There was no statistical difference between the mean of each of the coagulation parameters measured between the two wine-consuming groups.

This was surprising, since the red wine had been specially chosen for its rich content of resveratrol and other polyphenolic constituents that have been shown to exhibit strong inhibitory effects on platelet aggregation and eicosanoid synthesis in vitro (see ref. 68 for review). The failure to demonstrate in vivo activity could be due to poor absorption of polyphenols in the human intestinal tract, or to the possibility that thresholds for inhibition with regard to these phenomena were reached with the amount of alcohol given, so that no incremental inhibition occurred with the assimilation of polyphenols present in the red wine. With either interpretation, it is clear that the lowered platelet aggregatory potential induced by these wines can almost exclusively be attributed to ethanol, the concentration of which was identical in the two wines consumed. It is not known for certain how ethanol exercises its inhibition over eicosanoid synthesis, but its

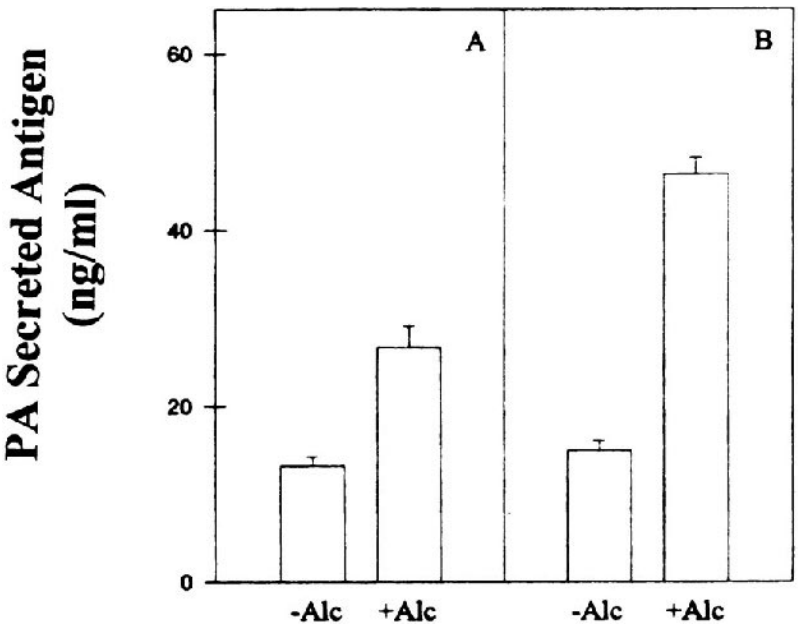

Figure 9 Effect of ethanol preincubation on 24-hr secretion of t-PA (A) and u-PA (B) in cultured human saphenous vein endothelial cells. (From ref. 72.)

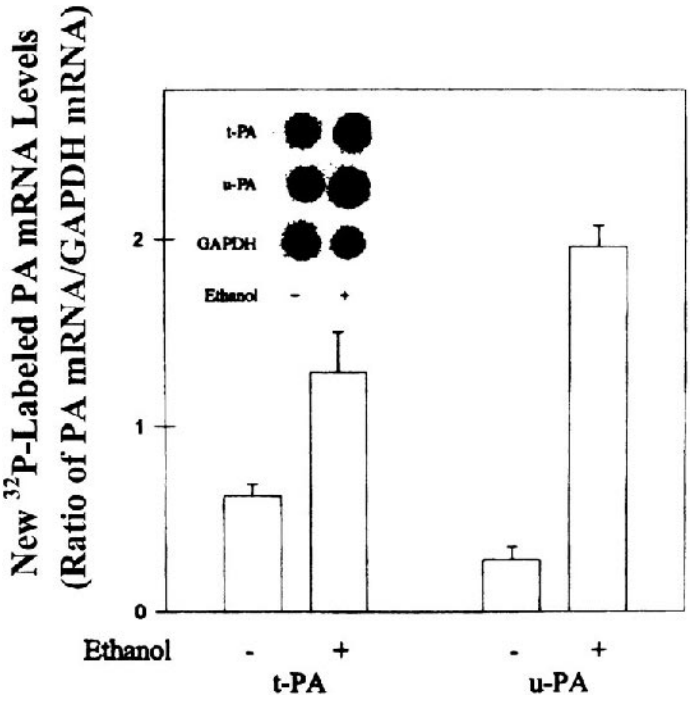

Figure 10 Effect of ethanol (alc) on transcription of t-PA and u-PA genes in cultured human umbilical vein endothelial cells. From ref. 73.

ability to reduce platelet stickiness can be attributed to the important changes that it generates in membrane structure and phospholipid content (69).

Epidemiological evidence has been provided (Fig. 8) in support of the notion that moderate alcohol consumption is associated with increased plasma plasminogen activator concentrations (70). A series of elegant experiments by Grenett and his colleagues, using cultured human endothelial cells, has clearly shown that relatively low concentrations of ethanol (0.1%) up-regulate t-PA gene expression, resulting in increased t-PA protein and mRNA content of treated cells, and increased fibrinolytic activity (71–73; see Figs. 9 and 10). This results in increased surface fibrinolytic activity measured as plasmin generation, an outcome that is also promoted by the ability of ethanol to lower gene expression of PA-in as revealed by decreases in its antigen and mRNA levels in the cells (74).

The third important contribution to overall blood clotting potential, plasma fibrinogen concentrations, are decreased by moderate alcohol consumption (75–77). Thus, by at least three mechanisms suggested by population studies and

established by intervention studies and laboratory experiments, ethanol has been shown to reduce coagulation of the blood or its potential to do so. This effect of ethanol can be predicted to greatly diminish the risk of acute coronary artery occlusion, accounting for about 25% of its overall reduction of CHD mortality and other sequelae (78).

IV. SMOOTH MUSCLE PROLIFERATION

This phenomenon represents an important intrinsic mechanism contributing to the progression of atherosclerosis, as described above. One potent stimulus is contributed by the adherence of platelets to damaged vascular endothelium or to underlying intima when the latter is denuded, resulting in release from the platelet of a number of cytokines and growth factors, of which platelet-derived growth factor (PDGF) is the most important. PDGF stimulates mitosis of medial smooth muscle cells, their migration into the intima, and their change from a contractile to a secretory phenotype (79). The reduction by beverage alcohol of platelet

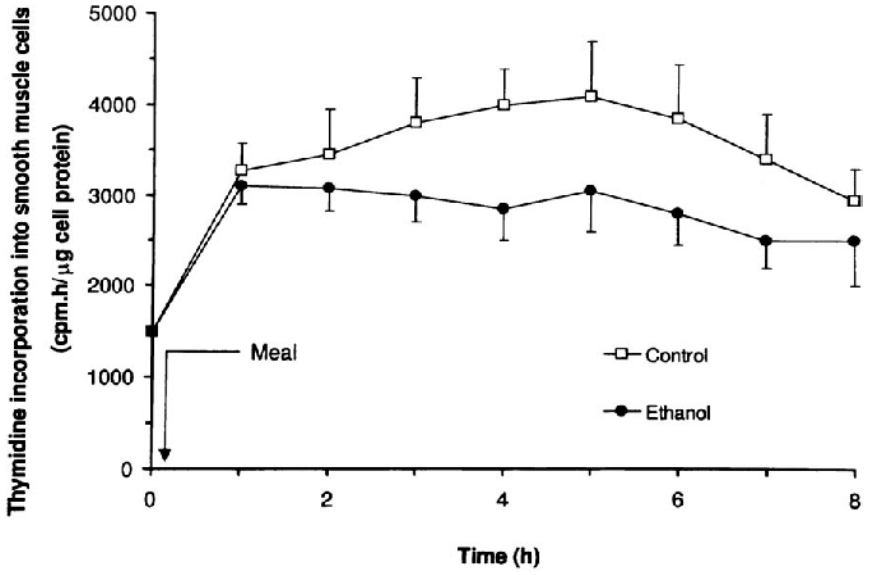

Figure 11 Time course of smooth muscle cell [^{3}H]thymidine incorporation after a fatty meal with and without (control) ethanol (0.5 g/kg body weight) during an 8-hr postprandial period. Data ($x \pm$ SEM) are presented as areas under the curve for the corresponding 1-hr period. (From ref. 80.)

stickiness, as already described, is almost certain to reduce the adherence of platelets to endothelium with consequent reduction in PDGF production. Locher and colleagues (80), upon measuring [^{3}H]-thymidine incorporation into rat vascular smooth muscle cells grown in culture, noted a 20% reduction in the presence of blood drawn from human subjects after a meal containing ethanol compared with that from subjects in whom ethanol was not included with the meal (Fig. 11). In addition, ethanol prevented the migration of human vascular smooth muscle cells that, grown in culture, display chemokinesis in response to pulsatile flow conditions (81). It remains to be determined whether ethanol also prevents phenotypic transformation of these cells.

V. ALCOHOL AND RISK FACTORS FOR CHD

Atherosclerosis is a multifactorial disease in which genes and environment interact to the disadvantage of the subject (82). Those major genetic factors that contribute to its development are unlikely to be modified by alcohol use. However, they are rather small in number, with familial hypercholesterolemia, combined familial hyperlipidemia, apo-E phenotype, and apo-B mutations being among the best recognized conditions in this category. Numerically speaking, environmental and life-style factors play a much more important role, or at least more knowledge has been acquired about their impact. Some of the more important of these factors are favorably influenced by moderate alcohol consumption. In this section these relationships will be described and analyzed, especially with regard to the quality of the evidence and the mechanisms supporting these associations.

A. NIDDM

As early as 1985, Baum-Baicker (83) pointed out that evidence existed to suggest that alcohol may improve glucose tolerance and the blood glucose response to ingested carbohydrates, citing an epidemiological investigation carried out in Yugoslavia that demonstrated a low prevalence rate for NIDDM in moderate drinkers (84). Many years later, Rimm and colleagues examined data collected in surveys by the Harvard School of Public Health and confirmed that those who consumed moderate amounts of alcohol were at lower risk for the development of NIDDM than abstainers or heavy drinkers (85). These findings were further supported by epidemiological studies from several countries including England (86,87), Austria (88), Japan (89), and the United States (90). A plausible mechanism has emerged from a clinical investigation performed in human subjects (91). These authors demonstrated that, in response to a glucose load, alcohol attenuates both the cumulative increase in blood glucose concentration and the accompa-

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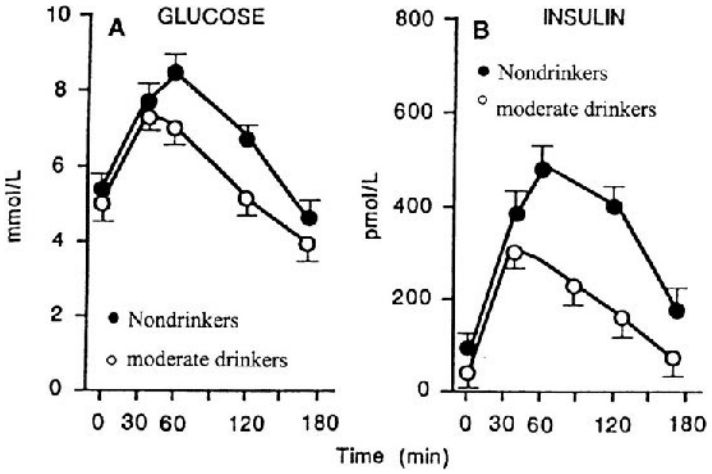

Figure 12 Response of plasma glucose (left) and insulin (right) to an oral glucose load of 75 g in 20 nondrinkers and 20 moderate alcohol drinkers. Data are mean ± SEM. (From ref. 91.)

nying increase in plasma insulin concentration (Fig. 12). This implies increased sensitivity to insulin in susceptible cells. A likely mechanism is enhanced binding of insulin to its receptor or enhancement of the signal transduction events that follow. This is an exciting and important new avenue through which to investigate the metabolic effects of alcohol.

B. Obesity

This is an independent risk factor for CHD, but it is also a feature of NIDDM. As outlined by Suter in this volume, opinions about the effect of alcohol upon body weight are divided almost equally. Many epidemiological studies point to a modest positive relationship or no relationship at all, with fewer population studies reporting a reduction in body weight with increasing alcohol consumption. A questionnaire-based study of a unique population revealed a J-shaped relationship, body weight decreasing at lower and increasing at higher alcohol intakes (92). Experiments involving the substitution of alcohol for carbohydrate are more consistent in showing a negative relationship between alcohol consumption and body weight (93). The kind of studies involving measurement of energy balance reported by Suter do not take into account the role of hormones, especially insulin, in determining the extent to which calories are converted to fat. Since obesity is an important component of NIDDM, the risk of which is attenuated by beverage

alcohol, with improvement in glucose tolerance and response to insulin, it is plausible that alcohol may reduce body weight when it is given as part of a diet standardized with respect to the intake of calories and fat, although the reduction is likely to be quite modest.

C. Hypertension

Heavy drinking is a well-accepted cause of high blood pressure (94). However, the relationship between blood pressure and alcohol consumption is J shaped; the lowest values are found in those consuming 1–2 drinks per day (95), and the reduction occurs in both systolic and diastolic pressures (Figure 13). The pres-

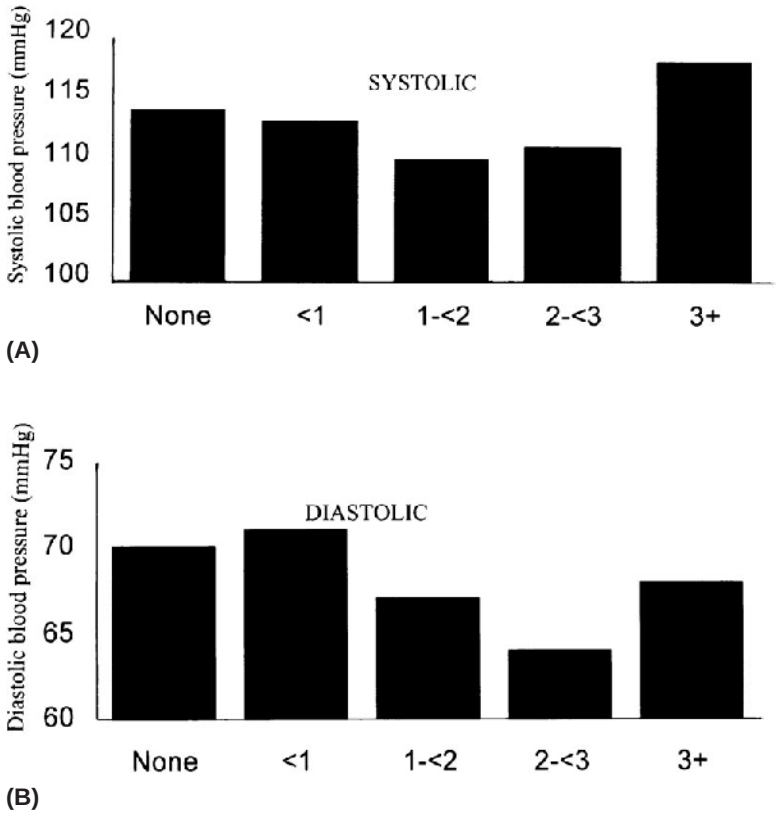

Figure 13 Relationship between intake of beverage alcohol and mean blood pressure in 316 young adults of both sexes, adjusted for age and Body Mass Index. (A) Systolic (top); (B) Diastolic (bottom). (From ref. 95.)

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sure-lowering effect of low-to-moderate alcohol consumption is more significant among women than men (96). The vasodilatory properties of some alcoholic beverages (97), as well as their inhibition of prostaglandin synthesis and enhanced production of the vasodilatory eicosanoid prostacyclin (98) and reduction of stress (vide infra), are plausible mechanisms underlying the pressure-lowering effects of moderate alcohol consumption. As yet, there is no mechanism to explain why heavy consumption should increase blood pressure.

D. Lp(a)

The protein component of this lipoprotein consists of apo-B covalently linked to a series of kringles homologous with those comprising plasminogen. Epidemiologically, Lp(a) is a strong positive risk factor for CHD, the presumed mechanism being inhibition of fibrinolysis by plasminogen. Population studies have yielded conflicting results, some apparently demonstrating a negative association between Lp(a) and alcohol consumption (99,100), while others have failed to find any relationship (101,102).

Intervention studies in human subjects support the notion that alcohol consumption reduces plasma Lp(a) concentrations. Most convincing are investigations (103–105) in which the cessation or reduction of beverage alcohol was accompanied by a significant increase in plasma Lp(a). In one such investigation, the increase was greater among binge drinkers than among regular drinkers following reduction in their alcohol intake, but with maintenance of the same drinking pattern (106). Experiments in which alcohol consumption has been increased have shown a tendency toward a reduction in LP(a) concentrations, but after several weeks the downward trend appears to be reversed and the previous concentrations were reached (107). On balance, alcohol may well reduce Lp(a), but this cannot be regarded as fully established and it is likely that different responses occur with different genotypes of this highly polymorphic protein.

E. Stress

Moderate use of beverage alcohol improves mood, enhances feelings of happiness and freedom from care, and decreases stress, tension, and depression (108–110). The mechanisms underlying these effects are unknown, but the phenomena themselves are widely accepted, and account for the almost universal use of beverage alcohol as a promoter of healthy social interaction.

F. Homocystine

This amino acid, present in the circulation but not in proteins, and an intermediate on the pathway from methionine to cystine, is one of the more recently recognized

risk factors for CHD. Its effects appears to depend upon its direct toxicity for endothelial cells (111). As described in detail in Leiber's chapter in this volume, ethanol blocks this pathway and reduces homocystine synthesis. This is postulated to be one of the mechanisms responsible for liver damage in chronic alcoholic abuse. Moderate alcohol consumption does not cause liver damage, but it is feasible that it could reduce blood homocystine concentrations. This proposal merits further investigation.

VI. COMMENTS

A number of statements made in this book are worthy of evaluation and reiteration. First, beverage alcohol is not synonymous with ethanol: as already pointed out, the former contains many biologically active constituents that are not present in the latter, although whether their in vitro effects are reproduced in vivo remains to be established. Second, definitions of *moderate consumption* and *dose-response relationships*, when they are based upon epidemiological population studies, are likely to be erroneous in light of the view, widely expressed in this book, that individuals responding to questionnaires or even personal interviews greatly underestimate their alcohol consumption. Limits of tolerance derived in this manner can safely be revised upward. Another notion coming out of the epidemiology literature is that very low levels of consumption (e.g., one or two drinks per week) provide as much protection against the risk of CHD as regular consumption on a daily basis. Until these benefits can be demonstrated by animal experiments or by intervention studies in human subjects, we have no way of knowing whether they are true or false. What is certain from the standpoint of theoretical pharmacology is that they are arrant nonsense. Unlike most drugs, alcohol does not accumulate in the human body and its biological half-life is very short. The concentrations reached by such low consumption will not come near those required for in vitro activity. It is well established that the effects of alcohol on blood coagulation are transitory, with return to baseline within 24 hr (112,113). In rats, heavier consumption is accompanied by rebound hypercoaguability (114). We are not suggesting that alcohol should be used therapeutically, but if this is the objective, it should be given regularly as is the case with all therapeutic agents.

More than one investigator has attempted to draw up a balance sheet illustrating the risks and benefits of alcohol consumption. Many of these risks, particularly traffic accidents, suicide, and liver disease, are the consequence of abusive rather than moderate consumption.

Finally, it must be emphasized that atherosclerosis is a disease process that begins early in life and follows an inexorable pattern of progression. Although its sequelae such as CHD, occlusive stroke, and peripheral vascular disease do

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not become manifest until the later years of life, the damage occurs many years earlier and is essentially irreversible. The idea that individuals in the second and third decades of life are not benefited by moderate alcohol consumption because heart attacks are rare below the age of 50 (115) is as illogical as the inference that the same individuals can smoke tobacco with impunity because lung cancer does not usually occur clinically until a person is beyond the age of 40. By the same argument, control of blood cholesterol would be deemed unnecessary and unhelpful before middle age since its benefits are not observed before that point is reached. Let us never lose sight of the maxim that underlies high-quality health care: prevention of a disease is better than its cure.

ACKNOWLEDGMENTS

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Regulation of the Quantity and Quality of High-Density Lipoproteins by Alcohol

A Review

Minna L. Hannuksela and Markku J. Savolainen
University of Oulu, Oulu, Finland

I. INTRODUCTION

The results of epidemiological studies indicate an inverse relationship between moderate alcohol consumption (one to three drinks per day) and the incidence of coronary heart disease and ischemic stroke. Although the mechanisms by which alcohol intake reduces the morbidity and mortality attributable to atherosclerosis are not fully known, there is substantial evidence that ethanol is protective, at least in part, through its effects on plasma lipoproteins, especially high-density lipoproteins (HDL). The principal alcohol-induced change is an increase in the plasma HDL cholesterol concentration. This effect is modulated by gender, drinking pattern, type of alcoholic beverage consumed, diet, smoking, exercise, and liver disease, and these complex interactions are discussed in this review. There are several mechanisms by which alcohol intake increases plasma HDL. Alcohol drinking increases the synthesis of apolipoproteins A-I and A-II, the main protein components of HDL, and slows down the catabolism of apolipoprotein A-II and whole HDL particles. Alcohol-induced changes in proteins and enzymes involved in HDL metabolism may also contribute to the elevated HDL levels. The effects of alcohol intake on these proteins are reviewed in detail, focusing on cholesteryl ester transfer protein, phospholipid transfer protein, lecithin:cholesterol acyltransferase, lipoprotein lipase, hepatic lipase, and proteins associated with HDL, such as paraoxonase and platelet-activating factor acetylhydrolase.

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II. ALCOHOL AND ATHEROSCLEROSIS

A. Alcohol and Coronary Heart Disease

Several studies have shown that moderate alcohol intake is associated with a reduced incidence of, and mortality from, coronary heart disease both in men and in women (for a review, see refs. 1–6). This association has also been found in subjects with adult-onset diabetes (7). Some studies, however, fail to confirm the current notion that moderate drinking is protective (8).

It has been estimated that men and women consuming one to three drinks per day have a 10–40% lower risk of coronary heart disease than abstainers (9). An extensive meta-analysis of 42 studies estimated that 30 g of alcohol per day (two to three drinks) would cause a 25% reduction in the risk of coronary heart disease (9). Heavy alcohol intake is associated with an increased risk of cardiovascular disease, resulting in a J- or U-shaped mortality curve, due to the adverse effects of high alcohol intake on arrhythmias, blood pressure, and the myocardium (4,5).

B. Alcohol and Stroke

Mortality from stroke represents the third leading cause of death after coronary heart disease and all cancers. Studies on alcohol consumption and the incidence of ischemic stroke suggest a distinct dose-response relationship (10–13). Moderate alcohol intake appears to be protective against ischemic stroke, although the benefit for stroke is less convincing than the benefit for coronary heart disease (14,15). Some studies also suggest a benefit of moderate drinking against intracerebral hemorrhage and subarachnoidal hemorrhage (10).

C. Antiatherogenic Mechanism of Alcohol

Although the exact mechanisms by which alcohol drinking protects from atherosclerosis are unknown, a major theory is that ethanol is protective through its effects on lipoproteins (16,17). Other possible mechanisms include decreased platelet activity (18,19), reduced fibrinogen concentration (20), and enhanced insulin sensitivity in moderate drinkers (21). It has been estimated that at least half of the beneficial effect of moderate alcohol intake is due to an increase in the HDL cholesterol concentration (22,23).

III. HIGH-DENSITY LIPOPROTEINS

HDL are a spectrum of spherical lipoprotein particles within the density range of 1.063–1.210 g/ml (24,25). The core of HDL particles contains mostly cholesteryl

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esters and triglycerides, while the surface consists of a phospholipid monolayer coat (primarily phosphatidylcholine) associated with free cholesterol molecules, and proteins. The two major proteins of HDL are apolipoprotein (Apo)-A-I and Apo-A-II, which comprise about 70% and 20% of the total HDL protein mass, respectively. HDL particles also contain Apo-E, Apo-C (Apo-C-I, C-II, and C-III), and small amounts of some other apolipoproteins (Apo-A-IV, Apo-D, Apo-F, Apo-H, and Apo-J).

HDL are heterogeneous and can be classified into two major subfractions: larger, less dense HDL_2 (density 1.063–1.125 g/ml) and smaller, denser HDL_3 (1.125–1.21 g/ml). HDL particles can also be divided into subpopulations according to their apolipoprotein composition. The two main subspecies of Apo-A-I containing particles are particles that contain both Apo-A-I and Apo-A-II (LpA-I:A-II) and particles that contain Apo-A-I but no Apo-A-II (LpA-I) (26,27). Most of the LpA-I particles are found in the HDL_2 density fraction, while most of the LpA-I:A-II particles float in the HDL_3 density fraction.

A. HDL and Atherosclerosis

Several studies (28–36) have shown that high plasma levels of HDL cholesterol and Apo-A-I are associated with a reduced risk of coronary heart disease (for a review, see refs. 37–39), but some controversy exists regarding the relative importance of the HDL subclasses. The protective effect of HDL has been related to high plasma concentrations of HDL_2 (40,41), but also to both HDL_2 and HDL_3 in some studies (42–44). In accordance with HDL_2, LpA-I has been proposed as the more antiatherogenic subpopulation within HDL (45–49).

B. Alcohol and HDL Subfractions

The effect of alcohol on HDL subclasses is controversial, most likely owing to differences in the amount of alcohol consumed and the length of exposure between the studies. The two main HDL subclasses, HDL_2 and HDL_3, behave somewhat differently: moderate alcohol intake has been reported to raise mainly HDL_3 (50–54), whereas heavy or chronic alcohol consumption elevates mainly HDL_2 (55,56) or both HDL_2 and HDL_3 (53,57,58). In some studies, however, moderate alcohol intake has raised both HDL_2 and HDL_3 (59,60). The increase in apo-A-I-containing HDL subpopulations also varies between studies. Some studies have reported an alcohol-induced increase of both LpA-I and LpA-I:A-II (60–63), whereas others have shown an increase mainly in LpA-I:A-II (64,65).

C. HDL as a Cholesterol Transport Mechanism

HDL protect against atherogenesis, but the mechanisms are not fully known. The antiatherogenic role of HDL has recently been reviewed (66–68). An attractive

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explanation postulates participation of HDL in the reverse cholesterol transport. HDL mediate the transport from peripheral cells to the liver, the major site of cholesterol removal from the body. A low plasma HDL concentration might reflect inefficient reverse cholesterol transport. This could explain the inverse relation between the plasma HDL concentration and atherosclerosis.

HDL particles may also have other functions. HDL and its components may influence the signal transduction pathways in vascular wall cells. Whether this is due to binding of HDL particles to cell surface receptors or perturbation of the signaling by lipid second messengers remains to be established. The net effect, however, may be alterations in the production of adhesion molecules (69) or growth factors (70), and these changes may have important consequences for the development of atherosclerotic lesions.

D. Effect of Alcohol on HDL Cholesterol

Alcohol drinking is associated with multiple changes in the concentration and composition of plasma lipoproteins (71,72). The principal change in alcohol drinkers is an increase in plasma HDL cholesterol. This effect has been consistently reported both in observational studies in a variety of populations (22,23,73,74) and in experimental studies (for a review, see ref. 9). A meta-analysis of 25 experimental studies estimated that an average individual consuming 30 g of alcohol per day would expect an increase in HDL cholesterol concentration of about 0.1 mmol/L, or 4.0 mg/dl (9).

In addition to the increased HDL cholesterol concentration, the concentration of phospholipids in HDL particles is higher in alcohol abusers than in control subjects, while the HDL triglyceride content is similar (57,72). The protein concentration in HDL_2 is also higher in alcohol abusers, while the HDL_3 protein concentration is similar to that in controls (57). These compositional changes are associated with a shift toward larger and less dense HDL particles in alcoholic subjects. During alcohol withdrawal, the concentrations of cholesterol and phospholipids in HDL are reduced (57), and there is a shift from larger HDL_2 to smaller HDL_3 particles (75).

IV. FACTORS AFFECTING HDL CHOLESTEROL AND CORONARY HEART DISEASE IN ALCOHOL STUDIES

A. Gender

Most studies on the effects of alcohol on HDL have been conducted in men, and there is very little information of whether the HDL particles of women and men differ from each other qualitatively with respect to protection from coronary heart disease. The meta-analysis of Rimm et al. reported that in experimental studies

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where alcohol consumption was less than 100 g/day, the increase in HDL cholesterol tended to be greater in men (29 data records) than in women (three data records), but this was not significant (9). In female alcoholics, alcohol intake has been reported to raise the HDL_2 subfraction as well as the activities of lipoprotein lipase and hepatic lipase (56), which is in accordance with the findings on male alcoholics (55). In moderate drinkers, gender is associated with differences in alcohol drinking patterns, binge drinking being more common among men than women (76).

B. Drinking Pattern

There is some controversy regarding the effect of drinking pattern on HDL and other plasma lipids and lipoproteins. Some studies have reported a more atherogenic lipid profile with episodic (weekend) drinking versus regular daily drinking (77–79), but this has not been confirmed in other studies (80). The influence of drinking pattern on cardiovascular diseases has recently been reviewed (76).

Weekend drinking has been suggested to be associated with sudden cardiac death (81,82), as well as with fatal myocardial infarction and all-cause mortality (83,84). In a prospective population-based study of 1641 men aged 42–60, the drinking pattern of beer binging was associated with an increased risk of death, independent of the total alcohol consumption (83). Drinking pattern may also modify the risk of stroke. Episodic drinking has been reported to be associated with a higher risk of stroke than regular daily drinking (85–88).

C. Type of Alcoholic Beverage

It has been suggested that wine is more beneficial for the heart than the other alcoholic beverages. The effect of wine on the plasma HDL cholesterol concentration, however, is similar to that of beer or spirits (9,89). Some studies have shown a greater increase in plasma HDL and Apo-A-I following consumption of red wine compared to white wine (90), whereas other studies have revealed no differences between red and white wine (91,92).

The hypothesis that wine is more beneficial in reducing the risk of coronary heart disease than other types of alcohol is still controversial. Most ecological studies (studies that compare populations rather than individuals) have suggested that wine drinkers have the lowest risk for coronary heart disease (93–96), whereas most case-control and cohort studies (97) have indicated that different alcoholic beverages are equally associated with a lower risk for coronary heart disease (for a review, see refs. 98–100). Some cohort studies, however, have suggested an additional beneficial effect of wine even after careful controlling for several risk factors (101,102).

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D. Diet

The relation between the type of alcoholic beverage and coronary heart disease may be affected by variation in the diet associated with the preferred drink. In a cross-sectional study of almost 49,000 men and women aged 50–64, wine intake, compared to other alcoholic beverages, was associated with a higher intake of healthy food items: fruit, fish, cooked vegetables, salad, and the use of olive oil in cooking (103).

Moderate alcohol consumption with dinner alters the chemical composition of HDL particles during the postprandial phase by raising HDL cholesterol, phospholipids, Apo-A-I, and triglycerides (104,105). These short-term changes are similar to those seen in chronic alcoholics, with the exception that HDL triglycerides usually remain unchanged in alcoholics (72,106). The effect of alcohol on HDL may be modulated by the dietary fat level. The HDL-increasing effect of moderate alcohol intake was seen in HDL_2 during a high-fat diet (38% of energy from fat), but not during a low-fat diet (18% of energy from fat) in women (107).

E. Smoking

Cigarette smoking is associated with opposite effects on plasma lipids and lipoproteins compared to those caused by alcohol drinking: smoking raises total cholesterol and LDL cholesterol and reduces HDL cholesterol (108,109). In a study of 46,750 men, smoking canceled the possible protective benefit in HDL cholesterol concentrations gained from moderate consumption of alcohol (110). Furthermore, smoking approximately doubles the risk of death from coronary heart disease, and moderate alcohol consumption may not compensate for this large increase in risk produced by smoking (111). A prospective population-based study of the combined effects of smoking, body mass index, and alcohol intake on mortality showed U-shaped mortality curves in relation to alcohol intake, with the lowest risk for lean nonsmokers and highest for obese smokers, and the relative risk of mortality in smokers was more than double the risk of nonsmokers in all categories of alcohol intake (112).

As an example of the complex interaction between diet, smoking, and alcohol, a meta-analysis of 51 nutritional surveys of smokers showed that smokers differ substantially from nonsmokers by their higher intake of alcohol, total fat, saturated fat, and cholesterol (113), which makes it difficult to estimate the net effect of diet, smoking, and alcohol on plasma lipids and on the development of atherosclerosis.

F. Exercise

Physical activity is associated with elevated plasma levels of HDL cholesterol (114–116), and exercise and alcohol intake contribute independently to a higher

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HDL cholesterol concentration (117). Physical activity may modulate the effect of alcohol on HDL and its subfractions. In habitual exercisers, moderate alcohol intake has been reported to raise mainly HDL_3 (50,118) or both HDL_2 and HDL_3 (59), whereas HDL_2 is increased in physically inactive men (50,118).

G. Liver Disease

The positive correlation between alcohol consumption and plasma HDL cholesterol is disturbed by the development of alcoholic liver disease. Patients with alcoholic hepatitis or cirrhosis have lower HDL cholesterol concentrations than alcohol abusers without liver disease, and the HDL level decreases parallel to the degree of liver function impairment (119–121). In patients with liver cirrhosis, withdrawal of alcohol is not sufficient to normalize the low HDL levels (122).

V. MECHANISM OF ALCOHOL-INDUCED INCREASE IN HDL CHOLESTEROL

Alcohol drinking may increase HDL cholesterol by several mechanisms (72,123). First, increased production of both apolipoproteins and lipids and/or HDL particles may occur as a result of the general induction of protein synthesis. Second, slower catabolism of HDL particles or their lipid components may also lead to an increase in HDL cholesterol. Third, alcohol-induced changes in proteins influencing HDL metabolism may result in increased HDL concentrations. These proteins include cholesteryl ester transfer protein, phospholipid transfer protein, lecithin:cholesterol acyltransferase, lipoprotein lipase, and hepatic lipase (Table 1). For example, the alcohol-induced increase in the activity of lipoprotein lipase may enhance the lipolysis of triglyceride-rich lipoproteins (chylomicrons and VLDL), which increase the lipid supply to immature HDL particles and raise HDL phospholipids and cholesterol (78). The mechanisms of alcohol-induced increase in HDL cholesterol are reviewed in detail in the following chapter.

A. Increased Production and Altered Clearance of Apolipoproteins

Alcohol drinking increases the plasma concentrations of Apo-A-I and Apo-A-II, the main apolipoproteins of HDL particles (9,51,61,124,125). It has been estimated that an average individual consuming 30 g of alcohol per day would show an 8.8 mg/dl increase in the plasma Apo-A-I concentration (9). The increased plasma Apo-A-I concentration is associated with an increased synthesis of Apo-A-I in the liver (126,127), which may be related to an induction of liver micro-

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Table 1 Effects of Alcohol Intake on Proteins and Enzymes Involved in High-Density Lipoprotein Metabolism

Protein or enzyme	Moderate or acute alcohol intake	Heavy or chronic alcohol intake
Cholesteryl ester transfer protein	No change in activity (54,140,141) or activity ↓ (142)	Activity ↓ (72,136–138) Concentration ↓ (136) Net mass transfer of CE from HDL to Apo-B-lp ↓ and HDL-CE ↑ (106)
Phospholipid transfer protein	No change in activity (141)	Activity ↑ (75,106) Net mass transfer of PL from Apo-B-lp to HDL ↑ and HDL-PL ↑ (106)
Lecithin:cholesterol acyltransferase	No change in activity (54,140,141) After alcohol intake with a meal, activity ↑ (104, 146)	Activity ↓ in squirrel monkeys (147,148)
Lipoprotein lipase	Activity ↑ (54,78,149)	Activity ↑ (56,57)
Hepatic lipase	No change in activity (51,54) or activity ↓ (78)	Activity ↑ (56,57)
Paraoxonase	Activity ↑ (154)	Not determined
Platelet-activating factor acetylhydrolase	Not determined	Not determined

References are in parentheses. ↓, reduced; ↑, increased; CE, cholesteryl esters; PL, phospholipids; HDL, high-density lipoproteins; Apo-B-lp, apolipoprotein B-containing lipoproteins.

somal enzymes (126,128). Increased synthesis and secretion of Apo-A-I by ethanol have also been reported in a HepG2 cell culture model (129). The fractional catabolic rate of Apo-A-I has also been reported to be increased, but to a lesser extent than the synthetic rate, leading to an increased plasma concentration of Apo-A-I (127). Some studies, however, have failed to detect changes in Apo-A-I metabolism (65). The increase in the plasma concentration of Apo-A-II has been shown to be associated with reduced plasma clearance of Apo-A-II and increased production of Apo-A-II (65).

In contrast to Apo-A-I and Apo-A-II, the plasma concentration of Apo-E has been reported to remain unchanged (125) or decrease (124). In studies with rats, chronic ethanol treatment leads to the appearance of sialic-acid-deficient Apo-E owing to down-regulation of the expression of sialyltransferase (130).

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This impairs the binding of Apo-E to HDL, and consequently, the Apo-E content of HDL particles is decreased (131).

B. Slower Catabolism of HDL

One mechanism by which alcohol intake could lead to elevation of the plasma HDL level could be delayed clearance of HDL. Studies on experimental pair-fed animals have shown that the removal of labeled HDL is slower in ethanol-fed baboons (132) and squirrel monkeys (133,134) than in control animals. This led to the suggestion that ethanol may inhibit the transfer of cholesteryl esters from HDL to other lipoproteins (132). The transfer of cholesteryl esters between lipoproteins is facilitated by cholesteryl ester transfer protein. The low transfer rate may reduce the reverse cholesterol transport, since much of HDL cholesterol is believed to be taken up by the liver in the form of LDL particles. This is, however, in contradiction to the role of cholesteryl ester transfer protein in reverse cholesterol transport.

C. Effects of Alcohol on Proteins Involved in HDL Metabolism

The effects of alcohol on cholesteryl ester transfer protein, phospholipid transfer protein, lecithin:cholesteryl acyltransferase, lipoprotein lipase, hepatic lipase, and other HDL-associated proteins are summarized in Table 1.

1. Cholesteryl Ester Transfer Protein

Cholesteryl ester transfer protein (CETP) participates in reverse cholesterol transport by transferring cholesteryl esters and triglycerides as well as phospholipids between plasma lipoproteins. In abstainers and subjects consuming moderate amounts of alcohol, the direction of the cholesteryl ester net mass transfer is from HDL to VLDL and LDL in exchange for triglycerides (Fig. 1) (106,135).

We have previously shown that alcohol intake reduces the activity of CETP in alcoholics' plasma, as alcohol abusers have 20–30% lower plasma CETP activity than control subjects (72,136–138). The specific activity of CETP (CETP activity/CETP concentration) was not reduced in alcohol abusers, indicating that there is no increase in the amount of CETP inhibitor in the plasma of alcohol abusers, and that the alcohol-induced decrease in CETP activity is most likely due to a reduction in the CETP concentration (72,136).

Our studies also showed that the alcohol-induced elevation in the plasma HDL cholesterol concentration reduced the rate of cholesteryl ester net mass transfer from HDL to Apo-B-containing lipoproteins or even reversed its direc-

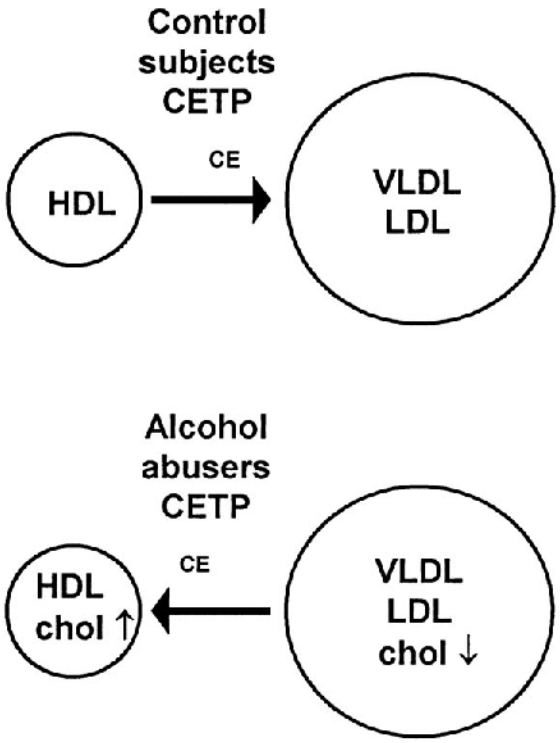

Figure 1 Cholesteryl ester transfer protein (CETP) transfers cholesteryl esters (CE) and triglycerides between plasma lipoproteins. In control subjects, the direction of the cholesteryl ester net mass transfer is from high-density lipoproteins (HDL) to very-low-density lipoproteins (VLDL) and low-density lipoproteins (LDL) in exchange for triglycerides. In alcohol abusers, the alcohol-induced elevation in plasma HDL cholesterol reduces the rate of cholesteryl ester net mass transfer from HDL to Apo-B-containing lipoproteins or even reverses its direction (106).

tion (Fig. 1) (106,139). In control subjects, the net mass transfer of cholesteryl esters is from HDL to VLDL and LDL, whereas in alcohol abusers, the direction is from VLDL and LDL to HDL (106).

Studies on alcohol abusers have shown an inverse relationship between alcohol intake and plasma CETP activity as well as between plasma CETP activity and HDL cholesterol concentration (72,136,137). During alcohol withdrawal, plasma CETP activity increases and HDL cholesterol decreases (56,72,75). In moderate drinkers, however, such associations are not always seen (54,140,141). A Japanese study of 317 men and 269 women showed that moderate alcohol

intake significantly reduced CETP activity, but no correlation was found between CETP activity and HDL cholesterol (142).

2. Polymorphisms of the Cholesteryl Ester Transfer Protein Gene

Several polymorphisms in the CETP gene affect plasma lipid levels and the risk of coronary heart disease, and these effects may be modulated by alcohol consumption. The B2 allele of the *Taq*I B polymorphism of the CETP gene is associated with an increased plasma HDL cholesterol concentration and reduced plasma CETP activity (138,143,144). In a large case-control study, the B2 allele was associated with a reduced risk of myocardial infarction in men consuming more than 75 g of alcohol per day (144).

3. Phospholipid Transfer Protein

The phospholipid transfer protein (PLTP) transfers phospholipids between plasma lipoproteins, accounting for two-thirds of the phospholipid transfer activity in plasma. PLTP, but not CETP, is responsible for the net mass transfer of phospholipids from Apo-B-containing lipoproteins to HDL (Fig. 2). Even though PLTP does not directly catalyze the transfer of cholesteryl esters, it is capable of enhancing the transfer of cholesteryl esters from HDL_3 to VLDL and LDL mediated by CETP.

Heavy alcohol intake increases the activity of plasma PLTP (75,106). We have previously shown that alcohol abusers have approximately 30% higher plasma PLTP activity than control subjects (106). During alcohol withdrawal, PLTP activity is reduced (75). Moderate alcohol intake, however, has been reported to have only a small or no effect on PLTP activity (141). In alcohol abusers, the net mass transfer of phospholipids is increased from VLDL and LDL to HDL, and the concentration of HDL phospholipids is also increased (Fig. 2) (106).

4. Lecithin:Cholesterol Acyltransferase

Plasma lecithin:cholesterol acyltransferase (LCAT), the enzyme responsible for esterification of cholesterol in lipoproteins, requires Apo-A-I for activity and acts preferentially on the smaller HDL particles (nascent HDL and HDL_3), leading to the formation of larger cholesteryl ester-rich HDL_2. The activity of LCAT is reduced in liver steatosis and alcoholic cirrhosis (145). In subjects without liver disease, LCAT activity has been reported to be unaffected after moderate alcohol intake (54,140,141). Alcohol consumption with a meal, however, is related to increased LCAT activity (104,146). In squirrel monkeys, LCAT activity has been

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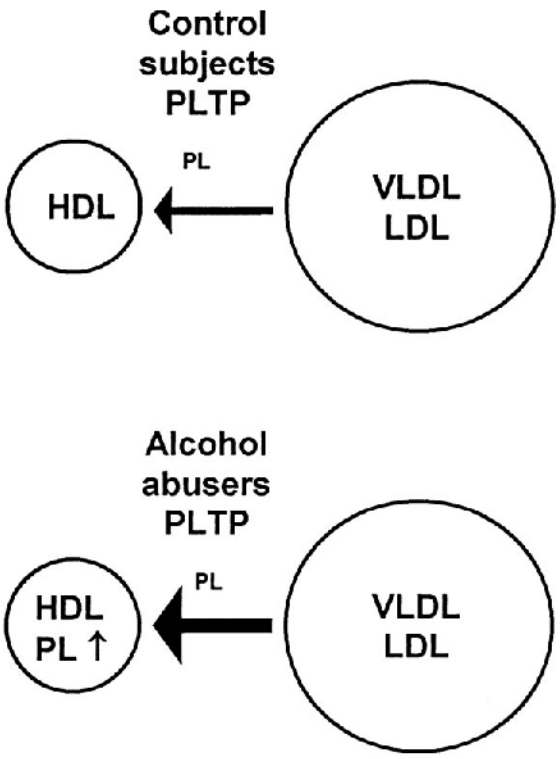

Figure 2 Phospholipid transfer protein (PLTP) transfers phospholipids (PL) between plasma lipoproteins. In control subjects, PLTP is responsible for the net mass transfer of phospholipids from very-low-density lipoproteins (VLDL) and low-density lipoproteins (LDL) to high-density lipoproteins (HDL). In alcohol abusers, the net mass transfer of phospholipids is increased from VLDL and LDL to HDL, and the concentration of HDL phospholipids is also increased (106).

reported to be normal or elevated after low ethanol intake, but reduced after very high ethanol intake (147,148).

5. Lipoprotein Lipase

Lipoprotein lipase is located on the surface of capillary endothelial cells of adipose tissue and muscles and hydrolyzes triglycerides in chylomicrons and VLDL particles, with Apo-C-II as a cofactor. Both acute (54,78,149) and chronic (56,57) alcohol intake enhances the activity of lipoprotein lipase. The high lipoprotein lipase activity results in rapid hydrolysis of newly secreted triglyceride- and phos-

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pholipid-rich VLDL particles; this secretion is stimulated by alcohol (78). Consequently, the transfer of VLDL surface components to HDL is increased, which is reflected in a rise of HDL phospholipids and cholesterol (72,78).

6. Hepatic Lipase

Hepatic lipase is associated with the surface membrane of nonparenchymal liver cells, uses Apo-A-IV as a cofactor, and catalyzes the hydrolysis of triglycerides and phospholipids in HDL particles, and also to some extent in chylomicrons and VLDL. The activity of hepatic lipase is unaffected (51,54) or reduced (78) by acute intake of alcohol, and the decreased hepatic lipase activity may promote an increase in HDL_2 particles. The activity of hepatic lipase is, however, increased in chronic alcoholics (56,57) and may counteract the effect of the high activity of lipoprotein lipase on the plasma HDL level.

7. "Oxidation-Protective Proteins" Associated with HDL

Two HDL-associated enzymes, paraoxonase and platelet-activating factor acetylhydrolase, have been suggested to protect against atherosclerosis by inhibition of the oxidation of HDL and LDL (66,150–153). Daily moderate alcohol consumption has been reported to increase serum paraoxonase activity, and the increase correlated positively with the increase in the plasma concentration of HDL cholesterol (154). No studies have thus far been published concerning the effects of alcohol drinking on platelet-activating factor acetylhydrolase.

VI. CONCLUSIONS

Moderate alcohol intake appears to be causally related to a lower risk of coronary heart disease. It has been estimated that at least half of the beneficial effect of moderate alcohol consumption is due to an increase in the HDL cholesterol concentration. Alcohol intake may raise plasma HDL levels by several mechanisms, which include altered synthesis and clearance of HDL and effects on proteins and enzymes influencing HDL metabolism. The molecular mechanisms are, however, complex and poorly known, and further studies are clearly needed.

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Alcohol, Lipoproteins, and the French Paradox

Michael H. Criqui
University of California–San Diego, La Jolla, California

I. INTRODUCTION

Data continue to accumulate on the relation of alcohol consumption to a number of major diseases and conditions, including coronary heart disease, stroke, cancer, cirrhosis, and accidents and violence. On the specific question of alcohol and cardiovascular disease, the recent literature has been truly prodigious. Despite this vast amount of information, ethical and pragmatic difficulties have precluded a specific experimental test of the effect of alcohol consumption on cardiovascular disease (CVD) events, such as myocardial infarction and stroke (1). Thus, the available observational epidemiological data are our best evidence on the alcohol-CVD question.

II. ALCOHOL AND CVD

Numerous case-control and prospective population studies have shown that light to moderate drinkers have, or will develop, on average lower rates of coronary heart disease (CHD) (2). This effect has been demonstrated in men and women, in black and white Americans, in Japanese-Americans, Britons, Yugoslavians, Puerto Ricans, and Australians (3). Recent studies have also indicated apparent benefit of light to moderate alcohol consumption in persons with known heart disease (i.e., secondary prevention) (4,5) and in adult-onset diabetes (6,7). Figure 1 comes from the American Cancer Society prospective study of 276,802 men aged 40–59, and each of the mortality risk ratios noted is adjusted for age and

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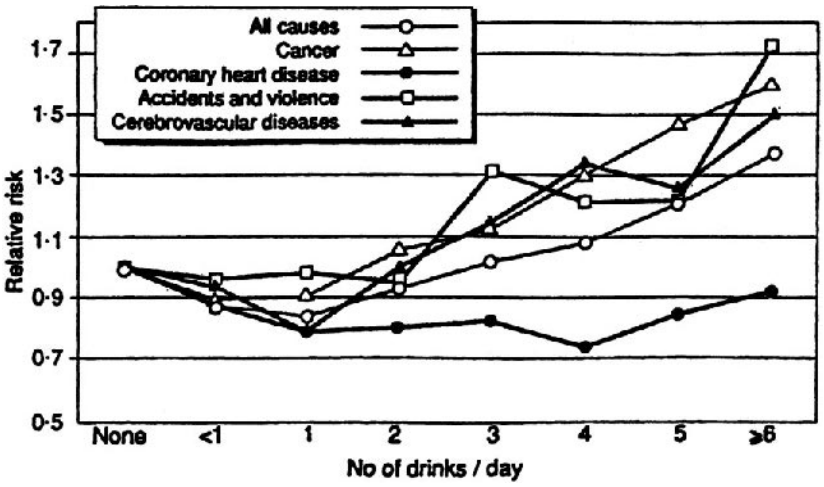

Figure 1 Relative risk of all-cause and cause-specific mortality in men by alcohol consumption category in drinks per day. (Adapted from ref. 8.)

for smoking habits (8). These findings are typical of those in prospective epidemiological studies. For CHD, a single drink a day was associated with a 20% reduction in CHD death. However, additional drinking did not result in any further benefit, and in heavier drinkers the risk began to move back toward unity. For cancer, the risk ratio was greater than unity for two drinks a day, and the risk of cancer continued to increase linearly with higher levels of drinking. For cerebrovascular disease (stroke), similar to CHD, there was a near 20% reduction in mortality risk at a single drink per day. However, unlike CHD, the risk for stroke quickly returned to unity at two drinks per day, and continued to increase with additional drinking. Finally, for fatal accidents and violence, the curve was flat for moderate (up to two drinks per day) drinking, and then increased sharply.

Total mortality includes each of the four cause-specific categories shown, as well as many other less common conditions. For total mortality, there was an approximately 15% reduction at one drink per day. At two drinks per day, however, total mortality was similar to that of nondrinkers, and increased linearly with each higher level of drinking.

The different findings for CHD and stroke closely reflect the known epidemiology of these outcomes. First, CHD is almost exclusively an atherosclerotic disease, with plaque formation in the coronary arteries followed by an acute thrombosis precipitating the clinical event. However, stroke can be atherothrombotic, embolic, or hemorrhagic. Atherothrombotic and embolic stroke make up

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more than three-fourths of all strokes in Western populations. Any protective effect of alcohol appears limited to atherothrombotic conditions, while, for example, hemorrhagic stroke increases linearly with higher levels of alcohol consumption (9).

The sharper increase in the mortality curve for stroke compared to coronary disease represents not only the heterogeneity of the stroke category, but also the particularly strong link between hypertension and the risk of stroke. Hypertension can be caused by higher levels of alcohol consumption (10,11), as discussed below.

A third major manifestation of atherothrombotic disease, peripheral arterial disease, has also been reported to be reduced in light-to-moderate drinkers (12).

Considerable evidence now is available that the above findings are consistent for nonfatal and for fatal CVD. Total mortality and morbidity seem to be minimized at about one drink per day. The upswing in total mortality beginning at two drinks per day and increasing strongly at higher levels of drinking reflects no further benefit for CHD, and an increasing risk of stroke, cancer, and accidents and violence.

Despite consistent findings in multiple studies, the findings for alcohol and CVD have been challenged as possibly artifactual, in that if the reference group of nondrinkers contained persons who had quit because of ill health from cardiovascular conditions, this could produce a spurious apparent CVD benefit for light-to-moderate drinkers (13). This issue has now been addressed in numerous prospective studies (14). To summarize, there is little evidence for such a ''sick quitter'' effect. Studies with careful exclusions of persons with known CVD at baseline still show a benefit for light-to-moderate drinkers, and never-drinkers have higher CVD rates than light-to-moderate drinkers (14).

III. ALCOHOL, LIPOPROTEINS, AND CVD

Alcohol has been associated with increased high-density-lipoprotein cholesterol (HDL-C) (15), and increases in the HDL-associated apolipoproteins, A-I and A-II (16,17). Is this increase in HDL-C real, or is it possibly due to other characteristics of light-to-moderate drinkers? The observational data have now been supplemented with several short-term experimental studies. A recent meta-analysis showed that on average, 30 mg of ethanol increased HDL-C 4.0 mg/dl, and increased Apo-A-I 8.8 mg/dl (18). Such an increase could be associated with a 10–20% reduction in CHD risk (19).

Low-density-lipoprotein cholesterol (LDL-C) has been reported to be lower in drinkers (15), but one study reported such an effect for women but not for men (20). To date, experimental data are inadequate for estimation of an average effect (18).

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Table 1 Alcohol, HDL, and CHD/CVD in Four Epidemiological Studies

	LRC study	Honolulu study	MRFIT study	Boston area study
Study type	Cohort	Cohort	Cohort	Case-control
Baseline status	No CHD	No CVD	No CHD	MI/controls
End-point	CVD death	Total CHD	CHD death	Nonfatal MI
Events/subjects	130/4105	124/1768	190/1688	340/680
Relative risk for alcohol				
Alcohol	0.80	0.83	0.89	0.60
+HDL	0.91	0.91	0.94	0.84
%Δ in alcohol coeff.	55%	47%	45%	60%

Source: Adapted from refs. 21–24.

We have used sequential multivariate models in two prospective population studies to see if the data were consistent with a protective effect of alcohol through increased HDL-C. We reasoned that multivariate models with CVD or CHD as the outcome variable and alcohol as the predictor variable would show a smaller effect size for alcohol if HDL-C were simultaneously considered in the model as a covariate. In the Lipid Research Clinics Prevalence Follow-up Study, we showed that about 55% of alcohol's effect appeared to be mediated through increased HDL-C (21). In the Honolulu Heart Study, using quite similar analyses we found this figure to be 47% (22). Table 1 shows the findings from these two studies, as well as findings from similar analyses in two other studies, the MRFIT cohort study (23) and a case-control study (24). The percentage effect that alcohol appears to have through increased HDL-C varied in a rather narrow range, from 45 to 60%, suggesting that about half of alcohol's protective effect for CVD might be mediated through increased HDL-C.

If HDL-C accounts for about half of alcohol's apparent benefit, what might account for the other half? Potential additional pathways include thrombotic (25,26) and fibrinolytic (27–29) factors, as well as insulin resistance (30–33). Pathway analyses similar to those for HDL-C above for these factors have not to date been available from population studies. However, meta-analyses of experimental studies show statistical significance only for increased plasminogen (18).

IV. ALCOHOL, BLOOD PRESSURE, AND CVD

As noted earlier, moderate and heavier drinking is associated with increased blood pressure, and this finding has been confirmed in experimental studies (34).

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To determine whether alcohol-associated higher levels of blood pressure in populations can be directly linked to CVD, we used multivariate models with again alcohol as the independent variable and CVD as the dependent variable, but this time with systolic blood pressure (SBP) as the covariate. We reasoned that if alcohol increased risk through increased SBP, models with SBP as a covariate would show greater protection for alcohol. We obtained exactly this result in both the Lipid Research Clinics Prevalence Follow-up Study (35) and the Honolulu Heart Study (22).

V. THE FRENCH PARADOX

The ''French Paradox'' refers to the relatively low rate of CHD mortality in France despite a diet which, because of its typically Western high saturated fat and cholesterol content, should be atherogenic. Ecological studies, where the unit of analysis is typically an entire country as opposed to an individual, have identified wine consumption as a strong inverse correlate of CHD risk in developed countries (36–39). Ecological studies need to be interpreted with caution because the analysis is not at the individual level; i.e., there is no direct linkage between an individual who consumes wine, or with any other specific dietary intake for that person, and whether or not they develop disease. Conversely, an advantage of ecological studies is that there are no nonrespondents—data for a country essentially include the entire population. Table 2 is adapted from an ecological study we published on the French Paradox question (39). We used United Nations Food and Agriculture Organization (FAO) consumption data and World Health Organization mortality data from 21 developed countries, and supplemented FAO alcohol data with data from the Finnish Foundation for Alcohol Studies and the Alcohol Research Foundation in Canada. We analyzed data for four discrete time periods, 1965, 1970, 1980, and 1988. Table 2 includes data from 1980 and 1988, and looks at the multivariate association of wine ethanol, beer ethanol, spirits ethanol, each in units of liters per capita per annum, and animal fat, vegetables, and fruit, each in units % Kcal., with both CHD mortality and total mortality in men and women aged 35–74. Both coefficients where the p value was significant (<0.05) or suggestive (>0.05, <0.15) are in italics. For CHD mortality in 1980, animal fat appeared to be positively related, while both wine ethanol and fruit appeared protective. For total mortality, which was not evaluated by the three earlier ecological studies (36–38), the findings were quite different. No alcoholic beverage showed benefit; only fruit was protective for total mortality. The data here underscore the findings from the prospective studies; populations include not just light-to-moderate drinkers, who may have an overall mortality benefit, but heavier drinkers at increased risk of total mortality from total CVD [including alcohol-aggravated arrhythmias (40) and cardiomyopathy (41)], cancer, cirrhosis, and accidents and violence.

Table 2 Multivariate Analysis of Dietary Items and Coronary Heart Disease (CHD) and Total Mortality, Men and Women Aged 35–74, 1980 and 1988

	CHD mortality				Total mortality			
	1980		1988		1980		1988	
	Coeff	*p* value	Coeff	*p* value	Coeff	*p* value	Coeff	*p* value
Wine ethanol	*INV*	*<.01*	*INV*	*.12*	POS	NS	INV	NS
Beer ethanol	INV	NS	POS	NS	*POS*	*.01*	*POS*	*.05*
Spirits ethanol	INV	NS	INV	NS	INV	NS	POS	NS
Animal fat, % kcal	*POS*	*.14*	POS	NS	INV	NS	INV	NS
Vegetables, % kcal	POS	NS	INV	NS	INV	NS	INV	NS
Fruit, % kcal	*INV*	*.08*	*INV*	*.06*	*INV*	*.03*	*INV*	*.05*

Source: Adapted from ref. 39.

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VI. THOSE WHO MAY NOT BENEFIT

A. Women

Women achieve a higher level of blood ethanol than men for a given amount of alcohol consumed (42). Also, alcohol is a dose-dependent risk factor for breast cancer (43). Thus, the risk-benefit ratio is less favorable in women than in men. Figure 2 shows results of a meta-analysis of 14 studies for the association of alcohol consumption and all-cause mortality, separately in men and women (44). Note the smaller maximum benefit and earlier upswing in risk in women compared to men.

B. Persons at Low Risk of CVD

It would be difficult indeed for alcohol or any other potentially protective factor for CVD to show benefit in persons who were unlikely to develop CVD. In the British Regional Heart Study, men at very low risk of CVD showed no benefit from any level of alcohol intake (45). In women in the Nurses Health Study, women free of CVD risk factors actually showed a significantly increased mortality risk with moderate drinking (46).

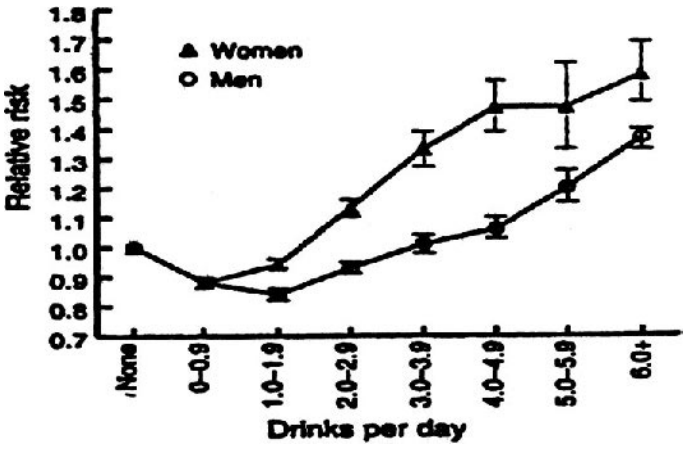

Figure 2 Relative risk of all-cause mortality in men and women by alcohol consumption in drinks per day—a pooled analysis of 14 studies. (Adapted from ref. 44.)

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C. The Young

No benefit of alcohol for mortality was found for those younger than 60 years in a large health plan in the United States (47), and the Nurses Health Study suggested a mortality hazard for women aged less than 50 years who were moderate drinkers (46). The hazard of drinking in younger persons is strikingly illustrated by evaluating, instead of morbidity or mortality rates, potential years of life lost (PYLL) as an outcome variable.

Figure 3 shows cause-specific contributions to PYLL before the age of 75 in men (48). Note that although CHD is in first place, motor vehicle crashes and suicide combined easily exceed CHD, because these conditions are common causes of death at younger ages. Motor vehicle crashes and suicide are strongly linked to alcohol consumption. Figure 4 shows similar data for women (48). Here, the data are even more problematic. Breast cancer, which has been linked to alcohol consumption, is the single largest cause of PYLL before age 75, followed closely by motor vehicle crashes. PYLL analyses highlight the dangers of alcohol, which are less evident in studies of middle-aged and elderly volunteers.

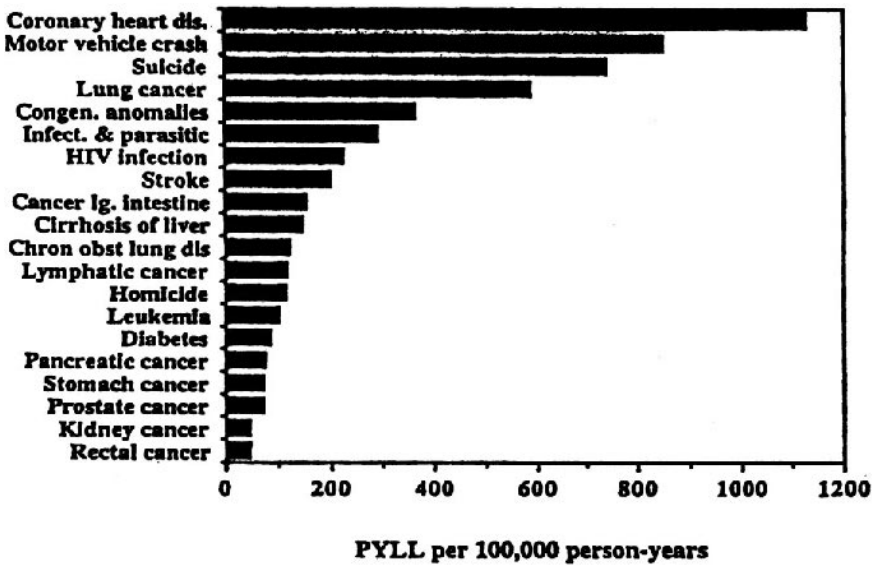

Figure 3 Rates of potential years of life lost before age 75, by cause of death, for men in Canada in 1990. (Adapted from ref. 48.)

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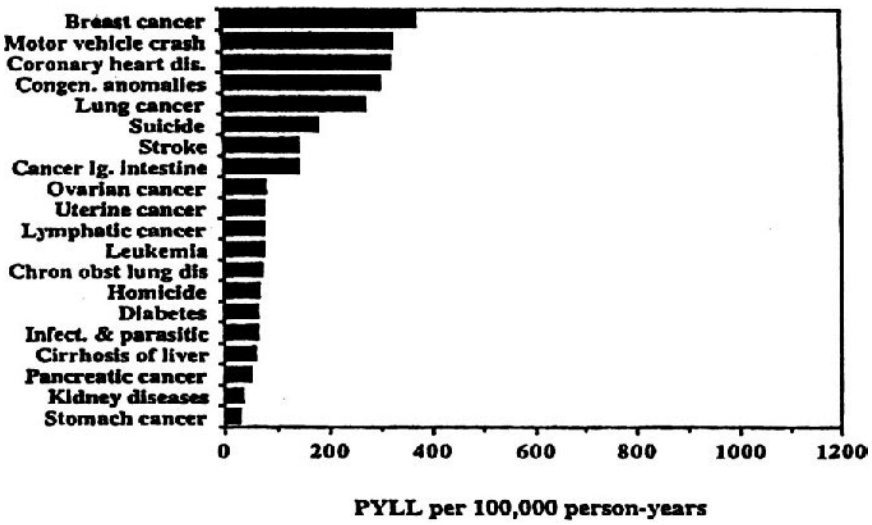

Figure 4 Rates of potential years of life lost before age 75, by cause of death, for women in Canada in 1990. (Adapted from ref. 48.)

VII. PUBLIC HEALTH CONSIDERATIONS

Candid discussion of the risks and benefits of alcohol by a health care practitioner with a responsible patient seems warranted in selected cases. But should there be a public health recommendation of moderate alcohol consumption for cardioprotection? Rose has shown in the Intersalt Study that the correlation coefficient between heavy drinking in a population and that population's mean alcohol consumption was 0.97 (49). Skog has shown that the percentage of a given population drinking twice the population mean is essentially a constant, 10–15%, irrespective of the mean population consumption, which can vary over 10-fold (50). This 10–15% group consumes half or more of the alcohol total, indicating the dependence of alcohol sales on heavier drinking. These population observations highlight the close link between the levels of consumption and the extent of abuse, and suggest that public health statements concerning the benefits of alcohol would lead to increased overall consumption and harm to the public's health.

It is interesting to consider that any favorable public health recommendation concerning alcohol would be equivalent to recommending alcohol as a pharmaceutical for cardioprotection. If alcohol were a new drug in the regulatory review phase, agencies would find in initial clinical trials a dose-related impair-

ment in coordination and cognition in all subjects, and eventual dependency in about 10% of those studied. Regardless of any cardioprotective effects, such a drug would clearly have a risk-benefit ratio that would disqualify it for licensure (51).

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