ADVANCES IN CANCER RESEARCH

VOLUME 41

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ADVANCES IN CANCER RESEARCH

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ADVANCES IN CANCER RESEARCH

VOLUME 41

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THE EPIDEMIOLOGY OF DIET AND CANCER

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I. Introduction

There is growing interest, both within the scientific community and the general public, in the relationship between diet and cancer etiology. Attesting to this interest is a rapidly expanding scientific literature as well as many articles in commonly read lay periodicals and newspapers. This literature, considered in sum, is complex and frequently contradictory and is thus confusing for the critical reader.

Much of the complexity in nutrition–cancer literature results from the multidisciplinary nature of the field. Nutritionists, clinical physicians, epidemiologists, biochemists, pathophysiologists, and a variety of other professionals, each sharing a common conviction that nutritional factors are of some consequence in cancer risk, must glean from the language and logic of each other's discipline those findings which integrate with their own. The complexity of the literature, however, is also reflective of the underlying

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complexity and uncertainty of the nutrition–cancer connection itself. At the present stage of incomplete understanding, we often must take large inferential leaps as we travel the road from incomplete biological understanding to personal dietary choices to public dietary policy.

Awareness of the possible relationship between diet and health is not new. Over 400 years before the birth of Christ, Hippocrates wrote (Adams, ed., 1939):

> this I know, moreover, that to the human body it makes a great difference whether the bread be fine or coarse; with or without the hull, whether mixed with much or little water, strongly wrought or scarcely at all, baked or raw. . . Whoever pays no attention to these things, or, paying attention, does not comprehend them, how can he understand the diseases which befall man? For, by everyone of these things, man is affected and changed this way or that, and the whole of his life is subjected to them, whether in health, convalescence, or disease. Nothing else, then, can be more important or more necessary to know than these things.

Despite Hippocrates' early interest in diet and health, research in the area of the nutritional factors in cancer etiology is still in its early stages of development, particularly investigations involving humans. Because of the advantages of experimental study designs, we know much more about the nutritional requirements and the implications of nutritional excesses and deficiencies in most experimental animals than we do about the human species. Understanding the relationship between nutritional factors and cancer risk in humans has been impeded until recently by the necessity to restrict research to observational rather than experimental studies. Within the framework of observational studies, there are many uncertainties, the most important of which in diet and cancer research is the valid measurement of diet.

In recent years there have been several assessments of the literature of nutritional factors and cancer etiology. These essays have varied in scope, detail, and emphasis. Table I lists a selected sampling of the more recent reviews on nutrition and cancer etiology, but it is by no means a complete listing. The purpose of this article is to review critically and in more detail than previously the various observational studies of diet and cancer etiology in humans. Because of this emphasis, we will mention only briefly the pertinent experimental research which generates or tests hypotheses for human epidemiologic research. The reader who wishes more detailed information regarding experimental or metabolic studies is referred to the appropriate references in Table I. Only nutritional factors which occur prior to the onset of cancer will be discussed. The many nutritional consequences of cancer, which are extremely important in the clinical management of the cancer patient, have been addressed by other authors (Van Eys, 1979) and will not be included in this review.

TABLE I
SELECTED ESSAYS ON THE RELATIONSHIP BETWEEN DIETARY FACTORS AND CANCER

Date	Principal author	Reference
	General	
1976	Alcantra	2
1976	Wynder	266
1977	Lowenfels	158
1977	Gori	70
1977	Wynder	267
1977	Modan	187
1978	Gori	71
1979	Wynder	268
1979	Doll	48
1979	Gori	72
1979	Shils	231
1979	Wells	261
1980	Alderson	3
1980	Werther	262
1980	Masek	174
1980	McMichael	176
1980	Reddy	221
1980	Graham	75
1980	Shils	232
1980	Miller	184
1981	Doll	49
1981	Correa	35
1981	Wynder	280
1981	Kritchevsky	152
1981	Graham	82
1981	Ellison	54
1981	Silverman	234
1982	McBean	175
1982	Newell	202
1982	Enstrom	59
	Focus on gastrointestinal cancer only	
1975	Wynder	265
1975	Graham	74
1976	Walker	252
1977	Reddy	219
1977	Kritchevsky	151
1978	Cummings	38
1979	Reddy	216
1979	Graham	81
1980	Zaridze	283

(*continued*)

TABLE I *(Continued)*

Date	Principal author	Reference
1980	Joossens	137
1980	Hill	106
1980	Cummings	39
1981	Joossens	138
1981	Reddy	217
1982	Burkett	22
1982	Weisburger	260
	Focus on breast cancer only	
1978	Hankin	97
1979	Dickerson	47
1980	Carroll	27
1982	de Waard	46
	Focus on experimental or metabolic studies only	
1975	Carroll	26
1975	Clayson	29
1979	Roe	223
1979	Weisburger	256
1979	Wattenberg	254
1980	Weisburger	259
1981	Stout	245
1981	Vitale	250
1981	Newberne	200
1982	Weisburger	260

We will first discuss the general methods of epidemiologic inquiry, focusing on considerations of study design which are specific to the study of nutrition and cancer and on problems of the measurement of diet. We will then review the evolution of observational studies of diet and cancer over the last 50 years and will integrate, site by site, the various human observational studies with pertinent findings from experimental studies, both in human and nonhuman systems. Finally, we will provide an overall assessment of the current "state-of-the-art" of epidemiologic inquiry in nutrition and cancer, and we will outline some strategies for the future.

There are a variety of means by which dietary factors might be related to cancer risk. Specific agents contained within foods, either naturally occurring (Hilker, 1980) such as mycotoxins, added during processing such as preservatives or colorants (Fairweather, 1980; Slaga, 1981), or unintentional contaminants such as pesticides may be either carcinogenic or anticar-

cinogenic (Conney, 1982). In these cases, food acts only as a vehicle by which agents affecting risk can be delivered to the individual. Cancer causation in these ways is a conceptually simple process and one which should be empirically testable given means of measurement of carcinogens in foods. It is quite clear that there are many hundreds of mutagens in foods commonly eaten by all of us with the potential of causing DNA structural changes which could result in tumor initiation (Sugimura, *et al.*, 1981). It is extremely important that we identify these potential carcinogens and limit their quantities in the foods that we eat, but specific carcinogenic contaminants of foods may not explain much of the overall variance in cancer incidence.

More important than simple foodborne contaminants may be nutritional factors themselves. Specific nutritional deficiencies and/or excesses may lead to somatic changes in body structure or function which increase the susceptibility to cancer development, either alone or in combination with other factors. It is clear from many experimental studies that dietary deficiencies of such nutrients as vitamins or inorganic ions, as well as dietary excesses of fats or total calories, may create a variety of somatic changes which can impair proper function. The immune system is a very important bodily function not only in combating infectious disease but also perhaps in preventing cancer. There have been several reviews of the experimental evidence that a variety of alterations in immune function occur with dietary excesses and deficiencies (Axelrod, 1980; Good, 1981; Gross and Newberne, 1980; Hoffman-Goetz and Blackburn, 1981). The concept of cancer causation by dietary factors is consistent with the clinical observation that nutritional deficiencies and excesses can lead to many clinically apparent somatic changes. It is also consistent with the commonly held notion that "we are what we eat."

Either additives to foods or nutrients themselves could theoretically act to increase or decrease risk either at the initiation or the promotion stages of carcinogenesis. In animal and *in vitro* carcinogenesis literature much attention is focused on the activity of carcinogens according to the stage of their action (Farber, 1982). In the epidemiologic literature, on the other hand, we have not yet developed enough precision in our methods to be able to define the time during which dietary factors may be acting. Although our inference from animal experimental studies is that dietary factors often seem to be more active in the promotional stages of cancer development, there are many reasons to believe that they may also be important in initiation as well.

As both scientists and citizens, those of us interested in nutrition and cancer find ourselves in a difficult position. Despite considerable progress in our understanding of the possible relationship between nutrients and cancer etiology and several exciting leads for future research, we find ourselves still in the early stages of research in this field. Many hypotheses and, in particu-

lar, investigative methodologies are still in the developmental stage. Drawing firm inferences from existing data is therefore very difficult. Nonetheless, chronic degenerative diseases including cancer continue to take their toll. In most of the Western world, cancer ranks as the second leading cause of death. Thus there is understandably a large public demand for dietary "prescriptions" for cancer prevention. Some have searched for the "optimal diet" (Hegsted, 1979), while others have attempted to define a "prudent diet" (Wynder, 1976). In the absence of definitive scientific evidence, however, public demand often results in both superstitious behavior by the public (Darby, 1979; Young and Newberne, 1981) and the overinterpretation of inconclusive studies by the scientific community.

In epidemiologic inquiry, there are a number of pitfalls which make causal inference difficult to draw. Associations observed in epidemiologic studies may be a result of a number of factors, including measurement bias, sampling bias, confounding, and chance alone, particularly when multiple factors are investigated simultaneously. Causal inferences can be made only after integrating epidemiologic information with existing knowledge from other scientific disciplines, which is a very difficult process. The judgment as to whether to take or, more importantly, to recommend preventive action on the basis of inconclusive data is a very difficult and complex one. The Committee on Diet, Nutrition, and Cancer of the Assembly of Life Sciences of the National Research Council has been charged with the task of reviewing all scientific evidence, both epidemiologic and otherwise, related to nutrition and cancer risk. Based on this comprehensive review (1982), it was their judgment that there now exists sufficient evidence of a causal relationship between certain dietary factors and cancer risk to make some specific recommendations on diet alterations for the American public. Their recommendations, presented as "interim dietary guidelines" are as follows: (1) decrease fat intake to 30% of total calories, (2) include fruits, vegetables, and whole-grain cereals in the diet, (3) minimize consumption of foods preserved by salt curing or smoking, (4) attempt to minimize possibly carcinogenic nonnutritive additives to foods, (5) increase testing of foods for mutagenicity, and (6) minimize the consumption of alcohol.

These recommendations seem rational and modest enough and, fortunately, are consistent with coronary heart disease prevention and sound nutritional practices in general. Nevertheless, future research may suggest important exceptions even to these modest recommendations. Other more radical recommendations for dietary changes and nutrient supplementation which appear in the lay literature are more difficult to evaluate with regard to their risk/benefit ratio. What is becoming clear from animal experimental work is that the relationship between cancer risk and many nutrients is not a simple one. For example, some vitamins may inhibit cancers in conjunction

with some carcinogens yet promote them with others, or they may inhibit cancer in one organ and promote it in another. We need to conduct a great deal more research *in vitro*, *in vivo*, and in human population settings to shed light on such conflicting findings and to arrive at more definitive grounds for prescribing diets which can enhance the public health.

II. Methods of Inquiry

Nutritional factors in cancer etiology can be studied by epidemiologic methods, which are largely observational, or by experimental methods in the laboratory. Laboratory methodology involving animals and *in vitro* methods in nutrition and cancer research have both distinct advantages and disadvantages as compared to human observational research. In animal systems and *in vitro*, exposure to nutrients can be carefully controlled and the outcome can be precisely measured while the risk of cancer can be experimentally manipulated with known carcinogens. Many animal models have demonstrated strong relationships between nutrient intake and cancer risk. Likewise, *in vitro* systems, which more recently have included human cell culture studies, have more precisely defined the relationships between micronutrients and the morphology and function of the cell and many subcellular components. Particularly exciting and relevant to nutrition and cancer work is the growing capability of researchers to study nutrient effects on DNA and its associated proteins.

However, extrapolation from effects measured on cells grown in culture to the *in vivo* situation or from an animal model to man, or even from one species of rat to another, is often very difficult. Despite the many similarities in the various systems, it appears that the relationship between nutrients and cancer risk may be highly dependent on the specific metabolic milieu examined. Marked differences in the apparent effect of nutrient deficiencies or excesses from species to species, from organ system to organ system, and from situation to situation have been observed. Despite differences, however, there are similarities in results among animal and *in vitro* models and the human system, such that laboratory experiments are an essential component of the entire research effort in human carcinogenesis. Experimental research complements human observational research, both by uncovering new leads of inquiry and by confirming the biological importance of observed associations. Integrating findings in animal and *in vitro* experiments with human observations is an important step in the complex process of drawing valid inferences from the general body of knowledge of carcinogenesis.

Human epidemiologic studies of diet as a causal factor in cancer have been generally of four types: ecological studies, case–control studies, prospective studies, and intervention studies. In the sections which follow, we will

discuss the methodologic considerations of each type of study as it relates to diet and cancer research, and we will review selected previous studies as examples of the ways in which the study designs have been employed.

A. Ecological Studies

Ecological studies are inquiries in which cancer rates for defined populations are correlated with rates of suspected risk factors as measured in these same populations. Thus these studies are based on populations as the units of analysis. Ecological studies have been useful in generating hypotheses regarding possible human dietary factors in cancer etiology. Stavraky (1976) presented a review and critique of the role of ecological studies of disease. Although this discussion focuses specifically on colon cancer, the concepts can be generalized to most ecological studies of human cancer and diet. More recently, Morgenstern (1982) has reviewed the methodological and statistical problems encountered in ecological analysis.

Associations between cancer risk and dietary factors observed in such studies are very difficult to infer as being causal, both because of the heterogeneity of diets within any defined population and because of the problem of many potential confounding variables. As we correlate cancer rates and dietary factors for defined groups of individuals, we cannot account for what is often rather marked individual variation of diet within each group and must, by design, make the implicit assumption that everyone within each group eats the same diet in order to draw inferences about cancer causation for individuals. This may be, of course, an untenable assumption. The Health and Nutrition Examination Survey (HANES) (Department of Health, Education and Welfare, 1979) in the United States, for example, showed that there is considerable variation in the diet of Americans, as shown in Table II. Ecological studies may only by chance describe the specific diet of cancer patients.

In addition to the problem of heterogeneity of diet within a population, ecological studies also are often not able to account for important confounding variables. Because diets of countries often correlate very strongly with many other factors which may be directly or indirectly related to cancer risk, there is an immense problem of potential confounding variables in such studies. Any factor associated with the population which experiences high cancer rates might, in an ecological study design, be implicated as potentially causal. In breast cancer, for instance, where the rates are highest in developed, Western nations, ecological studies based on international statistics would likely find strong associations with factors such as the per capita use of hair dryers and aluminum foil, though there are no compelling reasons to presume that these factors are causal. In addition, there are also strong

TABLE II
NUTRIENT INTAKE BY PERCENTILES OF THE POPULATION AS MEASURED IN THE HANES SURVEY[a]

	Percentile of Population								
	5th	10th	20th	50th	75th	90th	95th	$\bar{X}$	SD
Daily protein (g)									
8813 males	36	46	62	84	114	153	179	93	45
11930 females	25	31	43	59	79	102	119	64	31
Daily vitamin A (IU)									
8813 males	801	1184	2057	3503	5951	9796	13770	5138	7245
11930 females	575	872	1548	2714	4781	8581	12625	4431	8016

[a] Ref. No. 45.

associations between various elements of diet. Cereal consumption tends to be negatively associated with meat consumption so that international correlational studies find positive correlations with cereal (Hakama and Saxen, 1967) and negative associations with meat (Gregor *et al.*, 1969). It is often difficult to be sure, on the basis of ecological studies alone, which dietary factor, if any, is the causally important one.

There is striking variability from country to country in the international mortality and incidence rates for cancers of the various sites (Doll and Waterhouse, 1970; Segi and Kurihara, 1972). This striking variation, in conjunction with the observation that migrants tend to adopt cancer risks of the area to which they moved (within one or two generations) and leave behind the cancer risks from their native land, has led to the general belief that most human cancers seem to be environmentally caused. The use of the word "environment" in this context simply means "nongenetic," rather than implying causation necessarily by factors commonly held to be environmental, such as air and water pollutants (Higginson, 1979; Higginson and Muir, 1979). Adverse health behavior such as cigarette smoking and unhealthy diets, as well as the complexities of the entire social–psychological fabric, would be considered environmental causes in this context.

Ecological studies have been conducted using countries as the units of analysis, regions within countries, or special populations such as migrant or ethnic groups. Table III summarizes selected previous studies of human cancer which have been based on an ecological design. This table is not an exhaustive enumeration of all such studies but represents the major studies which are most frequently cited, as well as studies which are representative of their type. Included in this category, for the purpose of this review, are

TABLE III

SELECTED EPIDEMIOLOGIC STUDIES OF DIETARY FACTORS AND CANCER BASED ON ECOLOGICAL DESIGNS

Date	Principal author	Cancer site(s) studied	Country	Reference number
		Studies based on international cancer and food statistics		
1967	Hakama	Stomach	Many	96
1969	Gregor	All GI	Many	86
1973	Drasar	Colon, breast	Many	52
1973	Shennan	Kidney	Many	230
1973	Irving	Colon	Many	129
1974	Howell	Many	Many	125
1975	Wogan	Liver	Many	264
1975	Armstrong	Many	Many	7
1977	Wynder	Many	Many	267
1978	Hems	Breast	Many	99
1979	Gray	Breast	Many	85
1979	Lui	Colon	Many	161
1981	van Rensburg	Esophagus	Many	249
		Studies based on cancer and food statistics for regions within a single country		
1971	Alpert	Liver	Uganda	4
1973	Peers	Liver	Kenya	208
1975	(Study group)	Esophagus	China	34
1975	Jansson	All GI	United States	133
1975	Armijo	Stomach	Chile	5
1975	Howell	Colon, rectum	United States	126
1975	Enstrom	Colon, rectum	United States	56
1976	Cuello	Stomach	Columbia	37
1976	Peers	Liver	Swaziland	209
1977	(Study group)	Esophagus	Iran	136
1977	Enstrom	Colon, rectum	United States	57
1978	Enig	Many	United States	55
1979	Hill	Colon	Hong Kong	109
1979	Gaskill	Breast	United States	68
1979	Bingham	Colon, rectum	Britain	11
1980	Pawlega	Breast	United States (Iowa)	206
1980	Rawson	Many	United States (Southwest)	215
1980	Yang	Esophagus	China	281
1980	Hems	Breast	Britain	100
1981	Armijo	Stomach	Chile	6
1981	Ziegler	Colon	United States	285
1982	Nagi	Stomach, esophagus	Japan	199

TABLE III (*Continued*)

Date	Principal author	Cancer site(s) studied	Country	Reference number
		Studies based on migrant populations		
1968	Haenszel	Many	Japan to United States	91
1980	McMichael	Many	Europe to Australia	177
1981	Locke	Many	Japan to United States	157
1981	Kolonel	Stomach	Japan to United States	148
		Studies based on special ethnic or religious groups within a single country		
1976	Lyon	Many	United States (Mormons)	163
1978	Lyon	Colon	United States (Mormons)	162
1980	Phillips	Many	United States (Seventh-Day Adventists)	211
1980	Enstrom	Many	United States (Mormons)	58
1980	Hinds	Many	United States (Hawaii)	110
1980	Lyon	Many	United States (Mormons)	164
1981	Kolonel	Many	United States (Hawaii)	149
1981	Kolonel	Many	United States (Hawaii)	150
1982	Kinlen	Many	Britain (Religious orders)	145
1982	Gardner	Many	United States (Mormons)	65
1982	Gardner	Many	United States (Mormons)	66

studies which correlate changes in cancer rates with dietary changes that occur in a single population over a period of time. Although such studies are in fact longitudinal studies, because of differences in the ascertainment of both independent and dependent variables over time within a country, the problems in interpretation are similar to those encountered in international correlational studies. In addition, included in this section are studies based

on ethnic groups and migrant populations, which are extremely important in studying the relative contribution of environmental as opposed to genetic factors in the etiology of cancer.

In international studies, typically cancer incidence and/or mortality rates are compared directly to per capita consumption of various foods or nutrients as measured either in special surveys or, more commonly, as inferred from routinely collected food production or consumption data. Armstrong and Doll (1975) presented a comprehensive correlational study of cancer incidence rates for 27 types of cancer in 23 countries and mortality rates for 14 types of cancer in 32 countries with a variety of dietary variables including whole foods and nutrients. This study serves as perhaps the stereotypic example of international correlational studies of its type, and is frequently cited to support various hypotheses relating diet to cancer. Studies of this type generally present simple correlation coefficients or, in some cases, multiple regression analyses to control for possible confounding variables. Other types of higher order statistical analysis have been attempted as well. Howell (1974) produced what was termed a "Westernization factor" of foods associated with cancers common to Western countries employing a factor-analysis approach.

Correlational studies conducted using regions of a single country as units of analysis, although based on somewhat less variation in cancer incidence and/or dietary factors as compared to international studies are nonetheless important because, within a single country, there is less likely to be significant variation in the ascertainment of either independent or dependent variables. The studies of the correlation of dietary aflatoxins and liver cancer within African countries (Alpert *et al.*, 1971; Peers and Linsell, 1973; Peers *et al.*, 1976) and esophageal cancer in China (Yang, 1980) are good examples of the strength of correlational studies when a specific plausible etiologic factor can be measured.

When there is no clearly identifiable specific carcinogen in the diet, however, the interpretation of the significance of observed correlations is more difficult. Several studies have been conducted within the United States comparing food consumption data by state or region with cancer incidence or mortailty. Enstrom (1975) concluded that U.S. regional data were not consistent with a strong correlation between beef and fat consumption and colorectal cancer risk. He based this on the observation that colorectal cancer rates are relatively low in the Western United States where a high amount of beef is consumed. In a study relating food demand based on food marketing data to breast cancer mortality, Gaskill *et al.* (1979) found a particularly strong association between milk demand and breast cancer. An interesting study conducted by the American Cancer Society and presented by

Howell (1975) was based on the interview in 1957 of 1 million Americans as part of a national survey of cancer risk factors. Food frequency data for 16 items were collected. Regional comparisons were made between the northern and southern regions of the United States, which differ significantly in colorectal cancer rates. Additionally, these one million respondents were subsequently followed up in a prospective study, which will be discussed later.

Several similar correlational studies within regions of other countries have been conducted. Bingham *et al.* (1979) compared large bowel cancer mortality rates for nine regions of Great Britain with survey dietary data. The strength of this study is that dietary intake was measured by survey techniques and therefore may have greater reliability than studies based on population food consumption data. This allowed for more detailed analysis of the nutrient content of the diet. A similar study by Hems (1980) examined rates of breast cancer and consumption of fats or sugar in regions of Great Britain. Armijo and Coulson (1975) studied the variability of gastric cancer mortality rates in various regions of Chile. Similarly, Cuello *et al.* (1976) studied gastric cancer rates in Colombia. Both studies focused on the association between potential nitrate exposure (as inferred from regional agricultural activity) or water (as inferred for regional mean water nitrate levels) and cancer rates. A follow-up study by Armijo *et al.* (1981), which was based on individuals as the units of exposure instead of on regional groups, yielded results in the opposite direction as observed in the ecological study, demonstrating that inferences based on ecological studies alone must be guarded. Correa *et al.* (1983) are continuing their ecologic studies of diet and gastric cancer in Colombia by comparing nutritional survey findings in villages where gastric cancer rates are high with those where they are lower.

In studies involving a single population over a period of time, or in migrant studies, there is often an element of internal control not present in more typical ecological studies. This makes causal inference a bit easier to draw because of fewer concerns regarding measurement bias and confounding variables. Even these studies, however, are plagued by the same uncertainties inherent in correlational studies based on international statistics. Means of measurement of both independent and dependent variables may change over time, and the populations studied have usually been migrating from one country to another. Hence, problems of noncomparability of international statistics are considerations in these studies as well. Studies of a single population over time are often constrained because of the lack of good quality data for the distant past. Enig *et al.* (1978), however, studied U.S. cancer mortality trends as related to animal and vegetable fat consumption, and Hems (1980) and Ingram (1981) studied the correlation in England of fat

and sugar consumption one to two decades earlier and subsequent breast cancer mortality. However, it is very difficult to conclude that any such correlations over time are due to causal factors.

Studies of migrant groups are extremely important in cancer epidemiology, particularly in diet and cancer, as people tend to change their diet rather significantly after migration. Kmet (1970) presented an excellent review of the role of migrant studies in cancer research. More recent essays on this topic have been written by Modan (1980) and Miller (1982). An important study by Haenszel and Kurihara (1968) compared the mortality of Japanese immigrants in the United States with that of Japanese in Japan and with other United States citizens. They noted a tendency of Japanese immigrants to adopt mortality risks similar to the United States and dissimilar to their native Japan, which suggests that environmental factors are more important in cancer risk than genetic factors for many cancer sites. Dietary factors were proposed as being perhaps of principal importance in this regard. McMichael *et al.* (1980) studied migrants to Australia and presented data which demonstrated a similar phenomenon for European immigrants to Australia.

Studies of racial, ethnic, and religious groups are particularly important in hypothesis generation. This is because these groups often differ significantly in their diet as compared to others of the same general population. In the United States, members of the Mormon Church and Seventh-Day Adventist Church have been found to have lower cancer rates than the remainder of the United States. Lyon *et al.* (1976) in Utah and Enstrom (1980) in California compared cancer incidence rates of Mormons to non-Mormons and hypothesized that health behavior resulting from religious proscription, such as less tobacco and alcohol use, and also perhaps from less meat consumption, might account for the lower rates observed in cancers of many sites. Two studies presented by Gardner and Lyon (1982a,b) further examine the relationship between Mormonism and low cancer risk by studying subsets of the Mormon population in Utah by church activity level. Philipps *et al.* (1980) showed lower cancer rates among members as compared to nonmembers of the Seventh-Day Adventist Church, a religious group which generally does not smoke and is lacto-ovo-vegetarian in diet. Within the Seventh-Day Adventists population, comparisons have been made for vegetarians as compared to nonvegetarians.

Kolonel in Hawaii has studied cancer patterns in Japan and the United States, including Japanese and United States whites in Hawaii (Kolonel *et al.*, 1981b,c). Annually, a 2% random sample of Hawaiian households is interviewed by the Hawaiian Department of Health. In a followup to these interviews, Kolonel added a food frequency history containing approximately 80 items. Correlations were then made by ethnicity (Japanese born

in Japan, Japanese born in Hawaii, whites born in Hawaii, or whites born in the United States) and diet of each of the four ethnic groups. Cancer incidence rates from the Hawaii Tumor Registry were then compared to typical diets of each of the four ethnic groups.

As can be seen from this brief review, a variety of approaches have been used in conducting correlational and ecological studies of nutritional factors in cancer. It is not likely that improved computing capabilities or new statistical procedures will add materially to our knowledge of cancer based on this type of study design. Perhaps use of data from special dietary surveys in conjunction with biological markers of nutrient intake or food contaminants will lead to more progress in this area in the future. Regardless, findings from ecologic investigations need corroboration from those of other types of inquiry.

B. Case–Control Studies

Ideally in human observational studies, instead of using groups of individuals as the units of analysis, one would prefer to use individuals themselves. In this way potential confounding variables can be accounted for, and there is no need to make untenable assumptions of homogeneity of diet within a population. Both case-control (retrospective) studies and cohort (prospective) studies of diet and cancer avoid these difficulties, yet they also introduce their own unique problems. Case–control studies, being more efficient than cohort studies in both time and cost, have been predominant in the literature. In these studies, a series of patients with a specified type of cancer is assembled, either from a single hospital, a group of hospitals, or a total community and are questioned with regard to their diet as well as other potentially important nondietary cancer risk factors. Similar questioning is then performed on a control group of individuals drawn from the same hospital or community but not affected with cancer. Direct comparisons of the reported diets of cases and controls can then be made. Potential confounding variables can be controlled for, either by design in the selection of control patients through matching or by using statistical control methods in the analysis. Although very efficient in design, this type of study is plagued by uncertainties regarding the representativeness of both the case and control groups and regarding the validity of the dietary measures.

Although the findings from ecological studies have generated many widely accepted hypotheses regarding nutritional risk factors for human cancer, the findings from case-control studies have often failed to confirm them. This has led some investigators to question the adequacy of retrospective study methods in diet and cancer. Whether the failure to confirm the strong associations suggested in ecologic studies is a result of the many confounding factors

which may be present in them but which are controlled in case-control studies, or whether it is a result of the inability of case-control studies to make sufficiently valid measurements of diet, is yet to be seen. Various explanations have been advanced, including both that case-control studies are conducted in populations in which diet is too homogeneous so that no case-control differences can be expected and also that diet cannot be measured because it is, in fact, too heterogeneous. The general pattern, nonetheless, which has emerged over the last 20 years is that case-control studies tend to demonstrate much weaker dietary effects on cancer risk than might be predicted from correlational studies alone. Table IV summarizes the case-control research in nutrition and cancer in the last 50 years.

The study by Stocks and Karn (1933) in England is prototypical of all subsequent case-control studies of diet and cancer. In this study, cancer cases from several English hospitals were matched to controls by age and sex, and interviews were conducted using the food frequency technique. Although much of the analysis was presented for multiple cancer sites considered together, it is interesting that important findings demonstrated by Stocks and Karn were replicated in later work. For instance, there was a negative association observed between cancer risk and consumption of several vegetables, including carrots, cauliflower, and cabbage, as well as for an index of vegetable consumption which was based on seven vegetables. In addition, in this early study, foods were classified according to their relative content of the various vitamins, including A, B, C, and D.

The conclusion of Stocks and Karn (1933) would not be out of place in the conclusion of almost any diet and cancer study report today. They stated that "further research, experimental and statistical, should be carried out to confirm or refute (these preliminary findings). . . . Important practical effects in the reduction of cancer incidence might conceivably be brought about by a modification of diets, or better still, some substance preventive of cancer might be isolated." In retrospect, it is disappointing that, over these subsequent 50 years, we have not managed to improve our understanding of the epidemiology of diet and human cancer more substantially. Table IV presents a listing of case-control studies which have included dietary factors. This is not an exhaustive list, but it includes the more important and frequently referenced studies, as well as studies which represent special approaches and techniques. Inquiries which are limited to alcohol consumption as the only nutrient measured are not included in this table.

Most of the early work done in the 1940s through 1960s focused on dietary factors in gastrointestinal cancer. These sites were certainly the most logical place to first look for dietary factors and cancer risk because of the close contact of the gastrointestinal mucosa with foods. Much of the early work was designed to search for specific foods which might increase risk. The

TABLE IV

SELECTED CASE-CONTROL STUDIES WHICH HAVE INCLUDED DIETARY FACTORS

Cancer site(s)	Year of publication	Principal author	Study location	Number of		Approximate number of food items measured	Statistically significant findings		Reference
				Cases	Controls		Risk factors	Protective factors	
Stomach	1946	Dunham	Chicago	40	40	6	None	None	53
	1957	Segi	Japan	2000	4000	NP[a]	Rice, soy sauce, "miso" soup, "sake"		228
	1957	Stocks	England	200	9000	37	Fried foods		242
	1960	Pernu	Finland	1606	1773	NP	Meat, animal fat		210
	1963	Wynder	New York City	154	154	16	None	None	277
			Japan	268	362	16	Hot foods		
			Iceland	48	82	16	None	None	
			Yugoslavia	51	55	16	None	None	
	1964	Acheson	England	100	200	48	None	None	1
	1964	Meisma	Netherlands	340	478	7	Bacon	Citrus fruits	178
	1966	Higginson	Kansas	93	1020	22	Cooked fats	None	102
	1967	Graham	Buffalo, New York	276	2200	33	Cabbage		77
	1967	Hirayama	Japan	454	454	NP	Salted foods	Milk	113
	1972	Haenszel	Hawaii	220	440	100	Pickled vegetables and salted fish	Raw vegetables	92

(*continued*)

TABLE IV (*Continued*)

Cancer site(s)	Year of publication	Principal author	Study location	Number of		Approximate number of food items measured	Statistically significant findings		Reference
				Cases	Controls		Risk factors	Protective factors	
	1973	Bjelke	Norway	228	1394	50		Vegetables and fruits	13
	1973	Bjelke	Minnesota	83	1657	50	Cereals, smoked fish	Vegetables, fruits	13
	1974	Modan	Israel	166	498	243	Starchy foods		189
	1976	Haenszel	Japan	783	1566	100		Lettuce, celery	94
Colon	1969	Wynder	Japan	69	307	20		Rice, fruit, milk	278
	1973	Haenszel	Hawaii	179	357	100	Legumes, starches		93
	1975	Modan	Israel	198	396	243		Fiber-containing foods	190
	1978	Graham	Buffalo, New York	256	783	33		Vegetables	80
	1980	Jain	Canada	348	542	NP	Fat		131
	1980	Sorenson	Utah	300	600	NP		Vitamin C	238
Rectum	1969	Wynder	Japan	88	307	20	None	None	278
	1978	Graham	Buffalo, New York	330	628	33	None	None	80
	1980	Jain	Canada	194	542	NP	None	None	131
Colon and rectum	1957	Stocks	England	300	9000	37	Beer		242
	1960	Pernu	Finland	666	1773	NP	None	None	210
	1966	Higginson	Kansas	340	1020	22	None	None	102
	1967	Wynder	New York City	791	309	15	None	None	272

	1973	Bjelke	Norway	278	1394	50		Vegetables	13
	1973	Bjelke	Minnesota	373	1657	50		Vegetables, fruits	13
	1978	Dales	San Francisco	99	280	89	High fat foods	High fiber foods	40
	1980	Haenszel	Japan	588	1176	NP	Cabbage		95
Mouth	1957	Wynder	New York City	659	439	5	None	None	274
	1977	Graham	Buffalo, New York	584	1222	33	Alcohol		79
Esophagus	1961	Wynder	New York City	150	150	5		Vegetables, milk	269
	1973	Bjelke	Minnesota	52	1657	50	Alcohol		13
	1974	deJong	Singapore	131	665	13	Hot beverages	Potatoes, bananas	44
	1979	Cook-Mazaffari	Iran	344	688	36		Raw vegetables, fruits	33
	1981	Mettlin	Buffalo, New York	147	264	33	Alcohol	Vitamin A index	182
	1981	Ziegler	Washington, D.C.	120	250	31		Fish, meats, vegetables, dairy products	285
Mouth and esophagus	1957	Wynder	Sweden	?	?	5	None	None	275
	1969	Martinez	Puerto Rico	400	1200	10	Hot coffee, spices, alcohol		173
All GI sites	1968	Paymaster	India	1441	1314	NP		Vegetarian diet with dairy products	207

(*continued*)

TABLE IV (*Continued*)

Cancer site(s)	Year of publication	Principal author	Study location	Number of		Approximate number of food items measured	Statistically significant findings		Reference
				Cases	Controls		Risk factors	Protective factors	
	1981	Modan	Israel	406	812	243		Foods containing β-carotene	191
Lung	1977	MacLennan	Singapore	245	NP	100		Green, leafy vegetables	166
	1979	Mettlin	Buffalo, New York	292	801	33		Vitamin A index	181
	1980	Gregor	England	100	173	10	Vitamin A (females)	Vitamin A (males)	87
Larynx	1956	Wynder	New York City	93	209	5	None	None	273
	1981	Graham	Buffalo, New York	374	381	33	Alcohol	Vitamin A index	76
Bladder	1963	Wynder	New York City	250	250	NP	None	None	276
	1971	Cole	Boston	468	498	(Coffee only)	Coffee		30
	1975	Simon	New England	135	390	(Coffee only)	Coffee		235

	1979	Mettlin	Buffalo, New York	569	1025	33		Vitamin A index	181
	1980	Howe	Canada	480	480	NP	Coffee		122
Breast	1978	Miller	Canada	400	400	NP	Fat		186
	1981	Lubin	Alberta	577	826	8	Beef, pork, sweet desserts		160
	1982	Graham	Buffalo, New York	2024	1463	33		Vitamin A	83
Liver	1982	Bulato-Jayne	Africa	90	90	NP	Alcohol and aflatoxin-containing foods		18
Rhabdomyosarcoma	1982	Grufferman	North Carolina	33	99	12	Organ meats, cured meats		89
Glioma	1982	Preston-Martin	Los Angeles	209	209	6			213
Many sites combined	1933	Stocks	England	462	435	20		Vegetables, milk	243
	1978	Smith	Boston	800	3433	(Vitamin pills only)		Vitamin A	237

[a] NP, not presented.

selection of foods to be investigated was often guided by observations of international or interethnic variation in cancer risk. The comparison of one study to the next becomes very difficult because various investigators have chosen very different lists of foods to measure. Almost universally, the technique of ascertainment of diet in case-control studies was by the food frequency method. Nonetheless, just as there is not a standard list of foods, there is no generally accepted standard for measuring food frequency. Category options range from a simple dichotomous "ever" or "never" to (more commonly) three to six frequency options based on usual weekly or monthly frequencies. The analysis may be presented by all food frequency levels measured, or more commonly, food frequency categories may be combined into larger categories, or sometimes, simply the extremes of frequency are compared.

The combining of frequency categories in the analysis presents another problem with making statistical inference. With no clear a priori rationale for the combination of food frequencies, the way in which the frequency categories are combined may tend to artificially inflate or deflate relative risk estimates. This problem is always encountered in collapsing data in categorical analysis. Stable estimates of risk must be based on an adequate number of cases in each category, especially the base (reference) category. Thus the use of the same categories in two different studies where numbers in the base in one are not adequate to give a stable estimate will yield questionable, noncomparable estimates. This is equally true for studies of different sites or of the same sites in populations with different dietary habits. Investigators should, when possible, present numbers of cases and controls in the various exposure categories as well as relative risk estimates, and they should be careful to have adequate numbers of cases in base (reference) categories. With no clear a priori rationale for category definition, using tertiles, quartiles, or quintiles of the study population distribution would seem logical.

The largest case-control study of stomach cancer and diet to date was conducted in Japan (Segi *et al.*, 1957). In this study, more than 2000 patients with stomach cancer were compared with 4000 controls that were recruited from over 400 health centers. All patients were interviewed regarding frequency of consumption of various food items as well as preference for various food items. Another massive study, conducted in Finland (Pernu, 1960), was based on over 1600 patients with stomach cancer and 666 patients with colorectal cancer. Although these studies were strong in numbers of patients, there were some serious problems with the procedures used for the selection of controls. These problems may have resulted in selection biases which make the interpretation of the results difficult.

Among the earliest case-control studies of diet and cancer conducted in the United States were those by Wynder in New York City. Although other

investigators focused their attention initially on cancer of the stomach and colorectum, Wynder and colleagues investigated first the dietary factors of the upper alimentary tract and larynx. The first report for laryngeal cancer in a series of case-control studies of various sites was presented in 1956 (Wynder *et al.*, 1956). It is not clear exactly how many food items were included, but the dietary history was apparently only a very small part of the entire interview schedule. The analysis was based on five general categories of food, with case-control comparisons being made for the percentage of cases as compared to hospital controls that were deficient in each of the five categories, though "deficiency" was not precisely defined.

Later, Wynder presented studies of oral cancer (Wynder *et al.*, 1957a), esophageal cancer (Wynder and Bross, 1961), and bladder cancer (Wynder *et al.*, 1963a) in New York City and cancer of the upper alimentary tract in Sweden (Wynder *et al.*, 1957b). These studies, based on the same dietary questions used in the laryngeal cancer study, found that there was no significant difference between cases and controls in the frequency of dietary deficiencies for each of the five food categories, except for esophageal cancer in which a deficiency of milk and vegetables was seen in cases. Generally, only the conclusions were given and very little data were presented. In a four-country study by Wynder *et al.* (1963b), dietary data for sets of cases and controls in New York City, Japan, Yugoslavia, and Iceland are presented. After the completion of several studies which generally failed to demonstrate the expected case-control differences in diet as measured, Wynder concluded that the retrospective method may not be adequate in nutrition and cancer research because "a retrospective study cannot obtain quantitative differences in respect to dietary intake" (Wynder *et al.*, 1974).

Acheson and Doll (1964) conducted an important case-control study of gastric cancer and diet in England. Although based on only 100 cases and 200 controls, the study was designed with careful matching and, importantly, was methodologically rigorous, containing a built-in validity component. The dietary interview was based on the food frequency technique, which also included information regarding the means of preparation of each food item, the temperature of the food as eaten, and characteristics of eating patterns. An important element in this study is the reinterview of 63 of the study subjects in order to assess the repeatability of the food frequency questions. Although repeatability was certainly not perfect, it did appear to be sufficiently high to allow the investigators to confidently place individuals within broad categories of food consumption.

Higginson (1966) conducted a study of gastrointestinal cancer as related to diet based on interviews of cases and controls in the State of Kansas. Ninety-three patients with stomach cancer and 340 patients with colorectal cancer along with 1020 controls were interviewed based on the food frequency

technique. Thirty-seven of the study subjects were reinterviewed in a reliability study. Although there was some variability in recall, similar to that observed by Acheson and Doll, the author's conclusion was that the reliability was "good enough for group data." Nonetheless, Higginson, finding no apparent dietary risk factors, was led to question, like Wynder, the role of retrospective studies in diet and cancer.

Stocks and Davies (1964) presented an ingenious study of the mineral content of soils in the gardens of individuals who had recently died from gastric cancer as compared to that found for individuals who had recently died from other nonmalignant causes. Zinc/copper ratios were high in the soil from gardens of cases as compared to controls, particularly if the gardens were vegetable gardens and if the individual had lived at the house for over 10 years. Although this was by no means a definitive study, it employed an interesting study design which includes both case-control and ecological components.

The first in a series of studies based on data collected at the Roswell Park Memorial Institute (RPMI) in Buffalo, New York, was conducted by Graham *et al.* (1967). During the years 1957 to 1965, all patients admitted to RPMI were interviewed at the time of their admission regarding a variety of potential cancer risk factors including diet. This dietary interview was based on the food frequency technique for 33 selected food items. On the average, the dietary interviews took approximately 15 min. The interviewer was unaware of the patient's suspected diagnosis or chief complaint at the time of the interview, and most of the patients were unaware of their own diagnosis, because most of the admissions were for diagnostic workup for possible cancer. Subsequent analyses have compared the recorded dietary history of groups of individuals with cancers at various sites as compared to a group of patients who were subsequently found not to have cancer and matched for various characteristics.

The analysis of the first study (Graham *et al.*, 1967) was based on a comparison between gastric cases and controls of the reported frequency of ingestion for specific food items. A reanalysis of these same data by Graham *et al.* in 1972, however, which matched controls to cases for ethnic background, showed an opposite pattern of the association between cabbage and risk. The second report in the series of studies based on the RPMI data concerned oral cancer in men (Graham *et al.*, 1977). This study focused mostly on alcohol, smoking, and dentition factors but showed no significant differences between cases and controls when comparing frequency of ingestion of specific food items. However, a subsequent reanalysis of these data (Marshall *et al.*, 1982) in which indices of vitamin A and vitamin C consumption were generated from the food frequency data in combination with food composition data showed significant vitamin A and vitamin C deficiencies in

the diets of cases as compared to those reported by controls, even when controlling for smoking and alcohol ingestion using multiple regression techniques. Thus it is important to consider reanalyses of previously collected data.

Many subsequent case-control studies have been conducted based on the RPMI data collected between 1957 and 1965. A study of colon cancer (Graham *et al.*, 1978) used individual food items as the units of comparison. Nutrient indices have been compared to cancer risk for lung (Mettlin *et al.*, 1979), bladder (Mettlin and Graham, 1979), larynx (Graham *et al.*, 1981), esophagus (Mettlin *et al.*, 1981), and breast (Modan *et al.*, 1974). Similarly, case–control studies have been completed on cancer of the prostate (Graham *et al.*, 1983), ovary (Byers *et al.*, 1983a), cervix (Marshall *et al.*, 1983), and uterus (J. Marshall, personal communication). Studies on lymphoma, leukemia, Hodgkin's Disease, and other more rare sites are planned to be conducted soon.

Bjelke (1971) reported a case-control study of gastric and colorectal cancer in Norway which was later followed by a similar study that included esophageal cancer cases in Minnesota (1973). Bjelke's study of gastrointestinal cancers in Norway and Minnesota contained elements of both ecological studies and case-control studies and, later, prospective as well. His approach to the epidemiologic investigation of these cancers in these populations stands as a classic example of a rational comprehensive approach. The case-control component of his investigation was based on food frequency interviews covering approximately 50 food items. The analysis was conducted by comparing both individual food items and nutrient indices between cases and controls.

Haenszel, building on earlier correlational studies of Japanese migrants, conducted a case-control study of stomach cancer (Haenszel *et al.*, 1972) and colon cancer (Haenszel *et al.*, 1973) in Japanese immigrants in Hawaii. The ascertainment of diet was by the food frequency technique. Though the specific number of food items was not present, it was stated as "over 100." Later, Haenszel attempted to replicate these case-control studies on Japanese in Japan (Haenszel *et al.*, 1976, 1980). However, even though the same study design and questionnaire were used, the results observed in Hawaii were not replicated in the Japanese studies. Haenszel speculated that the reason for the negative findings in Japan might be related to more homogeneity in the Japanese diet which would tend to make case-control studies of dietary factors less sensitive.

Modan presented case-control studies of diet and gastric cancer (Modan *et al.*, 1974) and colon cancer (Modan *et al.*, 1975) in Israel. These studies, though similar in design to previous studies of gastric cancer and diet, involved much more extensive questioning regarding diet. The food frequency technique was used for 243 separate food items. The large number of food items was necessitated apparently by the tremendous cultural diversity

found in Israel, which results in an extremely diverse diet. For analysis, the food items were grouped into similar types of foods. Seventy-three food items, for example, constituted the group of foods containing significant amounts of dietary fiber. Similarly, Dales *et al.* (1978) presented data based on a case-control study of colorectal cancer in Blacks in the San Francisco area. The food frequency technique was used to ascertain diet, based on 89 food items. In an analysis similar to that by Modan, Dales grouped foods into those containing high levels of specific nutrients, especially fat and fiber.

Two interesting studies were presented in 1978 which are unusual in design. Smith and Jick (1978) conducted a case-control study comparing the frequency of supplemental vitamin usage in cancer patients as compared to controls. Although not precisely a dietary study, in that no dietary history was ascertained, this study is of importance because of earlier suggestive evidence that foods containing vitamin A might be protective against cancer. Nomura *et al.* (1978) conducted a unique type of case-control study of breast cancer. This study is unusual in that the cancer patients were not interviewed. Instead, two sources of existing data were combined in order to make inferences about the probable diets of breast cancer cases. Dietary histories of men which had been earlier collected for a heart disease study were combined with data from the Hawaii Tumor Registry so that the dietary histories of 68 men whose wives developed breast cancer could be compared to those men whose wives did not develop breast cancer. The assumption that must be made here is that diets of husbands are adequate surrogate measures of diets of their wives. Subsequent methodological studies, however (Lee and Kolonel, 1982), have suggested that, although there are certain similarities in the diets of husbands and wives, there also may be marked differences as well.

Miller *et al.* (1978) conducted a case-control study of dietary factors as related to breast cancer risk in four regions of Canada. This study is of importance because of the methodological rigor with which it was conducted. Controls were matched to cases by age, sex, race, and neighborhood of residence. More importantly, the dietary histories were carefully collected, and several different measures of diet were made in order to assess the validity of the various methods. Details of the validation studies have been presented by Morgan *et al.* (1978). These studies included comparison of 24-hr recall, 4-day diet diaries, as well as diet histories taken by the food frequency method based on both the last two months and on the two-month period ending six months prior to interview. The detailed dietary histories collected by Miller have allowed for a detailed analysis of nutrient intake, including various subtypes of dietary fat.

Gregor *et al.* (1980) conducted a case-control study of lung cancer and diet in England. Frequency of ingestion of various food items both immediately

before symptoms as well as 20 years prior to diagnosis in addition to personal preferences for various foods were measured by interview. Vitamin indices constructed from these food frequency responses were then correlated with case status.

Jain *et al.* (1980) presented the results of a case-control study of diet and colorectal cancer in Calgary and Toronto. Colon and rectal cancer cases from a population-based registry were compared to neighborhood age- and sex-matched controls as well as hospitalized controls who had previously undergone abdominal operations for nonmalignant disease. Using the same questionnaire evaluated by Morgan, the frequency of ingestion of selected food items for cases both two months prior to diagnosis as well as for a two-month time period ending six months prior to diagnosis was compared to controls. Another Canadian study conducted with very different methodology was presented by Lubin *et al.* (1981). Women with newly diagnosed breast cancer in Alberta were questioned regarding diet based on the frequency of consumption of only eight food items. Their responses were then compared to those of controls selected as a random sample of Alberta women.

A study conducted by Ziegler *et al.* (1981b) based on esophageal cancer in Black men in the Washington, D.C., area is of methodological interest because cases were never interviewed. Case diets were reported by the "next of kin" for individuals who were recently deceased of esophageal cancer. Diets for the control group were ascertained in a similar way by interviewing next of kin of men who had recently died of nonmalignant conditions. The usual diet prior to 1974 was specified. Although this methodology is attractive in diet and cancer research in that it may be a way to collect dietary information on patients who have rapidly fatal disease, or whose disease causes such disability that they cannot be interviewed, the reliability of ascertaining diet from the surviving next of kin must be better tested before this technique will gain wide acceptance.

A case-control study of primary liver cancer presented by Bulato-Jayne *et al.* (1982) was based on interviews conducted by a single nutritionist who was blinded as to the diagnoses of the patients. Although details of the dietary histories were not given, the interviews were said to last 1.5 hr and included details of dietary changes throughout the subject's life. Analysis was based on the estimated level of aflatoxin exposure in the diet as reported.

There are many case-control studies of cancer currently underway which include dietary measures. Table V summarizes these studies. Of particular importance because of their strong emphasis on dietary measures are the studies by Kolonel, Jain, Zaridze, Graham, Nomura, and Hinds. These newer studies generally use population-based case and control selection procedures and involve detailed dietary histories. In most cases, reliability studies are a part of the research design.

TABLE V
CASE-CONTROL STUDIES NOW IN PROGRESS WHICH INCLUDE THE STUDY OF DIETARY FACTORS IN CANCER ETIOLOGY[a]

Tumor type	Principal investigator	Location	Projected (approximate) number of cases:controls
Colorectal cancer	Potter	Australia	400:800
	Kune	Australia	750:1500
	Cornee	France	250:250
	Czygan	Germany	1000:1000
	Manouses	Greece	100:200
	Graham	United States (Buffalo)	900:900
Colorectal polyps	Zaridze	France	300:300
	Cornee	France	NP[b]
	Lambert	France	100:100
Stomach cancer	Frentzel-Beyme	Germany	NP
	Jain	Canada	400:400
	Kuratsune	Japan	150:300
	Juhosz	Hungary	NP
	Crespi	Italy	NP
	Tosi	Italy	NP
	Amadori	Italy	50:100
	Serrao	Portugal	NP
	Graham	United States (Buffalo)	250:250
	Correa	United States (Louisiana)	NP
Pancreatic cancer	Kune	Australia	150:300
	Durbec	France	150:150
	Norell	Sweden	100:100
	Gordis	United States (Baltimore)	200:400
	Mack	United States (Los Angeles)	NP
Kidney/bladder cancer	Correa	United States (Louisiana)	NP
	Knudsen	Denmark	200:600
	Jussawalla	India	950:NP
	MacMahon	United States (Boston)	350:700
	Connolly	Canada (Toronto)	350:700
	Jensen	Denmark	500:1000
	Vahlensieck	Germany	NP

TABLE V *(Continued)*

Tumor type	Principal investigator	Location	Projected (approximate) number of cases:controls
	Mack	United States (Los Angeles)	NP
Prostate cancer	Zaridze	Netherlands and Japan	150:NP
	Kolonel	United States (Hawaii)	500:1000
Lung/laryngeal cancer	Buffler	United States (Texas)	700:NP
	Koo	Hong Kong	200:200
	Hinds	United States (Hawaii)	500:1000
	Lynch	United States (Miami)	NP
	Jindol	India	NP
	Lopez	United States (Louisiana)	NP
	Zatonski	Poland	NP
	Graham	United States (Buffalo)	?
	Correa	United States (Louisiana)	NP
Breast cancer	Burns	Canada (Alberta)	NP
	Hislop	Canada (British Columbia)	1500:4500
	Marabini	Italy	200:400
	Socerdote de Lustig	Argentina	200:200
	Plesko	Czechoslovakia	1200:NP
	Le	France	1000:2000
	Modan	Israel	800:1600
	Kaloche	Brazil	NP
	Paffenbarger	United States (San Francisco)	2500:2500
	Nomura	United States (Hawaii)	600:600
Oral cancer	Notani	India	900:NP
	Fischman	United States (New York)	NP
	Graham	United States (Buffalo)	250:250

(continued)

TABLE V (*Continued*)

Tumor type	Principal investigator	Location	Projected (approximate) number of cases:controls
Penile cancer	Knudsen	Denmark	200:600
	Jain	India	200:NP
Malignant melanoma	Armstrong	Australia	500:NP
	Nathanson	United States (New York)	500:NP
All gastrointestinal cancer	Tuyns	France and Belgium	NP
Liver cancer	Mack	United States (Los Angeles)	NP
Endometrial cancer	Boonzanit	Thailand	150:NP
Nasopharyngeal cancer	Armstrong	United States (Hawaii)	125:125
Multiple myeloma	Grufferman	United States (North Carolina)	130:130
Miscellaneous cancer sites or sites not specified	Balakishnan	India	3700:2300
	Kaloche	Brazil	NP
Cervical dysplasia	Romney	United States (New York City)	NP

[a] Ref. No. 197.
[b] NP, not presented.

As case-control studies are based on the comparison of cases with controls, considerations relative to the selection of the control group are very important if we are to make inferences that case-control differences are due to attributes of the cases. In general, controls should be representative of the general population from which the cases come and not selected in a way which may be biased by risk factors which are to be measured. Ideally, therefore, cases should be a representative sample of all cases in a defined population and controls a representative sample of all noncases in the same population. This has not always been the situation, however, in case-control studies. Often, particularly in earlier studies, case-control studies were based on a series of cases from a single practice or a single hospital, and the control group was composed of noncases who happened to attend the same practice or the same hospital. With the use of such hospital controls, there may be selection biases which could either inflate or deflate relative risk

estimates, depending on the risk factor being studied. A classic example of this problem is the assessment of risk of lung cancer due to cigarettes using hospital controls. Such a control group would likely include many patients with other smoking-related diseases such as emphysema and heart disease, thus leading to an underestimation of the risk of smoking. Because nutritional factors may be related to many other chronic diseases, a similar problem may be present in studies of nutrition and cancer based on hospital controls. Some of the early case-control studies even included other cancer cases as controls, though we now suspect that different types of cancer may share similar risk factors.

An alternative means of selection of controls would be to randomly select nondiseased individuals from the general population from which the cases are derived. This method of control selection is being commonly used in more recent case-control studies. Controls can be randomly selected from community registries or by random digit telephone dialing (assuming telephones are nearly universally owned in the population). Because cultural and socioeconomic factors are associated both with diet and with cancer, it is also a frequent practice in more recent studies to select a control for each case from the general neighborhood of that case. In this way, neighborhood-related socioeconomic and cultural factors can be matched in the study design and are thus not likely to confound the diet–cancer associations observed.

Over the 50 years since the initial case-control studies of Stocks and Karn in 1933, case-control studies of diet and cancer have been conducted in several countries, have used several different means of ascertaining diet, and have examined many types of cancer. The marked variation in study design considerations, including the selection of controls, the construction of the dietary history, and the means of analysis make direct comparisons between the various studies virtually impossible, however. What is sorely needed in this area of research is for researchers to adopt more uniform means of ascertaining dietary history so that the results of studies can be more directly compared. Before this can be done, however, much more basic work is needed on the reliability of dietary histories. Eventually, after a more universally acceptable interview instrument or set of instruments is developed, researchers interested in this area will be able to more easily share ideas and learn lessons from the similarities and differences in the various studies.

C. Prospective Studies

Prospective studies are inquiries in which measures of potential risk factors are made on a large group of healthy individuals. Subsequent disease

rates then are observed among those with various levels of exposure to those factors over a period of years. In nutrition and cancer research, prospective studies involve questioning a large group of individuals regarding their usual diets, then comparing subsequent cancer incidence rates in those who report various levels of nutrient intake. Such studies would seem to be theoretically ideal, because there is no need in these studies to attempt to make retrospective measures of diet, and therefore no concern about the possibility of biased recall. Prospective studies are, however, plagued by their own inherent problems. These studies necessitate very large numbers of individuals who must be followed over a relatively long period of time in order for enough incident cancer cases to be collected to make confident causal inferences. Analyses even for large cohorts must often be limited to the more common types of cancer because of a small observed number of cases. Making careful and reliable measures of dietary intake on such a large group of individuals and adequately following up the entire group over a period of many years is methodologically very difficult and economically very expensive. In addition, nondietary measurements which may be potential confounders must also be measured, such as socioeconomic status and alcohol or cigarette use. Such possibly important confounding variables can be controlled statistically in the analysis only if they are properly measured at the beginning of the prospective study. Further, changes in diet during the study period may be difficult to account for. It is not surprising, therefore, that there have been very few prospective studies conducted on nutrition and cancer.

Despite their rarity, prospective studies of diet and cancer are extremely useful in order to further test hypotheses generated from case-control studies. By far the largest prospective study of diet and cancer has been conducted in Japan (Hirayama, 1979a,b; Hirayama, 1981). As part of the 1965 Japanese census, over 260,000 Japanese adults were given questionnaires which included questions regarding the frequency of intake of selected dietary items. This cohort is continuing to be followed for cancer incidence and mortality. The strength of these data and the importance of this cohort rests not so much in the completeness of dietary data which was, in fact, quite sketchy, but more in the tremendous size of the cohort and in the ability of the Japanese to accomplish nearly 100% followup.

In the United States in 1959, the American Cancer Society sent survey questionnaires to approximately 1 million Americans inquiring about a variety of potential cancer risk factors, including frequency of ingestion of 16 different food items. Although based on large numbers, this study, as compared to the Japanese study, is based on only a convenience sample of respondents and has less complete followup. There are no plans to continue

followup on this cohort subsequent to the report by Howell (1975). In 1982, however, the American Cancer Society embarked on its second effort to determine risk factors for 1 million Americans and to subsequently follow this group over time. Included in this effort, which is based on self-administered questionnaires by self-selected individuals, is a brief dietary history.

Bjelke (1975) conducted a five-year followup study of 8278 Norwegian males who had been mailed a dietary questionnaire which ascertained the frequency of approximately 50 selected food items in the diet. Followup on this cohort is continuing. Studies by Wald *et al.* (1980), Kark *et al.* (1981), and Shekelle *et al.* (1981) have more recently been reported. These studies report the assay of retinol in frozen serum specimens stored over 2 to 19 years. These specimens were obtained as a part of prospective studies originally designed to investigate risk factors in coronary artery disease. Serum retinol levels of cohort members who subsequently developed lung cancer were compared to those of members who subsequently did not develop lung cancer. Serum cholesterol and subsequent cancer risk has similarly been examined in prospective studies (Kark *et al.*, 1980; Feinleib, 1981; Sorlie and Feinleib, 1982; Williams *et al.*, 1981).

Historical cohort studies can be conducted based on cohorts of individuals who have previously had nutritional measures made for other purposes, as, for example, was the case with the retinol and cholesterol studies noted above. Old dietary surveys and studies should be found and converted into historical cohort studies. This would be a very economical way to conduct a prospective study and requires only a followup for cancer incidence or vital status for the previously studied group. Such studies are currently underway in many countries. Table VI summarizes selected on-going prospective studies of diet and cancer. Other nutritionists and researchers who are aware of previously interviewed cohorts should seriously collaborating with an epidemiologist to convert these cohorts into additional prospective studies of diet and cancer.

In addition, however, the collection of data on new cohorts must be considered. We have recently completed collection of dietary data based on the frequency of intake of approximately 50 food items for over 58,000 individuals in New York State. These individuals were selected from a list of people who have resided at the same residence for over 18 years. The dietary histories were ascertained by mailed questionnaires. Validity and repeatability studies are now in progress. It is our plan to follow these 58,000 individuals through the New York State Tumor Registry for subsequent cancer incidence. R. L. Phillips will be following a similar-sized cohort of Seventh-Day Adventists in California.

An interesting group collected by Burr and Sweetnam (1982) in England

TABLE VI
PROSPECTIVE STUDIES NOW IN PROGRESS WHICH INCLUDE THE STUDY OF DIETARY FACTORS IN CANCER ETIOLOGY[a]

Principal investigator	Location	Cohort being studied (*N*)	Year in which cohort was defined (comments)
Hirayama	Japan	Population sample (265,000)	1965
Bjelke	Norway and United States	Population sample (20,000)	1963
Kuratsune	Japan	Seventh-Day Adventists (10,000)	1975
Burr	England	Health food store customers (10,000)	1974
Phillips	United States (California)	Seventh-Day Adventists (60,000)	1978
Enstrom	United States (California)	Mormons (10,000)	1979
Kolonel	United States (Hawaii)	Population sample (45,000)	1975–1981
Graham	United States (Buffalo)	Population sample (58,000)	1980
Wigle	Canada	Population sample (20,000)	1970–1972
Ogawa	Japan	Civil service employees (16,000)	1970
Speizer	United States	Nurses (122,000)	1972
Howe	Canada	Population sample (14,000)	1972
Hill	England	Population sample (6,000)	1975 (fecal analysis performed)
Rang	England	Individuals postcolectomy (2500)	NP[b]
Tu	China	Population sample (13,000)	1979 (HCC only)

[a] Ref. No. 197.
[b] NP, not presented.

consists of nearly 11,000 customers of health food shops who filled out a food frequency questionnaire in 1974. This cohort will be followed in the future and, although a self-selected sample, may generate interesting hypotheses regarding nutrition and cancer, as the group is presumably heavily weighted with "health food" consumers.

D. Intervention Studies

Intervention studies are of experimental design. The prototypical intervention study is the randomized controlled trial in which study subjects are randomly assigned to treatment and control groups and then followed to observe differences in disease rates. This type of study presents numerous problems and therefore has not been common in the literature. First, there has to be certainty that the diet change is not pathogenic. Second, it is very difficult to assemble a sufficiently large group of individuals who are willing to have their diets modified and who are able to adhere to the modified diet. The simplest way in which to modify a diet in such a study design would be to add to the diet certain nutrients, such as vitamins or minerals, in a capsule form. This would eliminate the need for the individual to make changes in food eating habits, and it would allow for a blinded placebo group to be included in the study as well. Such an intervention study is, in fact, now underway. U.S. physicians who are smokers have been asked to volunteer for an intervention trial in which randomly half are administered β-carotene and half are administered aspirin. Both groups will be followed over time to determine their incidence of both cancer and cardiovascular disease. It is hypothesized that β-carotene will reduce the incidence of cancer while aspirin may reduce the incidence of myocardial infarction and/or cerebrovascular accidents.

A small-scale dietary intervention trial based on dietary change has been reported by Bright-See in Toronto (1982). In this trial, approximately 100 women with fibrocystic disease of the breast have been randomized into two dietary intervention groups. One group was counseled regarding a well-balanced diet with the usual amount of fat intake, while another group was counseled to follow a low fat diet. These two groups will be followed over time to determine both fibrocystic disease activity and, eventually, breast cancer incidence.

There is considerable interest in conducting randomized control trials of cancer chemoprevention in high-risk individuals. The National Cancer Institute is currently placing heavy emphasis on the development of such chemoprevention trials. These trials will likely include dietary modifications and/or nutritional supplements in randomized, controlled studies of individuals at high risk of cancer, such as those who have had previous colon polypectomies, minimal colon cancers, localized breast cancers, localized bladder cancers, or individuals at high risk of cancers because of family history or occupational exposures. Although the rationale for embarking on intervention trials seem compelling based on concurrent findings of animal, *in vitro*, and epidemiological research (Nigro, 1982), we must realize that,

though experimental study designs have their strengths, they may also have their weaknesses, particularly when the intervention is based on the modification of human behavior. The rather disappointing results of the MRFIT trial should serve as a reminder of the limitations of these trials. In the meantime, as we proceed with intervention trials, there is also a need to improve our ability to generate hypotheses and to make inferences about the diet–cancer connection in free-standing populations of people who are eating varied diets.

E. Metabolic Epidemiology

A relatively new type of epidemiologic study design which is becoming more widely used in nutrition and cancer epidemiology is "metabolic epidemiology." This approach combines many of the features of laboratory, correlational, and case-control study designs. Whereas the usual role of epidemiology is studying the distribution and determinants of disease, in metabolic epidemiology the distribution and determinants of various metabolic markers that are thought to be etiologically associated with disease are measured. Typically, a biological marker such as a serum hormone level or fecal biochemical characteristics are compared between two groups of individuals who are sampled from two separate populations that experience different rates of cancer. In this way, when there is reason to believe that the metabolic marker may be etiologically related to cancer risk, one is able to more sensitively measure preclinical effects of independent variables such as diet as well as to provide evidence regarding causal mechanisms of risk enhancement or reduction. This approach is a good example of the way in which epidemiologic methodology can be integrated with laboratory methodology. Such studies can also be of use to identify populations on which to devise specific dietary interventions, the effect of which can be assessed metabolically rather than waiting for cancer to appear or not. Despite its importance, metabolic epidemiology also has its limitations. Often, because of cost and compliance constraints, metabolic studies are conducted on only a very small number of individuals who are not randomly chosen. Thus the ability of the findings to be generalized for the larger reference population is often a problem in interpretation. Also, causal inferences regarding cancer risk can only be made insofar as we completely understand the precise relationships between the biological marker being studied and cancer. When these relationships are not fully understood, as is often the case, causal inference becomes much more difficult. In addition, this type of study, not being based on individuals with disease, shares with correlational studies the problem of the "ecological fallacy."

III. Methods of Measurement of Diet

The adequacy of current methods of measuring diet in epidemiologic studies is a subject of much current controversy. Many believe that current methods are of such low reliability and are so subject to potential biases that neither positive nor negative findings of epidemiologic studies can be interpreted. The various methods of measurement of diet have been reviewed by several authors (Marr, 1971; Bazzarre and Myers, 1979; James *et al.*, 1980); Jensen, 1981; Keys, 1979; Morgan *et al.*, 1978; Sorenson, 1982). We will focus this brief review on considerations which are of importance in the context of epidemiologic studies.

In ecological studies, estimates of per capita food consumption are determined from aggregate population-level data on food production or disappearance or from special nutritional surveys. On the international level, the Food and Agricultural Organization of the United Nations tabulates Food Balance Sheets for each nation based on its annual reported food consumption, food imports, and food exports. Many sources of error, of course, exist in this type of tabulation. One of the largest potential sources of error is in the use of food for purposes other than human consumption, such as animal feeding or food wastage. A few special dietary surveys are of use in correlational epidemiologic studies, such as the Household Food Surveys conducted in Great Britain, the HANES in the United States, and the Hawaii Department of Health Nutritional Surveys, among others. On the one hand, because special nutritional surveys that have acceptably rigorous methodology are generally not conducted in several countries simultaneously, inferences about the per capita food consumption of populations in international correlational studies generally have a rather broad confidence interval. On the other hand, correlational studies done within a single country, whereby regional cancer rates are compared with regional food consumption levels, are relatively less flawed by these problems and, in comparison with international correlational studies, are also less flawed by serious potential confounders.

Several methods of dietary measurement for individuals have been developed, but those which seem to be the most precise estimators of diet do not lend themselves well to epidemiologic study designs. Specifically, methods whereby subjects are asked to precisely record their food intake, using such techniques as keeping a detailed dietary diary or serving duplicate meals (one of which is submitted for assay), are not well suited for case–control studies. This is because, first, the measurement process itself likely interferes with the normal diet of the individual, as dietary patterns are extremely complex functions of social, psychological, biological, and economic vari-

ables. Second, even if these techniques did accurately measure current diet, it is very likely that newly diagnosed cancer patients, who would be serving as "cases" in case-control studies, might have significantly altered their diets in some ways by the experience of a recent hospitalization with the associated physical and psychological impact of cancer and its treatment. The recall method, in which the subject is asked to recall all foods eaten for a previous finite time period (usually 24 hr) tends not to interfere directly with dietary habits but is also of limited use in case-control study design because of the possible effect of the disease process on diet. In addition, dietary recall methods are also limited by potential problems of recall bias, whereby cases may have error rates in their recall which are different from the controls. For example, their recollection of past diet could be influenced by their current (changed) diet.

The method of dietary measurement most frequently employed in case-control studies of diet and cancer is the food frequency dietary history. In this method, which was originally developed by Burke (1947) as part of a comprehensive dietary assessment, individuals are asked to relate their usual frequency of ingestion of various food items for a selected period of time prior to onset of symptoms, such as the preceding 2 months, the preceding 6 months, or the preceding year. The frequency of ingestion of the various food items for cases can then be directly compared to the frequency reported by controls such that any case-control differences in reported frequency of consumption of specific foods can be assessed. In addition to analyses of case-control differences of individual foods, approximations of the nutrient content of the diet can be made for selected nutrients, based on reported food frequencies and the usual nutrient content of each food item. The U.S. Department of Agriculture has an ongoing nutrient data bank project which is continually updating information regarding the nutrient content of foods (Hepburn, 1982). There are, in addition, other important sources of this information. When there are still uncertainties about the nutrient content of foods consumed by a study population, direct measures of nutrient content can be made, as is being done by Sorenson (1980), although existing sources of data are usually sufficiently complete for purposes of epidemiologic inquiry.

In addition to assessing the frequency of consumption of various types of foods, use of a standard food serving picture allows the usual serving size to be estimated as part of the dietary history. This information, along with the usual means of preparation, can be combined with the food frequency information in such a way that a "nutrient index" can be further refined. Many investigators have used this approach in the analysis of food frequency data and have analyzed case-control differences relative to selected levels of these nutrient indices. It is important to point out, however, that most of the food

frequency dietary histories which have been used in case-control studies in the past have been based on a relatively small number of selected food items, and therefore these nutrient indices are not the same as the true nutrient content of the diets. Totals based on the nutrient indices may be significantly lower than the true total dietary intake. The assumption that must be made in the analysis and interpretation of studies based on nutrient indices is that, based on the foods whose frequency was ascertained in the interview, individuals can be properly classified into their appropriate relative rank order within the population with regard to the ingestion of each nutrient used as an index. Although the number of food items included in the food frequency history is, in most studies, relatively small (often less than 50), it turns out that many important nutrients are almost entirely contained in a relatively small number of food items. Modan *et al.* (1981) found, for instance, after conducting a case-control study based on the frequency of consumption of 243 food items, that 10 items explained 96% of the total dietary intake of retinol and another 10 items explained 87% of the total dietary intake of β-carotene.

Several validation studies of the food frequency technique have been conducted. Some of these studies compare the food frequency technique with dietary recall or diet diaries, while others assess validity by repeatability, reinterviewing study subjects after a period of time and measuring repeatability by percentage of agreement or correlation coefficients. Three of the early case–control studies of diet and cancer measured repeatability by reinterview of a selected sample of study subjects after several months. Acheson reinterviewed 63 subjects (Acheson and Doll, 1964), Higginson reinterviewed 37 (Higginson, 1966), and Graham reinterviewed 99 (Graham *et al.*, 1967). The general conclusion of all three authors was that although there is error in recall, repeatability is sufficiently good to allow proper crude rank order classification for the study population. A similar repeatability study by Nomura *et al.* (1976) showed good agreement in recalled food frequency for 109 men after 6 months and 111 men after 2 years, and reinterview of 60 subjects after 6 months by Reshef and Epstein (1972) showed good correlation for 162 foods. In summary, although many investigators have become disillusioned about retrospective measures of diet using the food frequency technique, it does appear that it may be an acceptable method for epidemiologic purposes.

Because cancer may take several years to evolve from the initiation stage to clinical diagnosis, it is not known whether the usual diet in the 2, 6, or 12 months prior to symptoms is critical with regard to cancer risk. Retrospective measurement of diet in the more distant past would be more relevant to studies of diet and cancer etiology, but uncertainties about the reliability of such measures have generally led us to attempt instead to make

more valid measures of diet in the more recent past and assume that this is reflective of diet in the more distant past. This "tracking" assumption holds that individuals tend, over time, to maintain a constant relative rank order within the population with regard to their ingestion of specific nutrients and/or foods, but it has never been empirically tested. There is very little longitudinal data of individuals within defined populations with which to test this assumption, and research on this question is sorely needed. Moreover, perhaps it is the dynamics of dietary change at critical periods in a person's life rather than the average intake of nutrients that is important in cancer causation. A significant dietary change, either an excess or a deficiency of a specific nutrient relative to the level at which an individual is adapted, may be an important consideration.

There is a paucity of empirical evidence that dietary histories taken from the distant past are valid. An early case-control study of gastric cancer (Dunham and Brunschwig, 1946) queried about dietary habits 15–20 years prior to diagnosis, but no reliability estimates were attempted. A later study (Acheson and Doll, 1964) also focused on diet 20 years prior to diagnosis. In this study, repeatability of responses for food frequencies recalled from the distant past by a subset of the study group were generally poorer than repeatability of responses for current diet, so it was concluded that dietary histories focusing on the distant past may not be reliable. Gregor *et al.* (1980), in a case-control study of diet and lung cancer, questioned subjects relative to both current diet and diet of 20 years ago. One study by Garland *et al.* (1982) and another by us (Byers *et al.*, 1983b), based on the reinterview of individuals over time, indicate that recollection of diet over 14 to 25 years seems to be biased by current diet. More importantly, however, in our study, correlations between current and past levels of diet are low. Recollections of past diets correlated with previously measured diets more closely than did current diets. Perhaps by combining information regarding current diet and perceived past diet, we may be able to generate an acceptably valid estimate of diet from the distant past. Much more work is needed in the area of developing methodologies (Moore *et al.*, 1982) to measure lifetime dietary histories retrospectively so that we can begin to evaluate the effect of diet as measured in a time period which may be more relevant to cancer risk. Whether that time period is the year preceding clinical diagnosis or 5 or 20 years preceding it, or even early life, is yet to be seen.

Food frequency dietary histories clearly have error. The misclassification which results from this error, if random for both cases and controls, would result only in an underestimation of the true relative risk and thus would be considered an error in the "conservative" direction with regard to causal inferences based on the research findings (Marshall *et al.*, 1981). Indeed, the failure of many case–control studies to demonstrate the hypothesized asso-

ciation between dietary factors and cancer risk has been explained by many investigators as being due to this random variation in the measurement of diet. If the true relative risk of cancer for a given nutrient or food item is quite low, such random misclassification may in fact completely disguise a true association. If, on the other hand, the measurement error were biased (affecting cases differently than controls), then the resulting estimate of the relative risk may either be inflated or deflated, depending upon the direction of the measurement bias (Marshall *et al.*, 1981). It is of some comfort, however, to note that many findings, from studies conducted using a wide variety of interview techniques in many different populations, are congruent.

One of the great needs in epidemiologic studies of diet and cancer is for better validation studies to be conducted in order to develop more reliable means of retrospectively ascertaining diet. Validation studies should more frequently be built into case-control investigations so that the degree and direction of misclassification for both cases and controls for the specific interview instrument can be estimated. One important means of validation of dietary intake is to make a second measurement of intake from an independent observer. In cancer research, where the typical case ranges in age from 40 to 80, a spouse is an important source of such information. Studies comparing dietary interviews of index cases and their spouses indicate that there is fairly good agreement between spouse pairs regarding food frequency (Kolonel *et al.*, 1977; Marshall *et al.*, 1980). Perhaps in the future measures of diet can be made with the individual at two points in time, as well as by a second observer such as a spouse, in order to generate more valid estimates of the true diet.

Theoretically, an ideal way to validate dietary questionnaire data would be to compare food frequencies with firm biological markers of nutrient ingestion such as might be found in blood, feces, urine, hair, nails, or other body tissues or excretions. Unfortunately, it appears that there are not many good biological markers of nutrient ingestion which are available for use in cancer epidemiology at this time (Mullen and Torosian, 1981) and that those biologic markers that do exist correlate rather poorly with dietary intake as measured by 24-hr recall (Kerr *et al.*, 1982). Hair and nail analysis, though at first considered to be a good potential marker of inorganic nutrient ingestion, are very difficult to perform reliably and seem to be subject to many potential contaminants and other sources of error, such as the rate of hair or nail growth. Serum levels of nutrients, though often easy to measure with very reliable and highly sensitive assay systems, tend to be only poorly correlated with nutrient ingestion, if at all. For example, levels of serum retinol are highly buffered by the large retinol pool in the liver so that there is virtually no correlation with retinol ingestion and serum level except in states of

extreme deprivation over several months. Liver stores are a better estimator of retinol ingestion but are not currently susceptible to estimation except in surgical or autopsy cases. Serum cholesterol, which has been found to be negatively associated with colon and breast cancer risk, likewise seems to be only weakly related to dietary intake for the general population. There is somewhat better correlation, however, for other nutrients, such as vitamin C, β-carotene, and perhaps selenium, in which blood levels vary more closely with dietary intake over the preceding several days.

Even if reliable biological markers of nutrient intake were available, they still might be of only limited use in case-control studies because cancer, which is often a systemic disease, creates many metabolic changes which may be reflected in abnormal levels of nutrients in the body. The development of reliable biological markers for the purpose of estimating nutrient intake must await a more thorough understanding of the determinants of serum and tissue levels for the various nutrients and a more thorough understanding of their basic biological activities. If in the future we better understand the determinants of levels of these nutrients, it may be that patterns rather than a simple one-to-one correlation between ingestion and tissue level will be of importance in epidemiologic studies. Unusual discrepancies between dietary intake and tissue levels, or between serum and tissue levels, may provide etiologic clues implying malabsorption or abnormal partitioning of nutrients within the body which may be associated with cancer risk.

IV. A Review of Dietary Factors and Cancer Etiology by Site

We have in previous sections reviewed the methods pertinent to epidemiologic research of diet and cancer etiology and have highlighted the evolution of epidemiologic studies of diet and cancer over the last 50 years. Coincidental with this has been a similar evolution in our knowledge of cancer causation via experimental studies in the laboratory. At times, discoveries from the laboratory have led to the creation of hypotheses which directed epidemiologic inquiries. Other times, discoveries from epidemiologic studies led into new areas of experimental inquiry. Sometimes findings have been congruent and sometimes they have not. In the sections which follow, we present brief reviews of the current state of knowledge regarding nutritional factors in the etiology of cancers of selected sites.

A. Cancer of the Oral Cavity and Pharynx

Epidemiologic studies have clearly shown that alcohol and tobacco are risk factors in human oral cancer (Decker and Goldstein, 1982; Keller and Terris, 1963; Lieber *et al.*, 1979; Rothman and Keller, 1972; Rothman, 1980; Schot-

tenfeld, 1979). There is an interesting incongruence, however, between the many case-control studies which have clearly shown alcohol to be associated with oral cancer risk and the inability of researchers to demonstrate that laboratory-grade alcohol can produce oral cancer in animals. This may be a result of some biochemical differences between man and animal, but more likely it is due to some as yet unexplained complexities related to alcohol ingestion in humans. It may be, for instance, that it is not ethanol per se that is carcinogenic but rather some other contaminant of alcoholic beverages. Investigations by F. Bach (personal communication) using bourbon instead of laboratory-grade alcohol demonstrate an increase in oral tumors with the application of bourbon. Interestingly, however, the dose–response curve is U-shaped, with the lowest risk appearing in those animals which are given a low dose and somewhat higher levels seen in those animals given no dose. Another of the complexities which is difficult to reproduce in animals is the interaction (Graham *et al.*, 1977) between tobacco and alcohol. It is difficult in laboratory animals to reproduce the alcohol and smoking behaviors of man, and in addition, because state of dentition also appears to be related to risk, animal models may not be entirely satisfactory for this disease.

Ecological studies are consistent with the alcohol and tobacco risk factors. There is marked international variation in the incidence of oral cancer, with rates being particularly high in India. In India it is a common practice to chew betel nut, often in the form of "pan," which is a complex mixture of betel nut, betel leaf, and lime. This observation, coupled with the observation that the cancer when observed is usually on the side of the mouth in which the pan is deposited (Paymaster *et al.*, 1968), strongly implicates a direct carcinogenic action by one of the constituents of pan. Even within the United States there is variation in oral cancer which is consistent with tobacco chewing habits. For example, higher rates are observed among women in the Southern United States where chewing tobacco "snuff" is more prevalent than in the remainder of the United States, where oral cancer rates are lower (Blot and Fraumeni, 1977).

Case–control studies have clearly demonstrated alcohol and tobacco to be risk factors, yet other dietary factors have also been investigated. Wynder *et al.* (1957a) concluded that there was no evidence that nutritional factors, as measured in his case–control study, were of importance in oral cancer risk. Likewise, Graham *et al.* (1977), basing the analysis on the frequency of consumption of 33 food items, demonstrated no significant case–control differences. However, reanalysis of these data by Marshall *et al.* (1982), in which vitamin A and vitamin C indices were calculated from the food frequency information collected, showed that cases reported significantly less vitamin A and vitamin C ingestion than controls, even after controlling statistically for alcohol and tobacco usage. Vitamin C in the diet reduces the level of nitrosamine formation, and vitamin A is important in the mainte-

nance of normal epithelial differentiation. Whether vitamin C or vitamin A are directly involved in protection against oral cancer or whether they are simply markers of a type of diet which is associated with individuals who are for other reasons at lower risk is yet to be seen. Supporting the view that vitamin A may be directly protective against oral cancer, however, is a finding by Ibrahim *et al.* (1977) that oral cancer cases, at the time of diagnosis, tend to have lower plasma vitamin A and β-carotene levels than do controls. There is always difficulty in making causal inferences based on biological markers in case–control studies because the disease can always cause changes in the biological markers and vice-versa. However, in oral cancer where the lesion is relatively small, one would expect that systemic metabolic changes at the time of diagnosis would be less than in other types of cancer.

B. Cancer of the Esophagus

Similar to oral and pharyngeal cancer, experimental studies of esophageal cancer have not been particularly enlightening and have tended to follow, rather than lead, epidemiologic inquiry. One of the first observations of the association between esophageal cancer and nutrition was made nearly 50 years ago, when it was observed that Swedish women suffering from cancer of the hypopharynx also frequently had anemia, which appeared to be secondary to iron deficiency and perhaps other multiple vitamin deficiencies (Jacobson, 1961). This syndrome, named the Plummer–Vinson syndrome, has since that time greatly decreased in incidence along with general improvement in iron and vitamin nutrition in that area.

Studies of the geographic variation of esophageal cancer reveal a striking variation both country-to-country and within countries, region-to-region. Of all the cancers, esophageal cancer probably shows the strongest geographical variation, which suggests the activity of strong environmental factors. Some of the highest incidence rates in the world are observed in South–Central Asia in the region between Turkey, Iran, China, and the Soviet Union. Ecological investigations of this region have shown that the high-incidence areas tend to have diets of almost exclusively bread and tea and which are severely deficient in vegetables (Joint Iran–International Agency for Research on Cancer Study Group, 1977). In addition, there is a suspicion that opium ingestion may also be high in these regions, but because the habit is illegal, it is difficult to study this factor with any degree of confidence in a survey design. In a case-control study conducted in Iran as part of the large WHO effort to study esophageal cancer in this area, Cook-Mozaffari *et al.* (1979) confirmed that cases in Northern Iran tended to ingest lower levels of vitamins and fruits as well as lower levels of animal protein as compared to

controls. This confirmed the suspicion generated from the ecological study that deficiencies of vitamins contained in these foods might be important in explaining the unusually high rate of esophageal cancer in Northern Iran. Examination of samples taken from the typical diets of cases and controls, moreover, showed no apparent differences in the levels of carcinogenic aflatoxins (known carcinogens in animal models) in the diet.

High rates observed in blacks in the United States are thought to be consistent with alcohol as a risk factor (Pottern *et al.*, 1981). Several case-control studies conducted in Europe and North America have demonstrated both alcohol and tobacco to be risk factors which appear to act multiplicatively in increasing risk for esophageal cancer (Day and Munoz, 1982). Interestingly, however, in areas that experience particularly high incidence rates of esophageal cancer, alcohol and tobacco seem to be extremely weak risk factors and relatively insignificant. That other factors, particularly non-alcoholic nutritional factors, might be important was suggested as early as 1961 in a case-control study (Wynder and Bross, 1961) in which milk and vegetable consumption was found to be diminished in cases as compared to controls. In studying blacks in the Washington, D.C. area, Ziegler *et al.* (1981b) also found that diets deficient in meat, fish, fruits, vegetables, and dairy products were reported more often for cases as compared to controls. Mettlin *et al.* (1981), in an analysis of data collected at Roswell Park Memorial Institute between 1957 and 1964, found that cases more frequently reported diets deficient in vitamin A than did controls.

Supporting the hypothesis of dietary deficiencies as etiologic factors in esophageal cancer is the finding by Mellow *et al.* (1983) that, in an uncontrolled study of esophageal cancer, serum levels of vitamin A and/or zinc were low in 15% of 17 cases. A case-control study conducted in three prefectures in Japan (Hirayama, 1979a) indicated that consumption of bracken fern, which has been found to be a risk factor for gastrointestinal cancer in animals, is also a risk factor in humans for esophageal cancer. An apparent synergistic effect was observed in those people who reported eating bracken fern and a daily consumption of chagayu (a hot tea gruel), and also in those who smoked. Chinese studies have provided evidence that aflatoxins may be etiologically important as well (Coordinating Group for Research on Etiology of Esophageal Cancer in North China, 1975).

Thus, epidemiologic studies strongly suggest that there are important dietary factors acting in human esophageal cancer. The demonstration that alcohol is a risk factor in populations which are relatively well fed might suggest that alcohol, rather than being a directly acting carcinogen, may act by metabolic means, depleting the body of essential vitamins, or may in fact be associated simply with nutrient-deficient diets. Nutrient deficiencies seem to be important in esophageal cancer in high incidence areas, but it

appears that there may be carcinogenic contaminants of foods eaten as well.

A massive screening program for esophageal cancer has been initiated in Northern China (Yang, 1980). This may provide additional valuable information regarding the determinants and natural history of this disease. Interestingly, a chemotherapeutic intervention using retinoic acid was begun here but was soon aborted. Excessive headaches as a side effect of the intervention was reported as the reason for discontinuing this intervention trial.

C. Cancer of the Stomach

Experimental studies of cancer of the stomach in animals have clearly shown that alkylnitrosoureas can induce stomach cancer. There has been considerable research, therefore, in the biochemistry of the related nitrosamines and nitrosamides, which are capable of being formed within the stomach in conjunction with nitrites and which are found in many foods and are a normal constituent of human saliva (Tannenbaum, 1983). Nitrates, which are commonly added to foods for preservation, have been found to be readily convertible to nitrites when foods are stored at room temperature but not when they are refrigerated or when vitamin C, BHT, or BHA are added to the food. Thus, experimental studies have led to the working hypothesis that derivatives of nitrates found in foods or formed from precursors found in foods may be etiologically important in human gastric cancer.

The most striking feature of gastric cancer in international studies is its strong inverse correlation with industrialization. Ecological studies consistently show that diets characteristic of developed countries tend to decrease risk of stomach cancer. Specifically, these are diets which are relatively high in fat and animal protein and low in cereal consumption. Within countries there appears to be a strong inverse relationship between gastric cancer rates and socioeconomic status.

Migrant studies have clearly shown that following migration from high-incidence areas to low-incidence areas, migrants within two generations adopt gastric cancer rates very similar to those of their new country. This has been seen in Japanese immigrants to Hawaii (Haenszel and Kurihara, 1968) as well as in Eastern European immigrants to the United States (Haenszel, 1961), who have adopted much lower gastric cancer incidence after migration though rates have remained high in their countries of origin.

In countries that have maintained reliable health statistics over the last 50 years (which are generally the more developed countries) there is a marked decline in the incidence of gastric cancer. The experimental studies relative to nitrosamines and the laboratory investigations of nitrates in refrigerated versus nonrefrigerated foods have led to the working hypothesis that im-

provements in means of preserving foods (specifically, refrigeration) have led to this observed decline and that they are also responsible for the large differences observed in international statistics (Weisburger and Raineri, 1975; Fraser *et al.*, 1980). Although this is an attractive hypothesis, it clearly cannot explain all phenomena, such as persistently high rates in Japan (although they are declining), which is a highly developed country.

Ecological studies in Chile (Armijo and Coulson, 1975) showed a strong correlation between the use of nitrate fertilizers for agricultural purposes and regional mortality rates for gastric cancer, further suggesting an important role for nitrates in this disease. Similar positive ecological studies have been conducted in Colombia (Cuello *et al.*, 1976) and England (Hill *et al.*, 1973) where nitrate levels in drinking water positively correlated with gastric cancer mortality rates. Interestingly, a followup study in Chile (Armijo *et al.*, 1981) was unable to confirm that the regional patterns of nitrate fertilizer use correlated with increased nitrate ingestion in individuals, either by assay of nitrates in vegetables or by nitrate excretion in urine. This may be a good example of the dangers of the "ecologic fallacy" and the difficulty in drawing causal inferences from ecologic studies.

Many case-control studies have been conducted of diet and gastric cancer. Indeed, many of the early studies focused specificially on this site. Stocks (1957) found fried foods to increase risk, and Pernu (1960) found that meat and animal fat consumption increased risk. Hirayama (1967) reported salted foods to be a risk factor, and milk to be apparently protective. Meinsma (1964), on the other hand, reported bacon as a risk factor and citrus fruits as protective, and Higginson (1966) reported cooked fats as a risk factor. However, other studies by Dunham and Brunschwig (1946), Wynder *et al.* (1963b), Acheson and Doll (1964), and Graham *et al.* 1967) reported essentially negative findings. Bjelke (1971) reported the apparent protective effect of fruit and vegetable consumption, and Haenszel *et al.* (1972) also reported an apparent protective effect of raw vegetable consumption, although pickled vegetables and salted fish increased risk in Japanese in Hawaii. In a later replication of this study of Japanese in Japan, this finding was not reproduced, although lettuce and celery were found to decrease risk (Haenszel *et al.*, 1976). Bjelke (1973) found cereals and smoked fish to increase risk and vegetables and fruits to decrease risk in a second case-control study conducted in Minnesota, and Modan *et al.* (1974) found that starchy foods increased risk in a case-control study in Israel. A large Japanese cohort study (Hirayama, 1979a) showed that milk consumption and green and yellow vegetable consumption were associated with decreased risk, while smoking was associated with increased risk of subsequent stomach cancer. In a later analysis of data from this cohort (Hirayama, 1982) soybean paste soup was found to be associated with lower risk, thus supporting the hypothesis that

protease inhibitors may protect against gastric cancer. Hirayama points out, however, that possible confounding effects of vegetable consumption cannot be ruled out.

Considering all case-control and prospective studies together, it is difficult to identify any consistent pattern of foods which seems to increase risk. The problem of integrating these studies may be related to the tremendous variability in the way in which diet was ascertained in the various studies, including the specific foods ascertained. The working hypothesis that nitrosamines may be etiologically related to human gastric cancer may not be testable with the data available in case-control studies previously conducted. Certainly, much more work needs to be done before we can understand the precise role played by diet in the etiology of gastric cancer.

D. Cancer of the Colon and Rectum

One of the problems in interpreting and correlating various studies of colorectal cancer is that risk factors for colon cancer as compared to rectal cancer may be quite different. Indeed, risk factors for cancer at various subsites within the colon may in fact differ. Many studies do not even distinguish between rectal and colon cancer. Even when they do, there is good reason to believe that there is considerable misclassification between rectal and colon cancers, as many cancers arise in the rectosigmoid area. Thus correlational studies of colon or rectal cancer based on international statistics must be accompanied with the caveat that there may be considerable variation from country to country in the classification scheme used.

Several laboratory animal models appear to be analogous to human colon cancer. These models, which are based on chemical carcinogenesis via oral, rectal, or subcutaneous routes of administration, are quite useful for studying dietary cofactors in colorectal carcinogenesis. The relationship between fat ingestion and colon cancer risk has been studied by Reddy *et al.* (1976) using several different inducing chemicals. Rats fed diets high in fats were found to be more susceptible to tumor induction than those rats fed diets containing lower levels of fat. It is significant that the type of fat, whether saturated or polyunsaturated, seemed relatively unimportant in these studies and that it was simply the total amount of fat in the diet that was related to risk.

This finding is consistent with international correlational studies which show that there is a strong relationship between colon cancer rates and industrialization. Industrialized societies tend to eat diets which are very high in fat in comparison to nonindustrialized societies. Colon cancer rates are particularly high in North America and Europe, whereas in Africa, South America, and Asia, rates are relatively low. This observation, in conjunction

with the animal studies linking fat ingestion to tumor promotion in dimethylhydrazine (DHM)-induced rats has led to the working hypothesis that fatty diets are a risk factor for colorectal cancer. Hill *et al.* (1979) found that high socioeconomic status (SES) groups in Hong Kong experienced over twice the colon cancer rates as low SES groups. Dietary surveys showed that the high SES group ate more meat, but also more of almost every other type of food as well, than the low SES group. Ecological studies in the United States also suggest meat to be a risk factor. Colorectal cancer is much lower in Seventh-Day Adventists, who are often lacto-ovo-vegetarian, as compared to nonadventists (Enstrom, 1980). Similarly, Mormons also have relatively low rates, yet a special dietary survey in Southern Utah, which is almost entirely Mormon, showed meat consumption levels to be virtually identical to the remainder of the United States suggesting that factors other than low meat consumption may be important in explaining the low colon cancer rates in this area. Similarly, a comparative study by Kinlen (1982) of strict religious orders in Britain showed that colon cancer mortality was not lower in an order which ate no meat as compared to one which did. In addition, there are some ecological patterns which are not entirely consistent with the fats hypothesis. Dietary fat intake is very high in Finland, yet colon cancer rates are relatively low, and within the United States there is no correlation between regional beef fat consumption and colorectal cancer rates (Enstrom, 1975).

Case-control studies have not always confirmed the suspicion generated from experimental and ecological studies that fat is a risk factor for colon cancer. Higginson (1966) reported no case-control differences in diet as measured. In a study in Norway, Bjelke (1971) found that vegetables and vitamin C apparently reduce risk; this was also found in a similar study conducted in Minnesota (Bjelke, 1973), but no association was seen with dietary fat. Haenszel *et al.* (1973) found only that starches and legumes were positively associated with risk in a case-control study conducted on Japanese in Hawaii, a finding which was not replicated in a later study of Japanese in Japan (Haenszel *et al.*, 1980). Dales *et al.* (1978), however, reported higher risk for those who ate diets that were both high in fat and low in fiber, and Jain *et al.* (1980) reported a study in Canada in which cases reported eating more fat than controls.

Burkitt (1971, 1978) proposed that dietary fiber is protective against colon cancer. This hypothesis was based on the observation that dietary fiber intake was considerably greater in areas of the world where colon cancer rates are low. Low fiber in the diet, he proposed, led to physical, chemical, and bacteriological aberrancies in stool composition which in turn could cause colon cancer. The ecological data are certainly consistent with this hypothesis, as are some case-control studies. In a case-control study in Isra-

el, Modan *et al.* (1975) found that colon cancer patients tended to report less frequent ingestion of foods high in fiber. Dales *et al.* (1978) reported increased risk with diets high in fat and low in fiber in a study of blacks in the San Francisco area. A study by Graham *et al.* (1978), however, found no relationship between fiber or fat ingestion and cancer risk, but they did find that cases tended to eat fewer cruciferous vegetables (e.g., cabbage, broccoli, and brussels sprouts). This led to the hypothesis that perhaps chemicals contained within the cruciferous vegetables, which have been found by Wattenberg and Loub (1978) to be capable of inducing arylhydrocarbon hydroxylase (AHH) activity in the gut, might thus be protective against colon cancer. Haenszel *et al.* (1980), in studying colon cancer in Japan, was unable to replicate his earlier findings of increased risk with starches and legumes from the Hawaiian study, but, consistent with the AAH hypothesis, he found that cabbage ingestion tends to decrease risk. However, preliminary results of a case–control study in Utah (Lyon, personal communication) do not indicate any effect on risk by cruciferous vegetables.

The large Japanese prospective study (Hirayama, 1979a) did not demonstrate any apparent relationship between the frequency of meat intake and colorectal cancer risk. In a correlational study, however, Hirayama (1979a) did demonstrate a strong positive correlation among 29 health centers between percentages of individuals who eat meat daily and the standardized mortality ratio for colon cancer in the district. In sum, case–control studies have shown inconsistent results. This could be a function of their methodological limitations and inherent lack of comparability, or perhaps it is indicative of other confounding factors in the apparent dietary fiber and fat risk factors in colon cancer as observed in the ecological studies.

Important activity in current research is in the area of metabolic epidemiology of colon cancer. Examination of stools of small numbers of individuals on various diets has shown that there is a relationship between the level of fat ingestion and the amount of bile acids and fecal sterols, as well as the fecal flora found in the stools (Reddy, 1981). For instance, Japanese in Hawaii have been found to have more deoxycholic acid in the stools than Japanese in Japan (Mower *et al.*, 1979). Also, Seventh-Day Adventists in New York City have been found to have more mutagens in the stool than non-Seventh-Day Adventists (Reddy *et al.*, 1980a). The stools of New York City residents, as compared to those of residents of Umea, Sweden, where colon cancer rates are lower, were found to contain the same total amount of bile acids, neutral sterols, and β-glucuronidase activity, although the stool concentrations were lower in the Swedes (Domellof *et al.*, 1982). It is hypothesized that higher levels of dietary fiber intake in Sweden may serve to dilute the concentrations of potential carcinogens in the stool.

Such correlational metabolic studies, however, have not always been con-

sistent with the hypotheses. Although there is a fourfold difference in colon cancer rates between Denmark and Finland, fecal sterols, bile acids, and oral-to-anal transit times are not different in the two populations (Jensen and MacLennan, 1979). In addition, such metabolic studies are usually based on a small number of subjects who are not always randomly selected from the populations being compared. In the future, work in metabolic epidemiology of feces may nonetheless be important in understanding the determinants of colon cancer, particularly as we gain more knowledge about the significance of various mutagens found in the stool, their sites of action (Hill, 1981), and the determinants of fecal microbial activity (Mackowiak, 1982). Such biological markers of adverse dietary effects are much more sensitive indicators than cancer and may be useful in understanding the determinants of disease and the effectiveness of interventions.

E. Cancer of the Larynx

There is not an entirely suitable animal model for laryngeal cancer. Most of the understanding of risk factors for laryngeal cancer have come from human investigations. Exposure to tobacco smoke and, synergistically, ingestion of alcohol are two major risk factors for laryngeal cancer (Flanders and Rothman, 1982). Therefore, laryngeal cancer rates in general tend to reflect lung cancer rates in international statistics. Very high rates in India, however, which are out of proportion to the Indian lung cancer rates, suggest other factors such as betal nut chewing as being etiologically important as well. Most case–control studies of laryngeal cancer have focused on alcohol as the only nutritional factor.

Wynder *et al.* (1956), in his early study of other dietary factors in laryngeal cancer, reported no apparent relationship between the ingestion of five food items and laryngeal cancer in his case–control study based in New York City. Graham *et al.* (1981), however, reported a relative deficiency in the vitamin A index in cases as compared to controls on the basis of an analysis of cases and controls from Buffalo, New York. This deficiency persisted even when controlling for cigarette smoking and alcohol. Interestingly, this same deficiency has been noted not only for laryngeal cancer but also for esophageal, oral, and (as discussed in the next section) lung cancer.

F. Cancer of the Lung

As is the case with laryngeal cancer, human epidemiologic studies have clearly led the way in the understanding of risk factors for lung cancer. Cigarette smoking is now a well-known strong risk factor for lung cancer. Nonetheless, there are still many cases of lung cancer which are unexplained

by cigarette smoking, and many smokers who do not acquire lung cancer, so there are important cofactors which act in concert with cigarette smoking in the production of lung cancer.

In international statistics, lung cancer rates seem to reflect cigarette smoking rates with a delay of approximately 20 years. Countries with populations who have been smoking for a generation, therefore, tend to have higher lung cancer rates. There is still much more to be learned about lung cancer, however. A variety of factors other than cigarette smoking, including occupational exposures and air pollution, have also been shown to be related to lung cancer risk.

MacLennan *et al.* (1977) conducted a study of lung cancer in Singapore, an area which has unusually high incidence rates in females. Studying this population, which was thought to have lung cancer for reasons largely unrelated to cigarette smoking, he found that cases as compared to controls tended to report less frequent consumption of dark green leafy vegetables, which is an important source of vitamin A in this population. Similarly, Mettlin *et al.* (1979) found that cases in Buffalo, New York, reported diets that were significantly deficient in vitamin A as compared to those of controls, even controlling for cigarette smoking. Likewise, Gregor *et al.* (1980) found that 100 cases in England reported eating foods containing vitamin A less frequently. Interestingly, however, Gregor's finding was true only for males; female cases actually reported significantly more vitamin A consumption than controls. A case-control study by Hinds *et al.* (1983) demonstrates an apparent increase in risk with dietary cholesterol intake.

Three prospective studies relating lung cancer to dietary factors have been conducted. In the large Japanese cohort study (Hirayama, 1979a) previously discussed, cases reported significantly less frequent consumption of green and yellow vegetables, which is the source of 44% of vitamin A in the Japanese diet. This effect was persistent at all levels of smoking. Shekelle *et al.* (1981) reported a strong linear decrease in risk of lung cancer with increasing dietary vitamin A in a 19-year prospective study of 1954 middle-aged men. Bjelke (1975) reported 5-year follow-up of a cohort of 8278 randomly selected Norwegian men. Based on health questionnaires which included a food frequency dietary history of approximately 50 items, the vitamin A index was significantly lower for cases as compared to controls. This effect persisted after control for cigarette smoking. A more recent report on this cohort (Kvale *et al.*, 1983), including an approximately equal number of individuals sampled by other than random means, details lung cancer risk at 11.5 years by histologic type. Squamous and small-cell carcinomas are seen more often in those who eat diets low in vitamin A, while adenocarcinomas seem not to be related to vitamin A ingestion levels. Again, the apparent vitamin A protective effect is independent of cigarette smoking.

The question arises as to whether "vitamin A" as estimated in these studies is, in fact, retinol or the retinol precursor β-carotene, each of which is included in vitamin A units in food composition tables. The vitamin A index used by Mettlin, since it does not include liver, is largely a β-carotene index, although both Mettlin and Bjelke found milk consumption to be associated with decreased risk. Likewise, the finding of Hirayama in the prospective study may be considered a β-carotene finding since there is no retinol in green-yellow vegetables. Because many nutrients are found in vegetables, we must be careful not to overinterpret such vegetable associations as necessarily due to any one of a variety of possible factors. The observation by Gouveia *et al.* (1982), however, that in a small uncontrolled trial, bronchial metaplasia seemed to be reversed with the administration of retinoids, lends some support to the hypothesis that vitamin A-related compounds may be protective against lung cancer.

Two prospective studies (Kark *et al.*, 1981; Wald *et al.*, 1980) showed that, many years prior to diagnosis, individuals with lung cancer have levels of retinol in their serum which are lower than expected. This is also clearly consistent with a protective effect of vitamin A, but at the same time it is not necessarily consistent with a role for β-carotene or retinol in the diet, as there appears to be very little relationship between ingested retinol or β-carotene and serum levels of retinol (except in cases of severe and prolonged deprivation). Therefore, we must look for other possible confounding factors in this apparent retinol–cancer link, such as LDL-cholesterol, or other determinants of serum retinol levels which also may be related more directly to cancer risk. Having identified cigarette smoking as a major risk factor in lung cancer, we should nonetheless continue to search aggressively for other cofactors in this important disease, particularly for histologic subtypes such as adenocarcinoma, which do not appear to be largely explained by cigarette smoking.

G. Cancer of the Bladder

Unlike most of the other sites discussed, there is relatively little international variation in human bladder cancer rates. Epidemiologic studies have demonstrated increased risk with exposure to certain industrial chemicals. The controversy that has surrounded the studies relating saccharine to bladder cancer risk is a good example of the problems in relating animal to human research. Although in animal experimental systems, saccharin does cause bladder cancer, large-scale human studies have not confirmed this finding (Howe and Bench, 1980; Newell, 1981). A study by Howe *et al.* (1977) indicated that artificial sweeteners might increase risk, but this was seen only in men and not in women. On the other hand, a study by Hoover

(1980), based on many thousands of bladder cancer cases and controls, did not detect such a relationship.

Other chemicals excreted in the urine have been implicated, however, in human bladder cancer. Excreted products of tobacco consumption are thought to explain the slightly increased risk noted in many studies for smokers. Coffee consumption has been demonstrated to be a risk factor in some studies but not all. A study by Cole (1971) showed a significant association between bladder cancer risk and coffee consumption. A similar study by Simon *et al.* (1975) again showed the association, but because there was no dose–response relationship, it was concluded that the association might be spurious. Mettlin and Graham (1979) found a modest elevation in risk with frequent coffee consumption, also with no dose–response. This study also included other dietary factors, demonstrating that risk increased with lower levels of vitamin A consumption. This finding is consistent with findings from experimental work with retinoids and bladder cancer, but it is increasingly clear that the effects of retinoids may be very complex in that they appear to be subject to effect modification by other cofactors (Lower and Kanarek, 1981). The only other case–control study of bladder cancer in human diet was presented by Wynder *et al.* (1963a) who reported no case-control differences in the frequency of ingestion of foods, but detailed data were not presented and the study was apparently based on a relatively small number of food items.

In future studies of bladder cancer, the use of techniques of metabolic epidemiology are likely to be useful, just as in colon cancer. Specific chemicals and nonspecific mutagens found in the urine can be used as readily obtainable biological markers in correlating bladder cancer risk to diet. Bladder cancer may also be a useful site for chemoprevention trials done on groups at high risk either because of a previously receded early cancer or because of occupational exposure such as aniline dyes, which increase risk.

H. Cancer of the Prostate

Until quite recently, there was no animal model for prostate cancer analogous to the human disease. Experimental studies based on prostate tissue cultures have been used extensively in the laboratory, however, and have provided useful information. It has long been noted that retinoids seem to have profound effects on the morphology of these tissue cultures, whereby the dysplastic changes created by mutagenic chemicals may be prevented with the prior administration of retinoids in some models. This has led to the hypothesis that vitamin A might be protective against prostate cancer and, perhaps, that vitamin A deficiencies might be etiologically important in prostate cancer. In addition, because prostate cancer is thought to be a

hormone-dependent disease, other dietary factors which affect the hormonal milieu, including overnutrition and high fat diets, are thought to be etiologically important.

International patterns of prostate cancer are strikingly similar to those of colorectal and breast cancer. These cancers are often considered together as a triad of cancers associated with "Westernization." Prostate cancer rates are quite low in Japan, however, and Japanese immigrants to the United States experience increased risk after migration. Armstrong and Doll (1975) found a strong positive correlation in international statistics between fat consumption and prostate cancer risk.

There has been to date only one case-control study relating dietary factors to human prostate cancer risk. Based on cases from Roswell Park Memorial Institute, Graham *et al.* (1983) found that cases tended to report a greater frequency of consumption of nearly all foods than did controls. This included foods high in fat, protein, as well as vitamin A. This finding is consistent with international correlational studies which suggest that some aspects of Western diets may be a risk factor. Specifically, overeating in general may be implicated, although any particular component of excessive food frequency could be important. The finding of a positive association between vitamin A ingestion and prostate cancer risk, on the other hand, is not consistent with the hypothesis that an absolute deficiency in vitamin A or β-carotene ingestion is etiologically important. Much more definitive case-control studies need to be conducted on this disease. Kolonel is currently conducting a large case–control study of prostatic cancer in Hawaii. A preliminary analysis of a subset of his study population shows (similar to Graham's study) that cases tend to report excessive consumption of fats and foods containing vitamin A (personal communication). One prospective study of prostate cancer has been reported to date. In the large Japanese cohort study (Hirayama, 1979b), the frequency of consumption of green and yellow vegetables was found to be inversely correlated with prostate cancer risk.

I. Cancer of the Breast

Experimental studies in the laboratory have been quite helpful in testing hypotheses relating breast cancer to nutrition. Animal models have been developed which clearly show that breast cancer risk in animal systems is related to genetic as well as environmental factors. Environmental factors include viruses, chemicals, radiation, and nutrition. Nutritional factors appear to act during the promotional stages of tumor development and, in some cases, are very strong cofactors. Mice fed high fat diets and who consequently become relatively overweight demonstrate a much higher rate of mammary tumor development after DMBA induction than do animals

who are fed lower levels of fats in their diet (Carroll and Khor, 1971; Hopkins and Carroll, 1979). This effect is particularly strong for unsaturated fats.

These experimental findings are consistent with the observation that breast cancer is positively correlated in international statistics with industrial development and, consequently, with fat ingestion and obesity. The "developed" countries of North America and Western Europe have relatively high rates as compared to Africa, Asia, and South America. One exception to the rule of industrialization is Japan, which, although quite industrialized and developed, still has relatively low breast cancer rates. Studies demonstrate that migrants tend to adopt the breast cancer risk of their new country, which argues strongly for some environmental (rather than entirely genetic) factors to explain the international variation.

One of the problems in international correlational studies is uncontrolled potential confounding variables. Hems (1978) presented an international correlational study focusing specifically on breast cancer in which animal fats, protein, and sugar were found to be positively associated with breast cancer risk. In the same study, childbearing, which is well known to be a protective factor, was demonstrated to be negatively associated with risk. Although this might be considered to be evidence for validation in this study, the methodological problems in the correct ascertainment of diet as compared to the correct ascertainment of childbearing history are quite different. Another ecological study of breast cancer (Gray *et al.*, 1979) further attempted to control for other breast cancer risk factors in assessing the relationship between per capita dietary consumption and international breast cancer mortality rates. After controlling for mean average weight, height, and age at menarche for women in each country studied, the observed positive association between animal protein and fat consumption and breast cancer risk still persisted. This implies that there are no strong confounding effects of these three variables with regard to the dietary risk factor. It is unlikely, however, that these three variables alone could account for much of the variation in breast cancer incidence, so this is at best a very incomplete control of potential confounders. Supporting the meat hypothesis is an ecological–metabolic study (Armstrong, *et al.*, 1981) which shows that postmenopausal women who are vegetarians have estrogen levels which are lower than expected. Contrary to the dietary meat hypothesis, an ecological study of religious orders in Britain by Kinlen (1982) found that women who strictly avoided meat in their diets did not experience lower-than-expected mortality for breast cancer.

A study by Miller *et al.* (1978) based on 400 cases with community-matched controls found a slightly higher intake of fat reported from controls as compared to cases. Similarly, a study by Lubin *et al.* (1981) in Alberta based on 577 cases and a very brief food questionnaire containing only eight

items found a higher frequency in reported consumption of beef, pork, and sweet desserts in cases as compared to controls. However, a study reported by Graham *et al.* (1982) based on 2023 cases from Buffalo, New York, did not demonstrate an increased frequency of meat or fat consumption in cases, but it did show a slightly decreased frequency of vegetable consumption, particularly those vegetables containing β-carotene, in cases as compared to controls. This effect was seen only in women in the postmenopausal age range.

Several case-control studies have demonstrated a positive relationship between body weight and/or various other measures of obesity and breast cancer risk, particularly in the postmenopausal age group (Kelsey, 1979). Certainly, body weight is related to nutritional factors. The precise mechanism of the increased risk observed with obesity in postmenopausal years is not clear (Mohla and Criss, 1981). It is hypothesized that increased risk may be related to the phenomenon of estrone production in adipose tissue from the androstenedione precursor. Adipose tissue in the obese postmenopausal female is therefore a significant source of estrogen. This raises the possibility that excess caloric intake leading to obesity may be etiologically important in postmenopausal breast cancer indirectly by affecting body fat levels.

Only one prospective study of breast cancer nutrition has been reported. The large Japanese cohort study (Hirayama, 1979a) showed that women who ate meat daily experienced higher breast cancer rates than those who did not. Interestingly, this effect was seen only in those women aged 55 and over. A prospective study of 2425 women who had colostomies for nonmalignant colonic diseases (Rang *et al.*, 1983) showed that breast cancer rates were not lower than expected.

In the future, dietary and chemoprevention controlled trials will be conducted for breast cancer. Preliminary work by Bright-See (1982) has shown the feasibility of lowering fat intake to 20% of total calories in a randomized trial. Experimental evidence for animal research may be strong enough to support a low fat diet therapeutic trial in conjunction with more traditional means of therapy (Wynder and Cohen, 1982).

J. Cancer of Other Sites

There are many types of cancer for which no epidemiologic work relative to nutrition and cancer etiology has yet been published. Female reproductive cancers other than breast would seem to be likely sites for a dietary effect, particularly since obesity is strongly related to endometrial cancer, and ovarian cancer is thought to be related to hormonal abnormalities similar to breast cancer.

We have completed analysis of data from the 1957–1965 RPMI series of patients for cancers of the ovary, endometrium, and cervix. We compared

the dietary histories of 274 patients with ovarian cancer (Byers *et al.*, 1983a), 502 patients with endometrial cancer (J. Marshall, personal communication), and 511 patients with cervical cancer (Marshall *et al.*, 1983) to those of controls. In each case, the consumption of vegetables containing vitamin A was reported to be slightly lower in cases as compared to controls, and in the case of cervical cancer, vitamin C ingestion was also reportedly lower, but no strong dietary effects were observed in these studies. One case–control study reported by Wassertheil-Smoller *et al.* (1981) in New York City of cervical dysplasia is perhaps pertinent to cervical cancer. A total of 169 women, approximately half of whom had dysplasia, kept a 3-day diet record from which various nutrients were estimated. The reported ingestion of vitamin C was significantly lower in the dysplasia group as compared to the group of women without dysplasia, suggesting that vitamin C deficiency may be a risk factor for cervical dysplasia. A similar effect was seen for other nutrients, including vitamin A, as 71 different nutrients were examined.

Because vitamin A and other retinoids have been found to be important in the maintenance of normal epithelial differentiation and because cervical carcinoma is a disease of undifferentiation, a clinical trial of topical retinoic acid (13-*cis*-retinoic acid) therapy in cervical dysplasia is underway at the University of Arizona, Tucson (Meyskens, 1982). Another nutritional intervention therapy for cervical carcinoma is underway at the University of Alabama, where systemic folic acid is being administered in a blinded randomized trial to women with cervical dysplasia (Krumdieck, 1982). Early results indicate that the folic acid group demonstrates greater improvement in dysplasia than the placebo group. Thus it seems that because the cervix is an organ which is very visible and has an easily measurable preneoplastic morphologic precursor, nutritional and chemopreventive intervention trials with cervical cancer will be proceeding well in advance of observational studies. There is, nonetheless, a desperate need for more case-control studies of endometrial cancer and ovarian cancer, as well as many of the common nonmalignant proliferative conditions of the female reproductive organs, such as endometriosis.

Dermatologists have known for many years that retinoic acid has profound effects on differentiation of the skin epithelium and that retinoids have a wide variety of clinical uses in dermatology (Bollog, 1983). Despite this, there has been virtually no observational research in nutrition and skin cancer. As is the case with cervical cancer, in which the affected organ is highly visible, nutritional research in skin cancer in the future will likely include an emphasis on activity in experimental study designs. For example, randomized control trials can be conducted in high risk populations such as those composed of elderly fair-skinned individuals in the South and indi-

viduals who have previously had one skin cancer and are therefore at high risk for another.

Human epidemiologic studies of cancers of mesodermal origin such as leukemias, lymphomas, and sarcomas have not been conducted to date, although we will soon be analyzing some data collected at RPMI between 1957 and 1965 on cases with these diagnoses. There is good reason to believe from *in vitro* studies that vitamins such as vitamin A and folic acid may be important in the differentiation process of white cell precursors and thus may also be important in the etiology of these neoplasms. This is an area of great need in epidemiologic research.

V. Conclusions

In the last 50 years, epidemiologists have investigated the relationship between dietary factors and cancer risk in a variety of ways. Studies have been conducted in many parts of the world, employing different types of study designs and focusing on many different types of cancer. This entire effort has not led to a single unequivocal conclusion regarding the relationship between dietary factors and cancer risk. However, there have been a few consistent patterns which have led to the formulation of some important hypotheses.

The few validation studies of dietary history methods that have been conducted indicate that these methods seem to be acceptable for epidemiologic inquiry, yet continuing uncertainty about the validity of these measures creates a shadow of doubt over almost all findings (or lack of findings) from epidemiologic research in this area. There are, nonetheless, a few examples of strong congruences between the findings of experimental research, ecologic studies, case-control studies, and prospective studies. However, there are in many more instances examples of lack of congruences as well. The same can be said for experimental research. It appears that dietary factors may act in complex ways, perhaps having different effects in populations with different profiles of other risk factors, and different effects in different target organs.

The coming era of nutritional epidemiology in cancer as well as other chronic diseases is certain to be marked by much more attention to methodological rigor. In our study of cancer in western New York in which we have been collecting during the last 5 years data from cases and neighborhood-matched controls regarding diet, we have expanded our dietary interview from what was a 15-min interview 20 years ago to a 2- to 3-hr interview which covers a wide variety of dietary items, including means of preparation and usual serving size. Built into this new study are validity studies of the

dietary measures, including reinterviews of a random sample of respondents, food diaries, 24-hr recalls, spouse interviews, and other special studies. We feel that careful attention to methodological detail will strengthen our findings. Other investigators are also beginning to follow more rigorous methodology. Even where rigor produces reliable findings, however, we should not be surprised to see contradictions between studies. There is a need for much better standardization of our measures of dietary intake so that studies will be more comparable.

There is also a need to better study interaction of dietary factors with other cancer risk factors. Where possible, dietary effects on cancer risk should be examined separately for different age groups as well as for different histological types and different anatomic subsites. Interaction of other important risk factors, such as alcohol, smoking, genetic predisposition, and occupational factors, also need to be investigated. Because dietary factors are thought to be active mostly in the promotional stages of cancer, it is quite possible that understanding the patterns of interaction with other known risk factors may shed more light on the nutrition–cancer connection than studying nutritional factors in only a univariate way.

An exciting area of current research in nutrition and cancer, and one which will undoubtedly expand in the future, is the area of "metabolic epidemiology." Biological markers of preneoplastic metabolic conditions are likely to be quite helpful in the future in defining the characteristics of individuals within populations of high risk and in understanding the mechanisms of action of diet in cancer causation and prevention. These studies, however, also have severe limitations. In the future, such studies should be conducted where possible on large numbers of individuals who are selected in such a way to be representative of the populations from which they were drawn.

More and more, there is interest in proceeding directly from dietary hypotheses based on experimental and ecological studies to intervention studies in man. The intervention study, when properly carried out, is as close as we can come to experimental studies in humans in this field. The potential benefits from such studies are enormous. However, there may also be risks, particularly when the biological effects of diet supplements are not completely understood for all target organs. Intervention studies should be carried out with great care and only after extensive research in animals and humans suggests that there is potential benefit and relative safety of the intervention. Intervention studies are not substitutes for more thorough investigation of other dietary factors such as may be found in normal diets that people eat every day. There is a continuing need, therefore, for observational studies in free-standing populations.

Finally, as "prudent" diets are discovered which can materially reduce

human cancer risk, much more research is needed in the area of human behavior, such that these diets will be made palatable and acceptable to the public. The "bottom line" in this entire research effort is the prevention of human suffering due to disease. These preventive efforts will of necessity involve not only etiologic inquiry but also an integration of scientific findings with the fields of social psychology, behavioral change, agriculture, economics, and business marketing so that the desired reduction in human cancer can be achieved.

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MOLECULAR ASPECTS OF IMMUNOGLOBULIN EXPRESSION BY HUMAN B CELL LEUKEMIAS AND LYMPHOMAS

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I. Introduction

A prime paradox in attempting a molecular or functional approach to the understanding of normal and oncologic systems is that examples of the latter often serve to enlighten our basic knowledge of the former. This can arise from one or the other of two diametrically opposing features of the neoplasia. First, gross disturbances to the fundamental make up of the cell may substantively interfere with these processes in such a manner as to shed light on these events not usually gleaned from normal situations. The second aspect is that malignant populations may instead remain remarkably faithful to the majority of the functions inherent to their normal counterparts and as such can provide model populations for probing phenotypic phenomena of defined cell types. While both considerations pertain to the subject of this review, it is the latter which will predominate in formulating our underlying concepts. The purpose of this article is to highlight a single, but major, molecular event of the largest single functional group of the lymphocytic neoplasia which is reflected in normal physiology by the capacity of cells of the B lymphocyte lineage to synthesize and process immunoglobulin molecules.

Since a concerted immunologic and functional approach was first applied to the study of lymphocytic tumors a little over a decade ago, it has become apparent that the overwhelming majority of these disorders represent malignant monoclonal proliferations of B lymphocytes (Aisenberg and Bloch, 1972; Payne *et al.*, 1977; Leach *et al.*, 1975). The hallmark of such cells is their commitment to the production of immunoglobulins, the antibodies of the immune system. This commitment is probably unique to B lymphocytes and represents their raison d'être. It is quite likely that all other attributes that we can ascribe to these cells reflect to a varying degree the means of modulating the primary processes of forming, handling, and regulating the antibody molecule. The identification of immunoglobulin as an integral

membrane protein of the precursors to the antibody-forming cells provided one of the most vital tools to the dissecting of the functional subtypes of the lymphocytic leukemia and lymphoma. Now, however (and this relates to our initial paradox), it is the probing of the precise modes of immunoglobulin expression by neoplastic cells which reveal a molecular and phenotypic complexity previously only hinted at by observations on normal polyclonal B cell subsets.

The current state-of-the-art in analyzing B cell processes probably rests at an analogous stage to that of serum immunoglobulin molecules 2 to 3 decades ago. The recognition that the paraproteins of myeloma represented single antibody species, which with some very minor preliminary steps only could be purified to molecular homogeneity, opened the way to the full elucidation of the structure, composition, and function of the immunoglobulins. Close scrutiny of the B cell subsets residing in normal tissue compartments reveals a degree of heterogeneity which renders precise functional analysis difficult at best. As with the myeloma proteins for antibody molecules, the B cell leukemia and lymphoma in many cases readily provide an immense number of cells which with minimal manipulation yield populations of clonal uniformity coupled with phenotypic and functional homology. It is these properties which have prompted the increasing use of neoplastic B cell populations as models for examining the molecular and cellular basis for immunoglobulin handling during normal B cell processes. Again by strict comparison with our myeloma analogy, where it was argued that the product of the malignant plasma cell probably only approximated normal immunoglobulin structures, it is often contended that the capacity of tumor B cell populations to mirror normal cellular functions is, at best, limited. A prime intention of this review aims at examining the reliability of the model in predicting normal immunoglobulin events. While occasionally "edict 1" of our opening sentiments may be conformed to, and as such open up new and often intriguing questions, the consensus studies point to the view that the fidelity of immunoglobulin expression preserved on malignant transformation is remarkably high.

While there exists a plethora of reviews covering the capacity of cultured human cell lines to synthesize and process immunoglobulins (see Nilsson, 1982, for examples), the information on these functions as reflected by freshly explanted human material has been relatively lacking. The exceptions here are the plasma cell neoplasms, including multiple myeloma and Waldenström's macroglobulinemia, and these disorders have been covered by some excellent reviews (see Mellstedt *et al.*, 1982). This article restricts itself to reviewing our current knowledge on immunoglobulin processing as reflected in the classically considered "nonimmunoglobulin-exporting" tumors. Partially as a reflection of my own interests over the past few years,

but primarily because these are the areas in which the most rapid progress has been seen, the chronic type lymphocytic leukemias and the non-Hodgkin lymphoma will be particularly highlighted as models for examining immunoglobulin production by developing B cell populations. However, this preference in no way precludes the discussion of other neoplasms of the B lineage from which molecular events at discrete levels of differentiation are being elucidated, and in this context the acute lymphoblastic leukemias and the Burkitt lymphoma receive close scrutiny.

Such a review may be particularly timely when at present much attention is being focused on the application of monoclonal antibodies to diagnostic systems, and here the hematologic neoplasms are no exception (Foon *et al.*, 1982). For a rational approach a framework is required in which to integrate new observations, and in the context of the B cell malignancies immunoglobulin provides the ultimate phenotypic marker for such a basis. It is the formulation of an integrated approach to the fundamental aspects of these areas which we attempt in the following sections.

II. Immunoglobulin Processing during B Lymphocyte Development

A. The Antibody Molecule

Antibody molecules can be considered as bifunctional structures incorporating within their basic four chain units (comprised of two identical heavy chains and two identical light chain polypeptides) the dual features of constancy and variability. The extent of the variability is immense, catering for some millions or even billions of potential antigens, and is carried at the N-terminus half of the light chains and an approximately equal span of the heavy chains, accounting for about one-fourth of their total length. A few stretches of hypervariability within these regions specify the antigen binding site while so-called framework sequences may confer on the combining regions their topographical disposition within the heavy and light chain union. Away from these areas of variability toward the C-termini of the heavy and light chains are protein sequences which, within each antibody type of which there are a strictly limited number, are as remarkable for their striking homology and lack of deviation in structure.

For the light chains only two clearly distinct forms exist while the heavy chain constant regions are specified by five major families: μ, δ, γ, α, and ϵ. It is the make up of the heavy chain constant regions which defines the class of antibody which in turn stipulates the adjunctive functions of the molecule. In man, the combination of two heavy chains of a given type or its subtype with two light chains of either κ or λ yields the basic units of the nine

different immunoglobulin isotypes designated IgM, IgD, IgE, IgG_1, IgG_2, IgG_3, IgG_4, IgA_1, and IgA_2 each carrying the structure defining its own effector capabilities, but all displaying the almost infinite options for antigen binding required for the effective discrimination of nonself from self. It is the genetic basis for this remarkable capacity to incorporate into a single structure such extreme diversity coupled with rigid constancy which is explored in the following sections.

B. Immunoglobulin Gene Rearrangements and the Generation of Diversity

1. Single Polypeptides from Several Gene Segments

Immunoglobulins are encoded by three unlinked gene families each located on separate chromosomes within every mammalian cell. In man the cluster of heavy chain genes is placed on chromosome 14 while the κ and λ light chains are on chromosomes 2 and 22, respectively (McBride *et al.*, 1982). For each immunoglobulin chain the resulting polypeptide is derived from a number of genetic elements defining parts of either the constant or variable portions of the chains. Within the germ line DNA there are probably several hundred separate *V* genes (Valbuena *et al.*, 1978). For light chains it was found that each *V* gene encoded for nearly, but not quite, all of the variable region of the molecule. The apparent deficit was made good by a short sequence of genetic information termed the *J* gene. In contrast to the hundred or so *V* genes, there are only five J sequences in the germ line for mouse κ chains (Seidman and Lader, 1978). The J segments lie in between the array of V sequences and a single *C* region gene as depicted in Fig. 1. For λ chains the precise arrangement is somewhat different with four or six Cλ genes for mouse and man, respectively, each linked to its own J sequence lying upstream toward the V sequences (Hieter *et al.*, 1981a). During early stages of development along the B cell lineage a single *V* gene is brought next to one of the J sequences with joining mediated by highly conserved sequences adjacent to the two segments. Intervening V and J sequences along with noncoding segments are deleted from the chromosome during these reshufflings. The joined V and J segments along with a single *C* gene forms the active light chain gene (Fig. 1).

An active heavy chain gene is formed by the same principle but here four separate genetic elements are brought together from the germ line. The fourth segment, termed D for diversity and only 13 nucleotides long, is juxtaposed between the V and J segments following the somatic recombination events of the heavy chain DNA sequences (Early *et al.*, 1980). As shown in Fig. 1, the recombined V-D-J sequences again lie upstream of the heavy

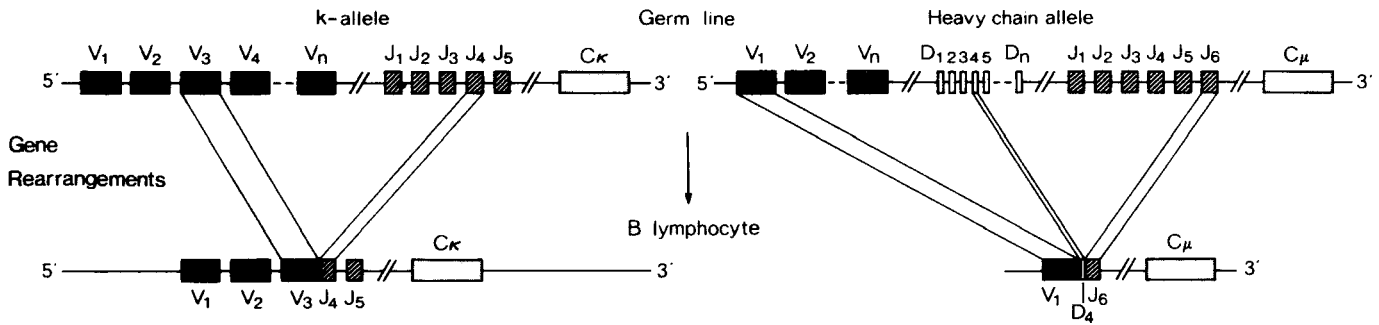


FIG. 1. Immunoglobulin gene rearrangements in early B cell development. The somatic recombination events occurring on a κ and a heavy chain allele are shown. Present in the germ line (top) are multiple copies of the genetic elements specifying the V, J, and (for the heavy chain) D segments which contribute to the variable regions of the two immunoglobulin chains. The constant regions are specified by the Cκ and Cμ genetic elements, respectively. During lymphocyte development rearrangements of these elements occur, commencing with the heavy chain, whereby one V gene is recombined with one of the J sequences (and D for the heavy chain) to form, with the adjacent C region sequence, an active immunoglobulin chain gene (bottom). Genetic elements and distances not drawn to scale.

chain constant region gene cluster. For each antibody isotype to be specified there is a corresponding coding sequence within the cluster, although for all developing B lymphocytes it appears that it is the μ chain *C* gene, Cμ, which is first transcribed along with the stretch of genetic information for the variable region encoded by the V-D-J elements (Maiki *et al.*, 1980).

2. *The Genetic Basis for Antibody Diversity*

The conceptual problem of how or whether the extreme diversity of the antibody repertoire for antigen binding could be incorporated into the genome has now been overcome with our recently acquired knowledge on the genetic arrangements which lead to the expression of functional immunoglobulins. Part of the flexibility of the system reflects simple combinatorial options for either V and J or V, D and J joinings for the light and heavy chains, respectively. At first glance this mode should account for about 500 to 1000 possible light chain variable regions, assuming about 100 V sequences and 5 J segments on a κ light chain allele. Extra diversity can be generated from these elements reflecting the option that the recombination site of V-J joining need not be precisely defined, with the crossover point varying over several nucleotides (Altenburger *et al.*, 1980). This area lies within the third hypervariable region of the κ light chain so that a single amino acid variation can have a profound effect on the antigen binding site. Such alternative joining site possibilities could then account for a further approximate 10-fold diversification. This mode of recombinatorial flexibility is further amplified for the heavy chain where variability in crossover can occur for both V-D and D-J joining. The number of D segments have not as yet been mapped in man but have been estimated at around 15 from analogy to the mouse. Together with the selection of one of an approximate 100 V and possibly 6 J heavy chain genes, the recombinational flexibility would generate on order of a million possible variable regions. Further extension of diversification can then be simply conceived as a consequence of optional light and heavy chain pairing from this vast array leading to the potential for approximately 10^9–10^{10} antibodies generated from only a few hundred starting genetic elements in the germ line DNA. From this already vast array of potentialities, additional mechanisms may generate further diversification of the antigen-recognizing system depending on single point mutations within the variable region coding sequences. Such a mechanism may have a particular significance for affinity maturation for antigen occurring during immunoglobulin class switching (Gearhart *et al.*, 1981).

3. *Allelic and Isotypic Exclusion*

For each of the heavy, κ, and λ chain genes there are two copies expressed on the different members of the chromosome pairs. In an antibody-produc-

ing cell only one or the other of these alleles is expressed to produce a functional immunoglobulin molecule (Hieter *et al.*, 1981b; Coleclough *et al.*, 1981). The simplest mechanism to account for allelic exclusion is that once V-J recombinations had occurred on one of the chromosome pairs the other would remain inactive in the germ line configuration and thus would not be expressed. However, in many cells producing antibodies of the κ type, for example, it has been shown that the recombination event has occurred on both chromosomes but that only one of the pairs is functionally expressed. This has been taken to imply that the recombination event is prone to error, sometimes giving rise to nonproductive rearrangements. This error probably reflects the price which must be paid for generating extra diversity through flexibility in the V-J joining in which untranslatable sequences will arise as a consequence of out-of-phrase recombinations. Nevertheless, this may be one of only several mechanisms which maintains allelic exclusion: it has been shown for a mouse myeloma that the κ chains from both alleles can be actively expressed (Kwan *et al.*, 1981). Here the V-J joining on one allele led to the deletion of two full codons which preserved the in-phase reading frame so that the gene product was expressed. To account for this apparent violation of the allelic exclusion principle, it was postulated that this short light chain could not contribute to the formation of a functional immunoglobulin so that the enzymatic machinery for V-J joining remained active until such a molecule was formed, in this case from the second of the κ chromosome pair. From these postulates it could be argued that the signal for turning off further immunoglobulin gene rearrangements is the appearance of a complete immunoglobulin molecule at the cell membrane (Kwan *et al.*, 1981).

While allelic exclusion for light chain may reflect both recombinational errors and phenotypic alteration, that of heavy chain, whose rearrangements precede that of light chains in B cell development, may reflect the former mechanism only. Thus in sharp contrast to light chain genes, heavy chains in the majority of antibody producing cells appear to have undergone rearrangements on both of the alleles (Early and Hood, 1981). Such high incidence of apparent error probably reflects the more complex structure of the heavy chain locus in which out-of-phase reading frames could arise from both the V-D and D-J fusions. This high frequency of nonproductive rearrangements suggests that many emerging B cells may be aborted if they produce errors on both heavy chain alleles. From this stochastic model allelic exclusion at the heavy chain locus is thus seen as a simple consequence of a high error rate. For those instances which by chance arise where a productive rearrangement has occurred on both alleles, the allelic exclusion principle may be preserved by elimination of the transgressors through heavy chain toxicity (see Section IX).

To extend the possibility that cells which have undergone appropriate heavy chain rearrangements produce a viable immunoglobulin, the options on the light chains are increased by having two chromosome pairs specifying, respectively, κ and λ chains. In both mouse and man the contribution of λ chain to the animal's antibody spectrum is minor to that of the κ chain and appears to reflect the relatively high success rate of rearrangements on the κ alleles. Several lines of evidence point to the λ genes acting as a safeguard in the event that both κ alleles are nonproductively rearranged. Most λ-producing cells have usually rearranged both κ alleles, and both of the κ constant region genes are often entirely deleted from the genome (Korsmeyer *et al.*, 1982). The deletion step does not appear to be an essential prerequisite for the onset of λ gene rearrangements but may occur as a secondary event to eliminate the possibility of aberrant transcripts from the genome. The existence of several J-C pairs in the λ gene cluster of both mouse and man probably emphasizes the compromise between the maximization of diversity through V-J joining flexibility and the occurrence of recombinational errors. Thus in man, each cell has 14 opportunities to "get it right," provided by two κ alleles and six *C*λ genes on each of two chromosomes.

C. The Formation of Membrane-Bound and Secreted Immunoglobulins

Interaction of an antibody with antigen for which it was encoded can occur in one of two primary modes during the lifespan of a B lymphocyte. Chronologically, the first of these is with the immunoglobulin molecule when it is membrane bound, and it is this interaction which transmits to the cellular machinery part of the information required for clonal amplification and cellular differentiation. The second of these processes results in the commitment of the developing cell away from surface expressed immunoglobulin structures to immunoglobulin primarily in a secretory phase. Divergence of the membrane and secreted forms may occur at transcriptional, translational, and posttranslational levels. All immunoglobulin isotypes appear to be governed by the same underlying principles in the creation of the separate forms. We will examine the differential processing of IgM which occurs to yield the membrane and secreted product.

Whether destined for the cell surface or for export, all immunoglobulins must travel from their sites of translation to be functionally active. The initial processes in the intracellular transport appear to be mediated by a hydrophobic leader sequence which is 17–20 amino acids long and lies at the N-terminus of the nascent immunoglobulin chains (Blobel and Doberstein, 1975). This leader is coded by a short DNA sequence which is positioned upstream from and separated by a noncoding segment to each of the *V* gene

sequences within the germ line. During the immunoglobulin rearrangements, the leader is transposed with its V sequence to the active gene site. The leader is cleaved away as the translation products of the immunoglobulin genes transverse the cell to become either surface expressed or secreted.

The discrimination between the two structural forms resides in the C-terminus of the heavy chain constant regions. Whereas the membrane-bound molecules end in a short sequence of hydrophobic amino acids at the C-terminus, secreted immunoglobulin lacks this and instead terminates with a hydrophilic peptide (McCune *et al.*, 1980). A close examination of an active heavy chain gene, as depicted in Fig. 2, reveals it to be the product of joining of a V sequence with its 5′-leader to a downstream D and J segment lying contiguous to the CH gene cluster but with intervening J segments and noncoding sequences still present. Each heavy chain isotype represented within the cluster is encoded by three to six coding domains, each in turn separated by short noncoding DNA stretches. For IgM there are six such coding domains for the heavy chain, two of which specify the hydrophobic

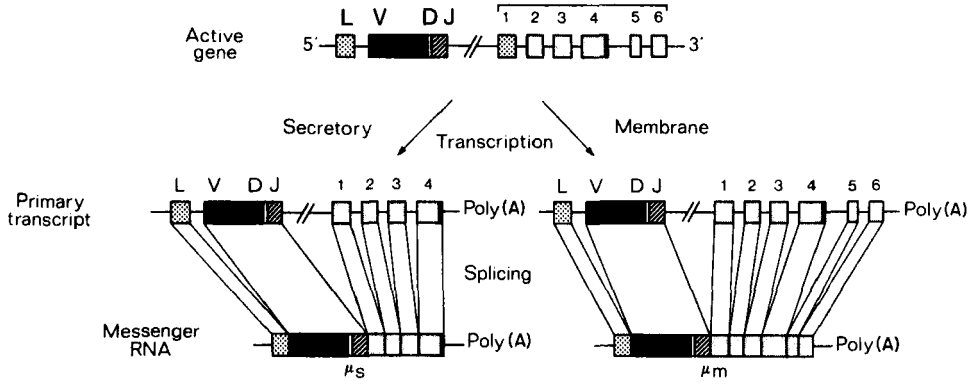


FIG. 2. Differential processing of membrane and secretory heavy chains. The top line shows an active heavy chain gene (not to scale). One V, D, and J sequence has recombined for the variable region with the leader sequence (L) lying 5′ to its V segment separated by a short intervening noncoding sequence. 3′ to these regions separated by an intervening sequence lie the exons for the heavy chain constant regions, of which there are six for the μ chains. For the secreted heavy chain, transcription ends at the fourth coding domain and includes the stop codon (indicated in black). Transcription up to and including the sixth coding domain forms the primary transcript for the membrane-bound molecule. Formation of the coherent mRNA occurs by splicing which, for the secretory molecule, removes the intervening noncoding sequence between L and V and between J and the first Cμ exons. When the transcript includes the fifth and sixth Cμ domains, then splicing also removes the stop codon but brings into the coherent messenger the last two exons which specify the anchor sequence of the membrane-bound molecule.

anchor sequence of the membrane-bound molecule. The information from these two domains are not present in the secreted form of the molecule, and this divergence arises from the translation of two separate messenger RNAs which differ accordingly at their 3′ ends. It is postulated that transcription of the heavy chain gene up to and including the fourth coding domain which contains a stop codon, which itself presumably specifies the small hydrophilic tail, would serve as the primary transcript for the secreted molecule. For the membrane-bound protein, transcription would continue to include the fifth and sixth coding domains. The formation of coherent messages from these primary transcripts is believed to occur by splicing mechanisms, indicated in Fig. 2. For both forms of the molecule, splicing removes unrearranged J segments along with the noncoding intervening sequences between L and V, J and the first heavy chain domain, and the short sequences between the first four heavy chain gene segments. Processing for membrane-bound μ chain would then splice out the stop codon of the fourth Cμ domain and bring the last two domains of the primary transcript into the message to include the anchor sequence. There is now good evidence that all isotypes are associated with their own sets of membrane exons specifying the hydrophobic tail (Alt *et al.*, 1980; Rogers *et al.*, 1980; Cushley *et al.*, 1982).

Antibodies are glycoproteins and the extent of glycosylation may differ depending on whether a molecule is secreted or membrane bound (Melchers and Andersson, 1973). This posttranslational processing also varies depending on the antibody isotype. While light chains are occasionally glycosylated, they are generally devoid of sugars, whereas carbohydrates are invariably added to the polypeptide backbones of normal heavy chains as they traverse the cell (Eagon and Heath, 1977). The assembly of the oligosaccharide units occur in a stepwise fashion during intracellular transport. The inner core sugars, *N*-acetylglucosamine and mannose are added as a single oligosaccharide, while the outer sugars are added sequentially as the immunoglobulin passes through the rough and smooth endoplasmic reticulum with galactose as the penultimate unit and terminating in fucose. While attachment of the outer sugars are probably not essential for the export from the smooth endoplasmic reticulum to the outside of the cell, glycosylation may be necessary for its preceding transport from membrane-bound polyribosomes. The extent to which an immunoglobulin is dependent on carbohydrate addition for its intracellular movement appears to be reflected by the number of oligosaccharides on the secreted product (Hickman and Kornfeld, 1978).

Whereas surface and the bulk of intracellular immunoglobulin of all classes are present as the basic four-chain H2L2 structure, the secreted forms of IgM and IgA exist predominantly as a pentamer and dimer, respectively, of the basic unit. Polymerization of these species occurs very close to the time

of secretion and is controlled by the intracellular level of a peptide-designated J chain (Roth *et al.*, 1979) (which is not to be confused with the J gene segment to which it is totally unrelated and which is encoded on a gene unlinked to either the heavy or light chain loci (Yagi *et al.*, 1982)). J chain initiates polymerization by forming a disulfide bridge between two of the basic monomer units and is incorporated into the secreted product at the ratio of one J chain per polymer. Production of J chain appears to be initiated as a consequence of B-cell stimulation; neoplastic counterparts of unstimulated cell contain little or no J chain whereas antibody secreting cells contain high levels (Mather *et al.*, 1981).

D. Multiple Isotype Expression and Heavy Chain Switching

At the phenotypic level, clonal integrity in B cell development is maintained by the expression of a single set of light chains and a single VDJ sequence at the heavy chain locus. In contrast, the effector capabilities of the B cell product can be modified by incorporating into the primary structure different constant regions from the heavy chain gene cluster. Such class switching occurs as a consequence of antigen stimulation in conjunction with the appropriate accessory signals. However, even prior to encounter with antigen, B cells can commonly express more than one immunoglobulin isotype. The capacity for multiple isotype expression and class switching while preserving the expression of a single set of variable regions has its molecular basis at two differing, though not necessarily independent, levels of processing. The order and frequency of isotype expression during B cell development (see next section) appears to relate to the 5′ to 3′ order of the *CH* genes within the heavy chain locus. For the mouse this order is now completely mapped (Shimizu *et al.*, 1982), as depicted in Fig. 3. In man it is only partially elucidated although two stretches of the sequence 3′ to the initial Cμ and Cδ genes have been identified. One of these sequences has the 5′ to 3′ genomic order of $\gamma_3 > \gamma_1 > \alpha_1$ while the second consists of $\gamma_2 > \gamma_4 > \epsilon > \alpha_2$ and is believed to lie downstream of the former (Ellison and Hood, 1982; Takahashi *et al.*, 1982; Max *et al.*, 1982; Flanagan and Rabbitts, 1982).

The most common example of dual isotype expression on B cells is that of IgM and IgD. The μ and δ genes are within relatively close proximity in the heavy chain gene cluster, and there is evidence to suggest that in cells expressing both isotypes, the two classes are generated from a single primary transcript which spans both of the *CH* genes and includes the single rearranged L-VDJ sequence (Fig. 3). Differential splicing of the transcript would serve to take out the appropriate sequences to yield the separate coherent messages for the μ and δ chains (Knapp *et al.*, 1982). Whether such a single transcript exists for other multiple isotype combinations, where the *CH* genes are separated by considerably longer distances, is unclear. Examina-

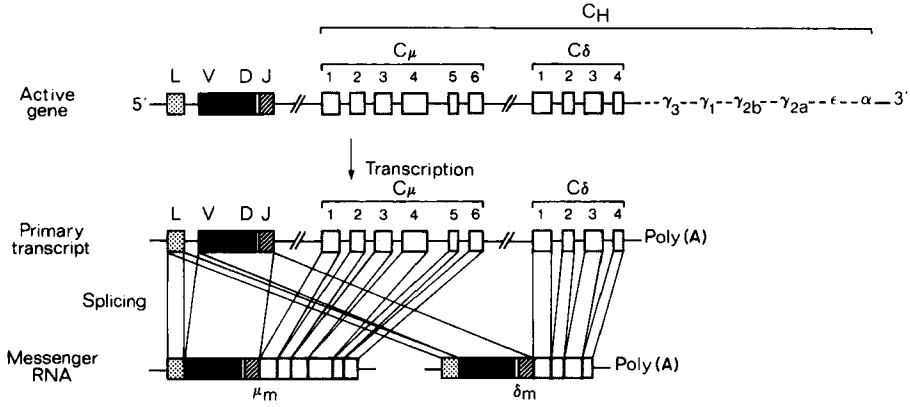


FIG. 3. Differential splicing of the heavy chain sequences for the joint expression of IgM and IgD; details as for Fig. 2 but with the exons for the δ chain constant region also shown. The relative positions of the remaining *CH* genes are indicated (as represented in the mouse). Here, the primary transcript includes the four coding domains of the δ gene. In this model, splicing would sometimes remove the μ gene from the coherent message so that the δ domains would join directly with the J of the variable region sequence and thus would be translated as the IgD heavy chain. Splicing up to the ultimate μ domain would again specify the μ chain.

tion of cells coexpressing IgM with IgE reveals that the heavy chain *C* gene remains intact, leading to the postulate that a single primary transcript does indeed span the entire length between the relevant sequences to undergo the splicing required to yield the expressed molecules with their common VH regions (Yaoita *et al.*, 1982). However, there is as yet no formal proof for such large multi-CH transcripts, nor has it been shown that such cells biosynthetically manufacture the dually expressed isotypes at the same time.

It has been suggested that dual isotype expression may be preliminary to the full differentiation of B cells involving heavy chain switching by deletions of the intervening sequences between the VDJ segment and the newly expressed heavy chain *C* gene (Honjo and Kataoko, 1978). In this model, DNA rearrangements occur during switching with the purpose of bringing a *CH* gene specifying an isotype other than μ in the proximity of the VH sequence. This probably occurs by a looping out followed by excision of the intervening information 5′ to the newly expressed *CH* gene. The recombination event is mediated by so-called switch regions (S) which comprise tandem repetitive sequences and lie in the 5′ flanking position to each *CH* gene with the exception of Cδ. It is interesting to note that for IgM secreting cells the switch region of Cμ is often deleted, which may promote high rate transcription for this area (Yaoita and Honjo, 1980). Observations on *CH* arrangements in immunoglobulin-secreting cells strongly indicate a unidirectional order of switching during differentiation which travels down-

stream from the *V*-regions. The finding of deleted switch regions even in IgM secretors argues that plasma cells are terminally differentiated with their genetic options on the immunoglobulin loci now completely closed.

E. Phenotypic Patterns of Immunoglobulin Expression during B Cell Development

The intention of this section is to provide a guideline for the events occurring throughout antigen-dependent and -independent B cell development with regard to immunoglobulin expression in order to provide a framework into which we can integrate our observations on neoplastic populations. The model outlined can be considered as only one of several possible descriptions based on our current knowledge of the B cell system. We shall return to these concepts in Section IX and examine to what extent the model can be vindicated or extended from patterns associated with B cell leukemia and lymphoma.

While the detection of endogenously synthesized immunoglobulins serves to identify a "B lymphocyte," there exists no consensus on the terminology to be used to describe these cells at their different stages of maturation and differentiation or at their varying levels of activation. This applies particularly to those cells which, although developing along the B lineage, have not yet commenced the synthesis of, or integrated into their surface membranes, functional immunoglobulins. For the purposes of this review, cells which do not phenotypically express any immunoglobulin but are nonetheless irrevocably committed to that function will be termed *pre-B progenitors*. Such cells arise from lymphoid stem cells, themselves probably a product of a pluripotent stem cell, and then (possibly as a consequence of microenvironmental factors) are channeled into the B cell line. With development along this lineage, the cells undergo the immunoglobulin gene rearrangements outlined previously and, reflecting the order of these rearrangements, begin to synthesize μ heavy chains which are initially retained within the cytoplasm. The presence of intracellular μ chains heralds the arrival of the pre-B cell first described by Raff and colleagues in 1976. A successful rearrangement on one of the light chain loci then permits the expression of complete IgM molecules at the cell surface following the transcription and translation of the active light chain gene. Such cells may be termed *primary-B cells*, and encounter with antigen at the stage when the immunoglobulin receptors are first emerging would lead to tolerogenesis probably through clonal abortion (Nossal, 1979). In the absence of a premature antigen confrontation these cells begin to transcribe and translate the δ chain units which, by combination with a possibly preexisting light chain pool, are coexpressed at the cell surface with the IgM. The emergence of IgD at the cell membrane may be intimately

involved with depressing the cell's susceptibility to tolerance induction, although the details of this process may depend on both the quality and quantity of the antigen signal received (Cambier *et al.*, 1977; Zan-Bar and Barzilay, 1982). The acquisition of IgD may also confer on the cell different migratory patterns and specify their tissue-homing potential (Gray *et al.*, 1982). Dual IgM- and IgD-expressing cells are often considered to be "mature" or "fully immunocompetent" B lymphocytes.

Following this stage, further B cell development is dependent on antigen triggering, with the precise sequence of events depending on the interplay between stimulating antigen and a variety of growth, replication, and maturation factors whose complexities are just beginning to be unraveled. An attempt toward a synthesis of the isotype changes occurring at these stages is depicted in Fig. 4. While the details are unclear, the essential features of the model include a dichotomy of developmental pathways to plasma cells or memory cells, a capacity for self-renewal of the latter, and the predominant phenotypic patterns accompanying these processes.

The predominance of IgM secretion following primary antigen challenges presumably reflects the direct maturation of an immunocompetent B lymphocyte to a IgM plasma cell. Here no isotype switches or further isotype expression would be required, and the primary processes involve a rapid loss of IgD expression (Preud'homme, 1977) and an alteration in the phenotypic disposition of the IgM from predominantly membrane bound to secretory. At some point during this linear maturation scheme and possibly interdependent on microenvironmental influences, a proliferative phase yields progeny, a proportion of which diverges along a distinct and separate memory cell lineage. There is evidence to suggest that memory responses of the IgM and IgG classes can be elaborated by populations bearing predominantly IgM or IgM along with IgD, while predominantly IgD- or IgG-bearing memory cells give rise to IgG but not IgM responses (Abney *et al.*, 1976; Zan-Bar *et al.*, 1978; Strober, 1975; Herzenberg *et al.*, 1980). Similar principles may apply to the generation of memory responses of other classes, with a general rule that those cells which have switched their expression to classes other than M and D give rise to progeny secreting that class (Gearhart and Cebra, 1981). Cells can, however, express more than one IgG isotype simultaneously. The order of their appearance along with the frequency of their expression appear to correlate directly with the 5′ to 3′ genomic order of the $C\gamma$ genes, whereas cells expressing and secreting IgA and IgE may evolve from quite distinct switching pathways (Mongini *et al.*, 1983).

These considerations at the cellular and phenotypic levels may relate to the homology of the corresponding switch regions in the heavy chain genome. The $S\mu$ region demonstrates striking homology to the $S\gamma_3$, $S\alpha$, and $S\epsilon$ in the mouse and only limited homology to other γ isotype switch se-

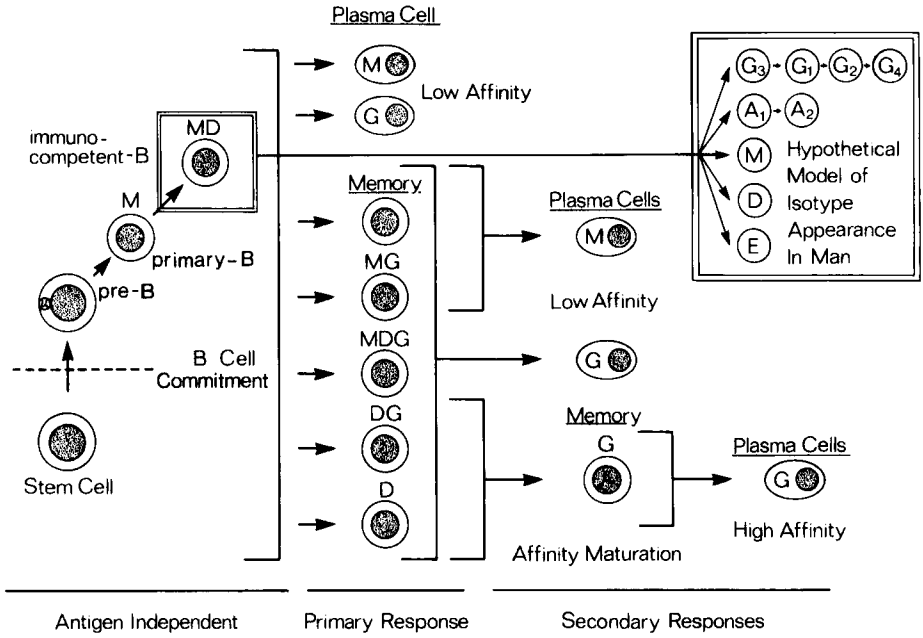


FIG. 4. A hypothetical model for immunoglobulin isotype expression during the development of IgG and IgM responses. In antigen-independent processes, emerging B cells sequentially express IgM and IgD at their surface following a pre-B stage identified by the presence of cytoplasmic μ chains. Antigen triggering elicits divergent responses either directly to plasma cells or to a memory cell pool whose phenotypic disposition is complex. Restimulation of these populations elaborates more low affinity plasma cells while those cells bearing predominantly IgD at their surfaces may also partake in the renewal of memory with IgG bearing cells then eliciting high affinity IgG responses. One possible sequence for isotype switching is indicated from the current information of the *CH* genomic order in man and the corresponding processes occurring in murine systems.

quences, the extent of which follows their downsteam order in the genome (Stanton and Marcu, 1982). There is some evidence to suggest that the quality of the activating signal may select for the predominating isotype responses to emerge from stimulation (Coutinho *et al.*, 1982). In addition, the tissue location for germinal center formation in the generation of memory cells may be crucial in determining the predominant isotype expressed in subsequent response (Butcher *et al.*, 1982).

The expression of IgD on memory cells may be involved both in their self-renewal and in the affinity maturation of the antibody response. Some experiments suggest that memory cells lacking IgD proceed directly to plasma cells and display no tendency for self-renewal, whereas those with IgD are capable of propagating further memory (Zan-Bar *et al.*, 1978). The loss of

surface IgD during self-renewal may select for cells capable of giving a high affinity antibody response (Herzenberg *et al.*, 1980). A general feature of memory cells may be a high density of surface immunoglobulin receptors (Strober, 1975) which are lost as cells respond to antigen by differentiation toward a predominantly secretory mode.

III. Common Acute Lymphoblastic Leukemia: A Model for Pre-B Development

A. Introduction

Approximately 80% of patients with acute lymphoblastic leukemia (ALL) have neoplasms in which the tumor population lacks the classical markers of surface membrane immunoglobulin (SmIg) or the capacity to form rosettes with sheep erythrocytes, which would serve to place them in either the B or T cell compartments, respectively. This so-called common form of ALL has thus been considered as a "null-cell" disorder or as non-T, non-B (Chessels *et al.*, 1977). The remaining 20% of cases appear to carry predominantly T cell markers, although a small number (<5%) can be confidently placed in the B cell category by the presence of readily detectable SmIg (Brouet *et al.*, 1976). A sizable percentage of the true B cell-derived ALL appear to involve cells of the Burkitt lymphoma type and can be considered as a distinct clinicopathologic group (Preud'homme *et al.*, 1981). These cases will be dealt with in Section IV where we discuss the phenotypic characteristics of Burkitt lymphoma in detail.

The obscure nature of the cell of origin in the common group of ALL has recently become unraveled by a combined approach utilizing monoclonal antibodies and studies at the molecular level. It is the latter of these approaches that we shall concentrate on in this section.

B. "Pre-B Leukemia"

The recognition that IgM appeared as a cytoplasmic protein before its emergence at the cell surface during B cell ontogeny prompted the screening of ALL for this early phenotypic marker. A number of independent studies have now clearly established that about 20% of null-ALL can be classified as pre-B malignancies primarily on the basis of cytoplasmic Ig (Vogler *et al.*, 1978; Brouet *et al.*, 1978). While the initial observations on normal pre-B cells suggested that the early Ig product was composed of both heavy and light Ig chains, the study of the neoplastic counterparts revealed an absence of light chains either on the surface or in the cytoplasm of the malignant cells. Careful studies on emerging B lymphocytes have now dem-

onstrated that this asynchrony of Ig chain expression is a normal physiologic event and that only at a relatively late stage of pre-B development are both the heavy and light chains found in the cytoplasm (Kubagawa *et al.*, 1982).

While an absence of surface Ig is one essential aspect of an operational definition for pre-B cells, it has been claimed that in some cases of pre-B ALL, a proportion of cells can carry at their surfaces scant amounts of μ heavy chains in the absence of Ig light chains (Vogler *et al.*, 1978). The appearance of these molecules may reflect a transient phase in the maturation of true pre-B to cells which begin to express both light and heavy chains, although the physiologic function of such a mode of Ig expression remains unclear. Nevertheless, to lend some support to this possibility, it has been demonstrated that exposure of a murine lymphoma cell line which has pre-B characteristics to the mitogen dextran sulfate leads to the expression of isolated μ chains at the cell surfaces. However, the surface heavy chains appear to be unstable, possibly reflecting that this is an aberrant mode of expression (Paige *et al.*, 1981). There is one study to suggest that normal pre-B cells can actually secrete their cytoplasmic μ chains (Levitt and Cooper, 1980), and this raises the possibility that the small amounts of these chains detected on the surfaces of some ALL cells represent molecules in transit from the cell to the extracellular environment. Such a speculation remains controversial, however, because other studies fail to point to a secretory mode for pre-B μ chains.

Another perhaps unexpected finding was that subclones of ALL populations with cytoplasmic μ chains were also capable of expressing γ and/or α chains in their cytoplasms, also without any evidence for the presence of light chains (Vogler *et al.*, 1978). A study utilizing monoclonal antibodies to analyze this phenomenon has suggested an isotype switch sequence occurring in subclones of ALL in the order of $\mu \rightarrow \gamma_1 \rightarrow \gamma_4 \rightarrow \alpha$ (Kubagawa *et al.*, 1983). Neither of the other two γ chain isotypes nor δ or ε chains were detected in any of the cells investigated. In this study, all apparently switched cells also expressed κ light chains in their cytoplasms; dual expression of heavy chain isotypes, particularly μ with γ_1, was also evident. These observations led the authors to suggest that events occurring on the light chain alleles could influence or control events on the heavy chain genes at the pre-B level, although the genes were located on separate chromosomes. For a murine pre-B leukemia line, isotype switching has been seen without either light chain gene rearrangements or deletion of the Cμ gene, suggesting that RNA splicing mechanisms may be responsible here (Alt *et al.*, 1982). In addition, a case of ALL has been described which expressed only γ chain within the cytoplasm and was found to have its Cμ segments on both alleles intact (Korsmeyer *et al.*, 1983). Careful probing failed to reveal such isotype switching occurring during normal pre-B development (Kubagawa *et al.*,

1982); thus it is concluded that this event is either extremely rare or that its presence in some cases of ALL may reflect chromosomal abnormalities.

C. "Pre-B Progenitor Leukemia"

Some of the most exciting findings to emerge from the recent studies on common ALL has come from observations of the immunoglobulin gene rearrangements in these populations. These results imply that, with few exceptions, common ALL represents neoplastic proliferations of cells, which are at some level committed to development along the B lineage. In a study of eight cases of non-T, non-B ALL, Korsmeyer and colleagues (1981) demonstrated varying degrees of Ig gene rearrangements in all but one case. Five of the cases had no detectable Ig product so that conventional phenotyping of these populations would fail to reveal an association with the B cell lineage. This study on the gene rearrangements in ALL was consistent with the hierarchal model outlined in Section II. Thus cases with light chains rearrangements had all undergone rearrangements on the heavy chain loci, while populations that had rearranged their λ genes had also rearranged the κ chain genes. This pattern was born out in a recent survey of a larger group of cases of "non-B, non-T" ALL (Korsmeyer *et al.*, 1983). In this study 25 out of 25 cases had undergone rearrangements at the heavy chain locus, while 11 of these had further rearranged at least one light chain allele. Despite an invariable occurrence of rearrangements, only 7 out of 17 cases tested expressed any detectable Ig gene product. This was explained in part by the probable high frequency of aberrant rearrangements on both heavy chain alleles. An alternative explanation is that a lack of phenotypic products in the presence of functionally active genes could simply reflect a transient differentiation stage immediately prior to their active transcription or translation. Some support for this possibility came from a study on a single case where it was shown that exposure of the leukemic cells to modulating factors *in vitro* led to the expression of both μ heavy chains and κ light chains from the previously silent, though rearranged, Ig genes (Cossman *et al.*, 1982). In those cases of ALL where abortive rearrangements appeared to have occurred on both heavy chain alleles, the possibility exists that it is this event which traps the leukemic cells at the pre-B progenitor stage of maturation arrest. Such cells are probably normally eliminated, but in the case of ALL malignancy may confer inappropriate survival. This may also apply to one case described in which only light chain products could be detected despite rearrangements having occurred at the heavy chain locus. Clearly ALL provides the ideal models for dissecting these processes in man.

The notion that common All represents malignancies of B cell precursors is being supported from studies utilizing monoclonal antibodies to probe

non-Ig surface antigens on these populations. These studies also indicate that the phenotypes represented may accurately reflect those of normal cells developing along the B lineage. For several years it has been realized that most cases of non-T, non-B ALL express a 100K glycoprotein termed cALLA (Greaves *et al.*, 1975). The nature of normal fetal cells expressing this antigen has been elucidated, and their phenotypic profiles appear to be faithfully mirrored by those of ALL populations (Hokland *et al.*, 1983). A new B cell antigen, B4, has been described which appears to be expressed across the entire spectrum of the B cell lineage, with the one exception of plasma cells (Nadler *et al.*, 1983). Using this monoclonal antibody as a probe, it has been shown that virtually all common ALL, including pre-B and putative pre-B progenitor types, express this B cell lineage antigen. The detection of other B cell-associated antigens on most common ALL is in agreement with this (Korsmeyer *et al.*, 1983). With this accumulated evidence there appears little doubt in including common ALL in that group of neoplasms which represent malignancies of the B lymphocyte lineage.

IV. Burkitt Lymphoma and Related Tumors

A. Introduction

In 1958 Burkitt described a malignant lymphoproliferative disorder which occurred at particularly high incidence in well-defined areas of the African continent and New Guinea. The etiology of this disease has been the subject of considerable speculation, much of it fueled by the observation that the malignant cells harbor the genome of the lymphotropic herpesvirus known as the Epstein–Barr virus (EBV) (Nilsson and Klein, 1982). While EBV is ubiquitous in man, outside of the endemic regions mentioned its appearance is usually asymptomatic or results in the self-limiting lymphoproliferation of infectious mononucleosis (Henle and Henle, 1973). The intimate association of the virus with the African Burkitt lymphoma (BL) strongly implies that it may be involved at some stage in the development of this malignancy in these cases (Nilsson and Klein, 1982).

BL commonly presents as a lymphoblastic lymphoma. Lymphomas with identical morphologies and histologies to those described by Burkitt do arise in nonendemic areas and are variably termed American BL, European BL, non-African Burkitt, or simply Burkitt-type lymphoblastic lymphoma (Burkitt, 1967). A further related disorder appears as ALL with Burkitt cells, classified primarily on morphologic criteria. The etiology of all these non-African types of BL contrast to the major group by the frequent absence of the EBV genome in the malignant population (Andersson *et al.*, 1976).

However, a molecular link exists between all groups of this morphologic entity which is, almost without exception, a specific set of chromosomal translocations involving chromosome 8 and the chromosomes carrying the immunoglobulin genes. A reciprocal translocation occurs between 8 and, most commonly, chromosome 14 which carries the heavy chain gene cluster. Less often, the reciprocal genetic element may be transposed from either chromosome 2 or 22 which carry the two light chain genes. There is accumulating evidence to suggest that these translocations may be intimately related to the target cell of transformation, the resulting processing of the Ig genes and the active expression of an oncogene (reviewed by Klein, 1983).

B. Phenotypic Expression of Immunoglobulin by Burkitt Lymphoma Cells

In striking contrast to the cells of most B cell malignancies, the tumor cells of BL adapt readily to long-term tissue culture. This had led to a wealth of studies on BL-derived cell lines in terms of Ig production and the expression of other phenotypic markers (e.g., see Nilsson and Klein, 1982). Such studies will only be briefly mentioned here; the prime intention is to survey our current knowledge on the capacity of freshly explanted BL cells to process and express Ig molecules.

African BL represents the first group of malignancies in which it was recognized that immunoglobulins were expressed on the neoplastic cells as constitutive membrane proteins (Klein *et al.*, 1968). Subsequent studies on large numbers of biopsies have revealed, with few exceptions, the presence of readily detectable surface immunoglobulins which are associated with a single light chain specificity (Gunven *et al.*, 1980). In contrast to the high frequency of IgM expression, few of the African BL populations appear to synthesize appreciable amounts of IgD for insertion into the cell membrane. In a recent survey it was noted that in only 2 of 53 biopsies studied could IgD molecules be detected at the tumor cell surfaces and then only at threshold levels on a minority of cells (Gunven *et al.*, 1980). The authors raised the possibility, however, that the conditions prevailing during the transport of the material from Africa to Sweden may have affected the presentation of the IgD molecules at the surfaces of the BL cells. While selective cleaving of the IgD isotype under mild conditions is not an unreasonable proposition, the inability to detect this class may be an accurate reflection of the phenotypic status of the fresh cells, as in established BL cell lines IgD expression is uncommon (Nilsson and Klein, 1982).

An interesting observation on African BL concerns the presence of IgG as a membrane constituent. In fact, IgG appears as an endogenously synthesized surface molecule only rarely in African BL as reflected in studies on

both fresh biopsies and cell lines. A particularly high number of biopsies do, however, seem to be associated with extrinsic IgG which shows a low level reactivity of staining and which can be eluted by low pH, accompanied by dual light chain specificity, and associated with the presence of complement components. This material probably reflects the presence of antibodies to EBV-associated membrane antigens known to exist in these patients, and its presence on fresh biopsy cells may relate either to direct binding to the target membrane antigen or the binding to shed antigen forming immune complexes attaching to the cell surfaces via either Fc or C3 receptors (Klein *et al.*, 1969).

Few cases of non-African BL have been studied in regard to their expression of immunoglobulin molecules but virtually all cases investigated to date have revealed unequivocal staining for membrane Ig. In addition, non-African BL appears to show a similar predominance in its commitment to the expression of the $C\mu$ gene as does its African counterpart (Mann *et al.*, 1976; Gajl-Peczalska *et al.*, 1975). This preference is also shared by those cases of ALL with BL cells. In a study of 25 cases of this type, Preud'homme and colleagues (1981) noted that 24 carried monotypic surface Ig which was typically IgM and was expressed at generally high density. Of 10 of the cases tested for the presence of IgD, 3 were positive but only weakly on a minority of cells. Among these cases, however, 3 had tumor cells which stained (apparently unequivocally) for IgG, while a fourth case demonstrated expression of IgA at the cell surfaces. The capacity of these cells to express classes other than M and D indicate that the spectrum of cell types represented by the BL morphology covers cells which have undergone class switching.

C. Biosynthesis of Immunoglobulin by Burkitt Lymphoma Cells

Except for some early studies where the clonal origin of the detected product is unclear (Osunkoya *et al.*, 1968; van Furth *et al.*, 1972), there have been few investigations on the capacity of fresh BL biopsy cells to actively synthesize Ig *in vitro*. For this group of neoplasms we make the exception of using established cell lines to elucidate some of the molecular processes occurring during the handling of Ig by the tumor cells. However, caution is taken to highlight only those examples where the results bear directly on features associated with other malignancies covered in this review.

A particularly intriguing observation comes from a study with the Ramos line which is an atypical African BL line lacking the EBV genome. The aim of the authors (Spira *et al.*, 1981) was to document phenotypic shifts occurring on EBV conversion of the line. Using a quantitative radiolabeling assay, they noted that EBV conversion resulted in the appearance of IgD molecules at

the surface of the previously IgD negative cells. It was also shown by biosynthetic labeling that the converted subline synthesized an Ig which under reducing conditions migrated slightly faster than μ chains with a mobility consistent with that for δ chains destined for surface expression. The authors also noted that an excess of light chains, present on the surfaces of the nonconverted cells, was much reduced on the cells of the converted line. They concluded that the results taken together suggested that EBV conversion induced these cells to take "a small step to the right" in differentiation.

In a comparative study on African versus American BL lines and their capacity to synthesize immunoglobulins, it was noted that in contrast to African BL, which is known to synthesize IgM primarily for insertion into the membrane, many of the lines derived from American BL released a large proportion of their IgM into the extracellular environment (Benjamin *et al.*, 1982). This was interpreted as active secretion of the IgM by a number of criteria including a high molecular weight, an association with J chain and the improbability that such a large amount of material could be associated with simple membrane turnover. Even among the African BL lines the authors noted some respectable levels of IgM exported in a few of the cases suggesting that the biosynthetic options of these cells are not totally compromised. A feature common to both geographic tumors was the secretion of free immunoglobulin light chains from the established lines.

Both African and non-African BL are often associated with hypogammaglobulinemia, and the presence of a monoclonal serum Ig is not a common feature. In contrast, five cases of ALL with BL cells reported from Preud'homme's group (1981) had a circulating IgM paraprotein. In addition, two of their IgG-expressing cases were associated with a Bence–Jones protein of identical specificity to the light chain of the proliferating lymphoblasts suggesting that the biosynthetic light chain excess noted in the BL lines is not a feature restricted to the IgM-producing clones.

D. A Normal Analog for the Burkitt Lymphoma Cell?

On the basis of morphologic and histologic criteria, it has been suggested that both African and non-African groups of BL are derived from cells of the germinal centers (Gajl-Peczalska *et al.*, 1975; Lennert, 1978). The large number of cases which are extralymphatic tends to compromise this hypothesis, however. The predominance of IgM-expressing clones and a usual lack of surface membrane IgD molecules argue equally for these cells representing either a very early stage in development, prior to the activation of the Cδ gene, or a later cell which has possibly undergone antigen stimulation and has lost the necessity to express IgD. The typically high density of IgM molecules at the BL cell surface, coupled with its lymphoblastic morphol-

ogy, tends to favor analogy to the antigen-stimulated cell, possibly a cell which has emerged from the germinal center and is the immediate precursor of the immunoblast. Also in favor of this later stage of differentiation is the occasional finding, particularly among the ALL variants of BL, of IgG or IgA expression predominating; such cells would only be expected following antigen stimulation.

Hypotheses concerning the phenotypic normal equivalent of BL cells must take account of recent studies at the molecular level concerning the rearrangements and translocations on the Ig loci in these cells. While we cannot yet extrapolate fully, the finding of specific translocations at these locations raises the possibility that the phenotypic profile of BL may be fixed or modified to such an extent as to render a search for normal equivalents inappropriate. It has now been shown for a few cases of BL with the 8:14 translocation that the breakpoint on chromosome 14 is on band q32 and cuts across the heavy chain gene cluster with the 5′ end of the VH being transposed to chromosome 8. Reciprocally, a small portion of chromosome 8, which is known to carry the c-*myc* oncogene, appears to be localized at the same region of chromosome 14 which is within the DNA restriction fragment encoding for μ chains, although the precise position may differ between individual clones (see Klein, 1983). For murine plasmacytomas, related sets of translocations appear to occur with a high incidence of c-*myc* translocating to the switch regions of the *CH* genes. The implications for the expression of either the oncogene or immunoglobulin remains somewhat enigmatic because the translocations seem to occur on the allelicly excluded partner of the chromosome pairs. Nevertheless, even the nonfunctionally rearranged allele may be transcribed, while some evidence points to the possibility that the rearrangements on the excluded allele may influence the expression of the functional partner.

In a study on the variant translocations of BL, Lenoir and co-workers (1982) demonstrated that the functionally expressed light chain isotype of individual clones correlated directly with the locus involved in the translocation; thus those with an 8:2 translocation expressed κ chains, and those with an 8:22 translocation expressed λ chain immunoglobulin. A mechanism to account for a functional correlation between the expressed and nonexpressed allele, which might also apply to the heavy chain locus, is that the timing of the translocation risk may coincide with that of the normal Ig DNA rearrangements during B cell development. Therefore, although in most cases BL clones express characteristics of mature cells, the oncogenic "hit" may have occurred at the pre-B or pre-B progenitor stage, and the direct involvement of the Ig loci may subsequently modify the phenotypic characteristics of the emerging populations.

V. The Chronic Type Lymphocytic Leukemias: Models of Small Resting B Lymphocytes

A. Introduction

The common feature of chronic type lymphocytic leukemia is that it represents neoplastic proliferations of small lymphocytes (Lennert, 1978). In addition, with very few exceptions, the tumor cells express immunologic markers which serve to place them in the B cell compartment (Aisenberg *et al.*, 1973). Otherwise, this group of neoplasia present a relatively heterogeneous profile with regard to patient survival, clinical activity, histology, and precise morphologic and immunologic features. Primarily from histopathologic criteria, this disease spectrum encompasses three main subtypes which, although at their extremes may reveal quite distinct features, should probably be considered as a continuum of a single underlying disorder. The Kiel classification (Lennert, 1978) of malignant lymphoma distinguishes between B-chronic lymphocytic leukemia (B-CLL), B-prolymphocytic leukemia (B-PLL), and immunocytoma (IC). B-prolymphocytic leukemia is considered to be a rare morphologic variant of B-CLL proper, distinguished from the common type by a relatively large cell with a moderate amount of cytoplasm and distinctive nuclear features. Immunocytoma is distinguished from both B-CLL and the B-PLL variant by a degree of pleomorphism within the tumor population which are usually composed of small cell types but which also contain some plasma cells and/or immediate plasma cell precursors. This differential diagnosis is based on lymph node histology, and the leukemic populations of IC may be quite indistinguishable from those of true B-CLL. For the purposes of this review, we shall consider the chronic type B lymphocytic leukemia (chronic B-LL) primarily as a single disease spectrum and highlight those features relating to the phenotypic expression of immunoglobulin which may differ at the extremes of the subtypes.

B. General Considerations

Classically, the B cell nature of the overwhelming majority of chronic B-LL cases has been recognized by an absence of receptors for sheep red blood cells and the presence of surface immunoglobulins which appear to have an intrinsic origin (Aisenberg *et al.*, 1973; Kubo *et al.*, 1974; Brouet *et al.*, 1975). The amount of Ig found at the B-LL cell surface is generally considered to be low and has been estimated to be at some 10% of the level of normal adult peripheral blood lymphocytes (Chen and Heller, 1978). This has led to the assumption that B-LL populations are defective in their han-

dling of immunoglobulin and that the malignant cells may be more akin to members of the T lymphocyte lineage. Some recent findings on the reactivity of monoclonal antibodies which define predominantly T cell populations with B-LL cells could be interpreted to support the latter of these hypotheses (Royston *et al.*, 1980). However, as we shall see in the following sections, this is clearly not the case, and most workers concede that B-LL can be placed confidently within the B cell classification. The more pertinent questions are what portion(s) of the B cell universe are represented and how do these cell types process immunoglobulin molecules?

C. The Expression of Surface Immunoglobulins by B-LL Cells

1. Dual IgM and IgD Expression

From the earliest studies IgM was established as a predominant isotype at the surfaces of B-LL cells (Aisenberg *et al.*, 1973). This has been borne out by numerous studies over the past decade and applies almost equally to B-CLL, B-PLL, and IC (Herrmann and Wirthmuller, 1982). In addition, all studies have revealed that the surface Ig of individual clones is associated with only one or other of the light chain isotypes, hinting strongly at endogenous synthesis of the product. The discovery of IgD as an important B cell surface antigen prompted studies of its presence on neoplastic cells. It was subsequently shown that many B-LL populations expressed this isotype, usually along with IgM. Both isotypes not only shared the same light chain class in individual clones but also apparently the same sets of variable regions. In those cases of B-LL which were associated with a related serum paraprotein (which by definition must be considered as IC), it was a relatively simple matter to raise antibodies and render them specific for those unique determinants that define the clonal product (the idiotypes). It was shown that the antiidiotype antibodies reacted with both IgM and IgD expressed on single B-LL cells (Fu *et al.*, 1975). The idiotypic identity of the two isotypes in single clones has been confirmed for those cases which are not associated with a circulating M component (Stevenson *et al.*, 1981). The relative levels of expression of the two isotypes can reveal considerable variation, both between individual populations and among different cells within the same clone, and probably reflect interclonal heterogeneity in the stages of maturation arrest and intraclonal shift within this restriction, respectively (Preud'homme *et al.*, 1977). While there are cases which appear to express only IgM (at least at the level of detection), B-LL expressing only IgD is extremely rare. The proportion of double isotype-expressing cells does not seem to differ significantly in B-CLL, B-PLL, and IC (Hermann and Wirthmuller, 1982). In contrast, the overall density of membrane-bound

Ig may differ considerably. B-PLL is associated with high level expression of SmIg and thus may represent more advanced stages of maturation arrest by analogy of normal B cell physiology (Buskard *et al.*, 1976).

2. *Expression of Isotypes Other Than IgM and IgD*

The frequency of expression on B-LL populations of isotypes other than M and D, particularly IgG, is controversial. There is clearly a subgroup of B-LL which predominantly expresses IgG, exhibits an atypical morphology, and should probably be considered as IC rather than true B-CLL. These cases may occasionally be associated with an IgG paraprotein (Rudders, 1976). Whether IgG is commonly expressed by true B-CLL is more questionable.

We and other researchers (e.g., Stevenson *et al.*, 1981) often find IgG on the surfaces of B-LL cells isolated directly for peripheral blood. In contrast to the usual uniform circumferential staining associated with IgM and IgD, the pattern of staining for IgG often appears speckled or spotty. This is often accompanied by a similar staining pattern for both light chain isotypes simultaneously, denoting a probable exogenous origin for this material. If the simple precaution of incubating the cells at 37°C in medium free of human serum prior to staining is taken (Lobo *et al.*, 1975), the extrinsic material is usually dissociated so that subsequent staining reveals an absence of IgG and the presence of a single light chain isotype only. However, despite this caution, methods of detection more sensitive than immunofluorescence can often reveal that some IgG remains associated with the cell surfaces. Here, the isotype may be expressed with other immunoglobulins (usually IgM and IgD but also occasionally IgA) and appears to be restricted to one light chain class (Dahliwal *et al.*, 1978). A claim for such multiisotype expressors has been made for normal B lymphocytes where it has been suggested that this represents an early stage of differentiation prior to the emergence of the fully competent B lymphocyte expressing only M and D (Abney *et al.*, 1978).

Partly in order to resolve these controversies, Stevenson and colleagues (1981) examined the idiotypic restriction of the different immunoglobulin isotypes isolated from a number of B-LL populations. The approach relied upon the retention of the various isotypes on columns to which antiidiotype antibodies generated for the individual clones had been immobilized. While the overwhelming bulk of IgM and IgD was retained, the majority of the IgG passed through unimpeded, indicating an origin other than the tumor clone. Nevertheless, while these experiments convincingly demonstrate an extrinsic origin for the bulk of IgG associated with B-LL cells, it does not exclude the possibility that trace amounts of clonally restricted IgG could be expressed. To further cloud the issue, a recent study has added more weight to the contention that multiple isotype expression is a common feature of the B-LL (Hsu, 1981). Here, the author utilized a novel approach of first incubat-

ing the cells with fluorescein-labeled antibodies at 37°C for several days to remove all SmIg by stripping. Following this procedure, all Ig isotypes originally detected, including IgG, were reexpressed suggesting that they did derive from the tumor clone. Again, the light chain restriction noted in this study would tend to support such a notion.

In conclusion, from the survey of the current literature, it seems impossible to exclude the presence of small quantities of endogenously synthesized IgG on B-LL cells. However, by comparison with the major isotypes IgM and IgD, the bulk of IgG usually detected on these populations is probably of extrinsic origin. This conclusion is supported from our own studies where biosynthetic labeling of B-LL populations generally failed to reveal peaks of radioactivity associated with reduced IgG chains on SDS gels, while material in the region expected for μ and δ chains was readily detected (Gordon, 1979; Hannam-Harris *et al.*, 1980).

In contrast to the typical situation outlined above, there are clearly exceptional cases where the leukemic populations coexpress IgM with IgG in amounts which are readily detectable and which share a common light chain isotype. For one such case we demonstrated that the neoplastic cells actively synthesized both isotypes *in vitro* (Gordon and Smith, 1980). More recently we identified a case where the neoplastic cells predominantly expressed IgM at the cell surfaces, while IgG was detected in the cytoplasm and was actively secreted *in vitro* (Gordon *et al.*, 1983b). Such double IgM- and IgG-expressing cells are likely to represent stages of maturation restriction at varying levels in the switch from IgM to IgG production (Rudders and Howard, 1977). It will be of considerable interest for future study to determine whether the capacity of B-LL clones to express both isotypes simultaneously is due to separate long-lived messengers transcribed from the heavy chain cluster or to differential splicing of a single primary transcript spanning the Cμ to Cγ gene segments. Those cases expressing membrane IgM while secreting Ig specified by downstream genes on the CH cluster may be particularly valuable for examining the DNA arrangements at the transition from a primarily membrane-bound mode of one isotype to a secretory mode of another. The considerations outlined for B-LL cells expressing IgG with or without other isotypes may equally apply to other downstream specified Ig classes, although the apparent infrequency of their occurrence would limit their potential usefulness.

D. Cytoplasmic Immunoglobulins in B-LL Cells

It follows that all actively Ig synthesizing cells contain intracellular immunoglobulins so that the consideration of whether B-LL, or any other cell-type, is positive is essentially quantitative. The reported frequency of neo-

plastic clones in B-LL where cytoplasmic immunoglobulin (cIg) can be detected varies considerably between different series, which presumably reflects the sensitivity of both the microscope being used and the antisera, coupled with the experience and discriminatory capacity of the observer. While many consider B-LL as expanded clones of cIg-negative B lymphocytes, virtually all cases appear to be positive to some researchers, and the detection of cIg has even been claimed to be a more reliable probe for conality than SmIg in B-LL populations (Han *et al.*, 1982). This may reflect the possibility that, while B-LL cells often express only sparse amounts of membrane-bound material, the actual Ig content of the cells (or at least the clone) is similar to, or even higher than, that of normal adult B lymphocytes (Johnstone, 1982). Thus, many of the populations in B-LL may comprise dominating subclones where the cells reflect a stage of maturation arrest representing the transition from manufacturing and assembling the constitutive immunoglobulin molecules to transporting them to the cell surface.

The patterns of both intracellular Ig distribution and quantitation, in terms of the intensity of staining and the number of cells registered as positive, vary considerably among the B-LL and provide a pointer to the complexity of cell types and modes of Ig handling reflected in this histopathologic category. In a study of 48 cases of B-LL, which included almost equal numbers of true B-CLL and IC, we determined that slightly less than half were composed of subclones where the cells stained clearly for identifiable cIg (Gordon *et al.*, 1983b, and unpublished observations). These subclones represented from as little as 5% of the total malignant population up to virtually 100% (in some cases) of the recognizable leukemic clone. In five of the cases, despite a clear unequivocal staining for Ig heavy chains, cytoplasmic light chains were either not detected or were present only at comparatively low levels. The heavy chain isotype was μ in four cases and γ in the fifth, and all examples contained amounts of surface Igs that were either undetectable or just barely detectable. These and other phenotypic markers pointed to a stage of differentiation for these populations which could be most readily reconciled to the transition from pre- to primary-B lymphocyte. In three of these cases, the intracellular localization of the heavy chain was distinctly granular, while in all cases staining was confined to an extremely narrow rim of cytoplasm (Gordon *et al.*, 1983a). The possibility that rare cases of B-LL may be frozen within a narrow window of differentiation corresponding to the late pre-B compartment has been raised by other authors (Guglielmi *et al.*, 1982a).

The remaining cIg-positive cases of B-LL were clearly neoplastic expansions of more mature B cells, evidenced from the expression of readily detectable SmIg and intracytoplasmic staining for both the heavy and light chains equally. Of these 19 cases, 17 contained IgM positive cells and 2 were

IgG positive. While both cases of cIgG-positive B-LL were actually IC and were associated with a serum paraprotein, only 4 of the IgM cases had a related paraprotein, although the diagnosis of IC predominated, some cases were clearly of the "true" B-CLL type. It is worth noting that those cases having an associated paraprotein usually contained a higher percentage of cIg-positive cells and also showed a stronger intensity of staining. Five of the nonparaprotein-associated cases revealed patterns of cIg distribution that were clearly unusual. These and other examples of atypical Ig-packaging modes in B-LL are covered in more detail later.

While IgD is heavily represented as an integral membrane protein, it is rarely found as a detectable intracellular isotype either in B-LL or in normal cells. To the best of my knowledge, there appears to be only a single case of this type reported in the literature for B-LL. The patient had no unique features although the neoplastic clone lacked detectable SmIg of any isotype while the intracellular IgD was found to be present as predominantly δl half-molecules (Gordon *et al.*, 1977a). Although we initially suggested that it might be the failure of these subunits to combine which resulted in the lack of secretion noted, it is now known that in IgD-producing cells this form is a common intermediate. Thus the apparent block on secretion in this and other nonsecretory cIg-positive clones may be a reflection of a stage of B cell development just prior to active secretion of the molecules rather than any inherent secretory defect. The only other exceptional feature of our cIgD-positive case was its association with κ light chain, because most IgD myelomas and normal plasma cells show a strong preference for λ light chains (Pernis *et al.*, 1969). The possibility thus exists that cells which accumulate IgD of the κ type either fail to develop toward plasma cells or lack the cellular apparatus required for secretion. These points will be discussed in the following section.

E. Immunoglobulin Secretion by B-LL Populations

1. IgM Secretion

Although immunocytoma may be associated with a readily detectable M component in about 25% of cases (Lennert, 1978), B-CLL has been widely regarded as a neoplasm of non-Ig-exporting B lymphocytes. Some workers take this to represent a defect in the secretory apparatus of these cells, but it more likely represents the handling of the Ig molecules by normal counterparts at equivalent stages of maturation. In a study of 30 cases of B-LL not associated with an M component, we found that metabolic labeling procedures, while readily revealing the secretion of Ig light chains (see following sections), rarely showed detectable output of Ig molecules containing

heavy chains (Gordon, 1979; Hannam-Harris *et al.*, 1980). In those cases where labeled heavy chain determinants could be detected in the supernatants from short-term cultures, the amounts were usually small, suggesting a possible derivation from surface membrane material which could have been shed during the time of culture or from cell death with the resulting spilling of the contents to the extracellular environment. Clearly more sensitive assays were required to resolve these questions.

In conjunction with the use of antiidiotype antibodies to confirm the clonal identity of the detected product, Stevenson *et al.* (1980) demonstrated by radioimmunoassay that the export of small quantities of IgM was a common feature of B-LL populations. That the product was actively secreted was strongly indicated by its high molecular weight which was consistent with the pentameric IgM form. Subsequent studies have confirmed that B-LL populations often produce extracellular IgM which can be detected by sensitive immunoassays and is restricted to the light chain isotype of the neoplastic clone (Gordon *et al.*, 1983a,b; Johnstone, 1982). These populations contain both the secretory and membrane forms of messenger RNA, further indicating that the extracellular product arises from active secretion (Cossman *et al.*, 1983). However, in all these studies, both at the level of the messengers and the translation products, there has been no opportunity to determine whether the secreted IgM derives equally from all cells within the population or is the product of minor subclones with more differentiated features than the dominating population. The two alternatives each pose some important questions. The former implies that the dominating morphologic subtype in B-LL possibly transcribes two primary transcripts from the Cμ gene segment or undergoes differential splicing of a single primary to yield the two forms of messengers for the different types of μ chains and, in addition, presumably synthesizes the J chain required for polymerization of the basic Ig units. Both of these features would either serve to place the B-LL at a more advanced stage in maturation than previously considered or, alternatively, to ascribe both molecular events to earlier points in B cell development than commonly supposed. On the other hand, if the pentameric IgM is the product of a minor subclone, then this implies that the so-called maturation block in chronic B cell leukemia is simply quantitative rather than absolute; this, in turn, has some important ramifications for the underlying pathology of these disorders. As an approach to these questions, we have recently instigated a study of B-LL populations which combines the detection of Ig secretion by immunoassay with the enumeration of the number of high-rate Ig-secreting cells by a plaque-forming cell (PFC) assay (Gordon *et al.*, unpublished observations).

Although only a limited number of clones have been studied so far, there appears to be a strong, direct correlation between the presence of extracellu-

lar Ig from short-term cultures of B-LL populations and the number of PFCs (Fig. 5). While idiotype-specific antibodies are required for definitive proof, the clonal identity of the product and the PFCs are strongly indicated by the strict light chain restriction noted. These observations suggest that the Ig exported from B-LL populations may be a consequence of a few cells escaping from or "sneaking through" the differentiation block imposed on the bulk of the clone to give rise to a few high-rate Ig-producing plasma cells. From quantitation in the ELISA assay, it can be estimated that each PFC exports on the order of 10^6 pentamers of IgM per hr. While this is in keeping with the extremely high secretory capacity of plasma cells, we cannot exclude the possibility that other members of the clone are placed at intermediate stages of differentiation actively secreting Ig but that they are not in sufficient amounts to form a plaque. Recent studies on chronic B lymphocytic leukemia populations at the ultrastructural level provide support for the possibility that minor subclones with more differentiated features coexist with the more abundant small lymphocyte clonotypes (Goudin *et al.*, 1982).

In striking contrast to the apparent intraclonal variation in the capacity of

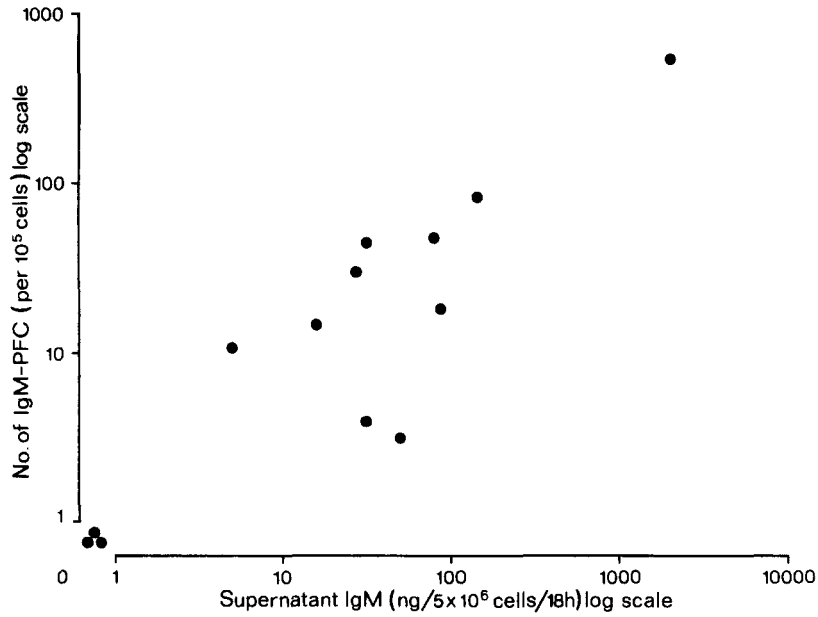


FIG. 5. Correlation between IgM secretion and the number of PFCs in chronic B lymphocytic leukemia populations (logarithmic scale). The quantities of IgM (ng/5 × 10^6 cells) released from B-LL populations into the culture supernatants over an 18-hr period are shown for 13 cases. At the same time, the number of IgM PFCs were enumerated.

B-LL cells to process Ig in its varying molecular forms at different rates, cases where double isotype (other than IgM with IgD) secretion can be detected, either at the level of PFC or by assay of the culture supernatants, are rare. This implies that the majority of B-LL populations are either intrinsically "fixed" into one route of development or that, in most cases, the clones have not yet received, or are refractory to, the signals required for undergoing the deletion events on the CH locus which may be necessary for switching to the secretion of isotypes other than IgM. For those exceptions where secretion of IgG is detected, IgM usually can not be found in the culture supernatants, confirming the notion that B-LL populations are channeled individually into a single branch of B cell development.

2. *IgD Secretion*

An intriguing observation has recently been noted on the processing of IgD molecules by B-LL populations (Stevenson *et al.*, 1983). In a study of 23 cases, 10 showed significant output of IgD into the supernatant from short-term cultures. The remarkable feature here was that all these cases were associated with λ light chains while κ-bearing clones, although expressing IgD at the cell surfaces, never released this material from the cells. The authors of this study argued that the extracellular IgD was probably secreted because it lacked any association with vesicles which might be expected for shed membrane material. Although they have not been sought, it is less likely that the extracellular IgD arose from plasma cell members of the malignant clones as we suggested might be the case for secreted IgM from B-LL populations. The distinct possibility then exists that the IgD may have resulted from the differential processing of the Cδ gene sequences within individual leukemic cells to yield messengers for the membrane and secreted molecules, respectively. Whatever the cellular origin of the secreted IgD, it is of extreme interest that the λ chain isotype preference for this secreted Ig class should be manifested at the level of the clonotypes represented by the B-LL. This may indicate that the machinery controlling IgD secretion can only develop in the λ chain-bearing clones or, alternatively, that IgDk molecules destined for secretion are prone to rapid intracellular degradation. The possibility also exists that events on the various light chain loci may control transcriptional processes on the heavy chain gene cluster. Obviously the monoclonal populations represented by the B-LL will provide ideal models for dissecting these separate molecular possibilities.

3. *The Export of Free Immunoglobulin Light Chains*

In two independent studies it was demonstrated, by metabolic labeling techniques, that B-LL populations synthesized light chains in excess of their requirement for the formation of complete Ig molecules and that the free

light chains were actively exported from the cells (Maino *et al.*, 1977; Gordon *et al.*, 1978). For the majority of cases in our study, radioactive precursor labeling of the cells followed by immunoprecipitation and gel analysis of the labeled products revealed that free light chains were usually the sole detectable secreted Ig product and that they were always present in vast excess of labeled heavy chains. Labeled intracellular material almost invariably comprised both heavy and light chains, although the latter was always present in molar excess. Representative examples of this pattern of Ig synthesis are given in Fig. 6. The association of a large excess of secreted light chain over heavy chain has now been confirmed in a number of laboratories

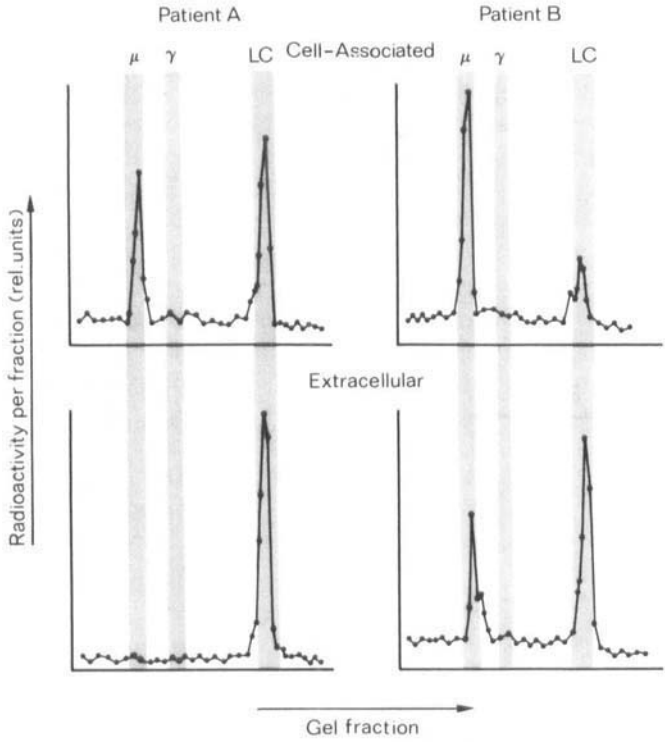


FIG. 6. SDS gel analysis of labeled immunoglobulin from chronic B lymphocytic leukemia populations. The gel profiles of metabolically labeled immunoglobulin specifically precipitated from two B-LL populations are shown following reduction and alkylation. Patient A represents the more typical pattern with only light chains in the supernatant material, while the pattern of Patient B, with some heavy chain, is representative of about 30% of cases studied (see e.g., Gordon *et al.*, 1978). In all cases studied, a more balanced level of heavy and light chains was noted for the lysate material. The position of the reduced chains from IgM and IgG markers are indicated.

(Stevenson *et al.*, 1980; Johnstone *et al.*, 1982), and there seems little doubt in considering this phenomenon as an integral component of the B-LL phenotype.

In more recent studies we have utilized an ELISA assay in order to quantitate the free light chains secreted from B-LL and other B cell populations (Gordon *et al.*, 1983b). The assay relies on the reactivity of the secreted molecules with antibodies raised against Bence–Jones proteins and rendered specific for free light chains by absorbing unwanted activities against combined Ig. The residual specificity thus defines those determinants, which although present on all light chains, are only revealed when dissociated from heavy chains. A survey of 48 cases of phenotypically and clinically well-defined B-LL revealed that, in all but two cases, monotypic free light chain secretion could be detected in appreciable quantities (Gordon *et al.*, 1983b, and unpublished observations). The amounts detected were, in general, remarkably constant among individual clones and did not vary appreciably for different phenotypic, histopathologic, or clinical subgroups. Even those clear cases of IC associated with a serum M component and demonstrating high rate Ig secretion *in vitro* exported readily detectable quantities of free light chains. The two exceptional cases in our series appeared to be of the pre-B type and probably represented a stage of differentiation prior to effective rearrangements on the light chain loci or at least preceding either the transcription or translation of the light chain genes. The finding of surplus light chain production in B-LL in the early studies suggested to Maino *et al.* (1977) that this feature may have been underlying to the disease process in this group of neoplasia. We, however, have preferred the notion that its occurrence is more representative of the modes of Ig processing exhibited by the normal equivalents of the leukemic cells. Further consideration of the relationship between surplus light chain production and the molecular and cellular events during early B cell development will be given in later sections.

The ability to detect in some apparently SmIg-negative leukemic populations the high rate secretion of free light chains suggested to us that this molecule must have an extremely transient surface phase (Gordon *et al.*, 1978). Attempts to radioiodinate surface free light chains proved fruitless (unpublished observations). Because light chains do not appear to possess the C-terminus anchor sequences present in the membrane-bound forms of the heavy chains, it is not too surprising that this should be the case. Nevertheless, under some conditions it may be possible to identify the molecules during their transition from the intra- to the extracellular environment, and the presence of free light chains on B-LL cells has been claimed (Fu *et al.*, 1974b). While we have not strived to determine whether the surplus light chain is exported in a monomeric or dimeric form, by analogy with

Bence–Jones proteins, it may be predominantly the latter. Whatever the molecular structure, we have estimated that the free light chain output of single cells from a typical B-LL clone is on the order of 10,000 dimer equiv. per hour. This contrasts with estimates of about 100 to 1000 molecules per hour for IgD and pentameric IgM assuming equal secretion from each cell (Stevenson *et al.*, 1983). Clearly, the production of light chains by chronic B lymphocytic leukemia represents a considerable excess of their requirements for immediate combination with heavy chains.

F. Unusual Packaging of Immunoglobulins by B-LL Clones

In most cases of B-LL studied by us and others, cIg when present usually has a diffuse or sometimes granular appearance. An unexpectedly large number of cases, however, reveal cells with patterns of intracellular staining which suggest that the immunoglobulin synthesized by these cells is packaged in distinctly atypical modes. These instances are exemplified by cells where the immunoglobulin is concentrated either as crystalline inclusions (Clark *et al.*, 1973; Cawley *et al.*, 1976; Gordon and Smith, 1979) or as amorphous globules (Hurez *et al.*, 1972; Smith *et al.*, 1977), although other packaging modes also exist (Guglielmi *et al.*, 1982b). The incidence of such cases has been variably reported up to 10% of all B-LL. Although the Kiel scheme for the non-Hodgkin lymphoma suggests that such cases should be considered as lymphoplasmacytoid immunocytomas, we have found cases which have been unambiguously classified as true B-CLL. In addition, positive cells often possess a distinctly small lymphocyte morphology. In our recent series, the incidence of cases with Ig inclusions concurred with the upper limit so far quoted in that 5 of 48 cases studied revealed such accumulations. All of our cases were of the crystalline type, which appears to predominate among the different packaging modes. One of the intriguing features is the exclusive association of the crystalline inclusions with Ig-bearing λ light chains. The globular inclusions appear to show a preference for κ light chain Ig, although the restriction here does not seem to be so rigid (Berrebi *et al.*, 1983).

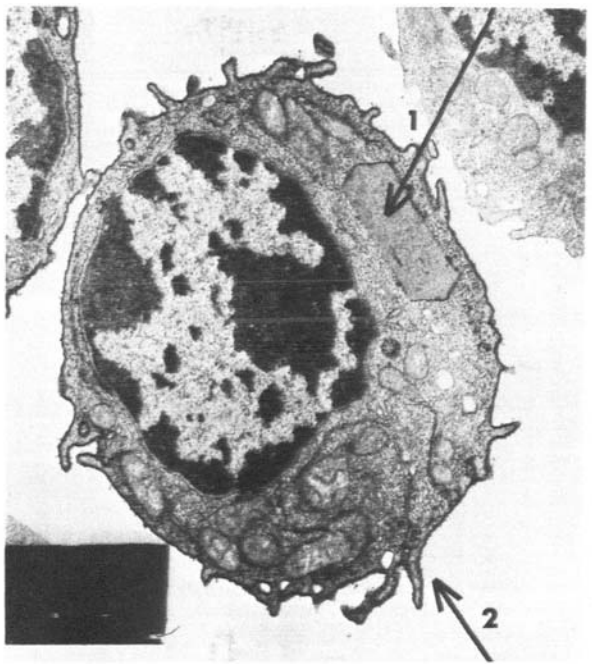
We have noted two types of patterns for the crystalline inclusions occurring in B-LL. One of these manifests as a single crystal, or very few large crystals, within the cytoplasm of positive cells, while in the other the cytoplasm contains numerous (10–20) fine needlelike structures. The former appearance seems to be the more common and an example is illustrated in Fig. 7. Within the crystal itself it is possible to note linear parallel arrays which stain for immunoglobulin and exhibit a marked periodicity.

Consistent with the B-LL phenotype in general, the intracellular accumulations are predominantly associated with IgM which may comprise

both the globular and crystalline forms and accounts for the bulk of the latter type. While IgG globules have been described (Nies *et al.*, 1976), no IgG crystals have yet been identified. In contrast, there appears to be an unusually high incidence of crystals associated with IgA (Cawley *et al.*, 1976; Gordon, 1979), an isotype generally not well represented in B lymphocytic leukemia. The presence of surface Ig on cells containing inclusions is variable. In some cases it is completely undetectable while in others the accumulated isotype may be represented at the cell surfaces along with other Ig classes, most notably IgD. For the particular cell, shown in Fig. 7, the density of staining for surface Ig is extremely high; this implies that if there is a block in the intracellular movement of Ig, it is restricted to the secretory form and does not impinge on the membrane form of the molecule. The suggestion that a secretory block may be inherent in these cells has been made on the basis of the invariable absence of a serum M component in spite of the high levels of intracellular material detected. The cells, however, do not lack the machinery for the transport and export of light chains, because in nearly every case investigated, free light chain secretion has been detected (Gordon *et al.*, 1983b, and unpublished observations). Thus the "block" may be restricted to the heavy chain containing Ig and as such could reflect either an abnormal mode of carbohydrate addition to the polypeptide backbone or structural defects within the C-domains of the heavy chains (Nies *et al.*, 1976; Roberts *et al.*, 1979). While such aberrations may occur, we have not as yet detected any gross abnormalities in the heavy chains derived from such populations.

The possibility then exists that such inclusions may result from defects in the secretory mechanism which impinge only on the heavy chain containing structures or, alternatively, that the cells reflect a normal, but perhaps particularly brief, stage of B cell development during which Ig is rapidly synthesized and concentrated into intracellular packages just prior to its secretion. The crystal shown in Fig. 7 is completely bordered by endoplasmic reticulum, which supports the concept that active synthesis at these sites may result in local accumulations of concentrated material. The association of the different light chain isotypes with different structural forms of inclusions may thus simply reflect the physicochemical properties of the different isotypic chains.

We have attempted to clarify the "normal equivalent," or at least the approximate stage of maturation arrest, represented by the Ig crystal-containing leukemic clones. First, it should be noted that not necessarily all members of the positive clones contain inclusions. In some cases only 5% of the cells were positive while remaining cells were either negative for cIg or contained intracellular Ig which was diffuse in appearance. This in itself suggests that the occurrence of inclusions does not necessarily reflect intrin-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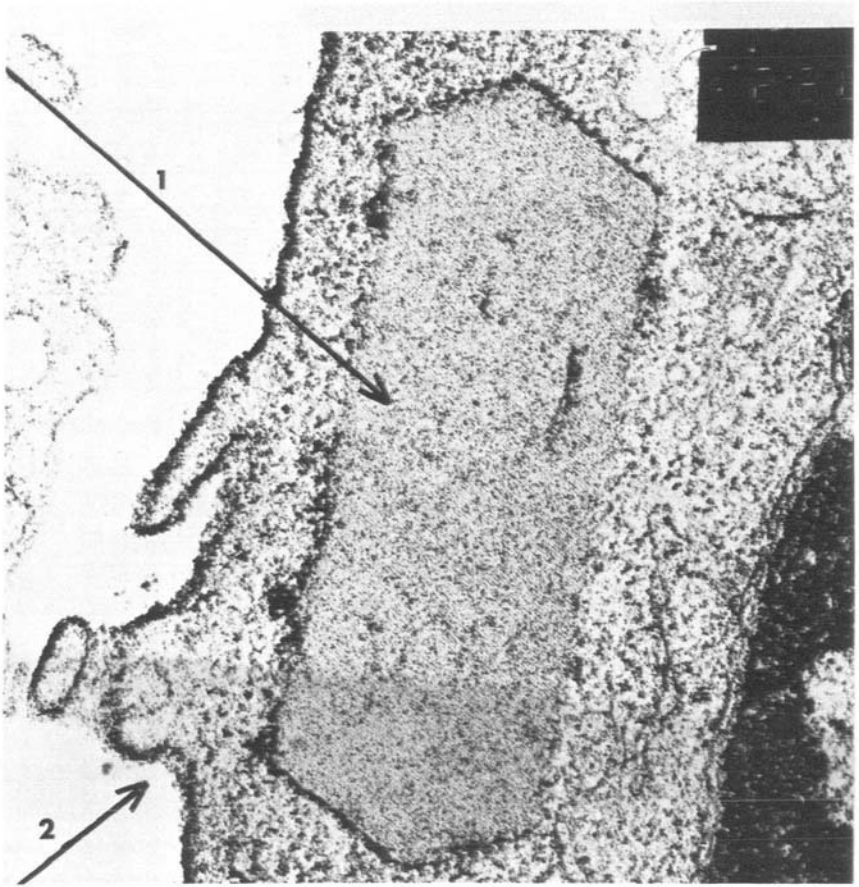
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sic defects in the neoplastic clones. This notion was substantiated by the finding of small amounts of IgM secretion in three of five cases which could be substantially amplified by exposure of the cells to phorbol ester. Also, following phorbol ester treatment, most cells acquired detectable cIg which manifested as diffuse or granular in distribution rather than crystalline. In some cIg-negative B-LL populations a transient pattern was sometimes noted, prior to the appearance of diffusely staining Ig in the cells, which resembled the intracytoplasmic Ig inclusions seen in the atypical cases and preceded the high rate Ig secretion induced by the phorbol ester. Similar patterns have been noted by Guglielmi *et al.* (1982b) when stimulating typical B-LL cells with mitogens.

Further probing of the clonal phenotype of B-LL populations containing Ig crystals with a battery of monoclonal antibodies suggested a stage of differentiation arrest intermediate to M and D coexpressing immunocompetent cells and recently antigen-stimulated mature B cells. These observations coupled to the lack of plasmacytoid features of the inclusion-positive cells point to the possibility that the unusual Ig packaging occasionally observed in B-LL clones does not arise from biosynthetic defects but instead highlights a presecretory phase of immunoglobulin processing briefly appearing in normal B-cell differentiation. It will be of interest to explore normal lymphoid tissue carefully for those few cells which may exhibit such transient modes of Ig localization.

VI. Hairy Cell Leukemia: A Proliferation of Immunoglobulin Synthesizing Cells

A. Introduction

Hairy cell leukemia (HCL), sometimes inappropriately referred to as leukemic reticuloendotheliosis (LRE), is characterized by an infiltration into the spleen and bone marrow of mononuclear cells usually displaying the prominent vilous projections for which it is named but definitively diagnosed by the presence of a tartrate-resistant acid phosphatase marker (Yam *et al.*, 1971). Despite its recognition as a distinct hematologic and pathologic entity for many years (Bouroncle *et al.*, 1958), the cell of origin in this chronic

FIG. 7. Immunoglobulin crystals in a case of B-LL. Shown is a transmission electron micrograph of a cell from a case of B-LL where the cells were stained with peroxidase-labeled antibodies to light chains (magnification ×4200 and inset ×20,960). Note the strong staining for surface light chains (2) and the shape of the crystal (1). The crystal is bordered by endoplasmic reticulum and displays a distinct periodicity with fine parallel arrays. (Reproduced by kind permission of Dr. J. Hata.)

leukemia has been the focus of considerable debate. It has probably been only in the last few years that this question has been adequately resolved, although there are features of these cells which remain intriguing.

The enigmatic nature of the hairy cell is reflected in part by its apparent dual association with features of both the lymphocytic and monocytic lineages. It was characteristics of the latter, an extreme "stickiness" of the cells coupled to a phagocytic potential, which hampered and confused early attempts in obtaining an accurate phenotypic profile of the malignant clones in this disease. Thus the detection of antigens on hairy cells, and immunoglobulin in particular, was often effectively dismissed as being of extrinsic origin. Formal proof of their B cell nature awaited studies on the capacity of the cells to actively synthesize the Ig being detected at their surfaces.

B. Surface Immunoglobulins on Hairy Cells

The problems encountered in the detection of Ig on B-LL cells are considerably amplified when examining the neoplastic cells of HCL. Almost without exception, freshly isolated hairy cells carry polyclonal immunoglobulins at their surfaces as evidenced by the capacity to stain the cells with antibodies to both light chain isotypes simultaneously. A minimal prerequisite in the study of these cells' immunologic phenotype is an overnight incubation at 37°C in medium free of human serum. Even with this precaution applied, precise characterization can be hampered, particularly when double antibody labeling procedures are employed, by the nonspecific attachment of the antibodies themselves to the hairy cell surfaces, possibly via particularly avid Fc receptors. Nevertheless, with extraneous material displaced, a majority of cases reveal monotypic staining patterns for the surface membrane Ig, which strongly points to an intrinsic origin (Fu *et al.*, 1974a; Burns *et al.*, 1978; Jansen *et al.*, 1979, 1982; Burns and Cawley, 1980).

In contrast to the clones typically represented in B-LL, hairy cells usually display strong staining for SmIg. The heavy chain isotypic distribution also tends to be more diverse than the monotonous IgM or IgM/IgD restriction of B-LL. While IgM is a prominant isotype in HCL, it is rarely expressed alone and is often accompanied by IgG or IgA instead of, or as well as, IgD (Jansen *et al.*, 1982). The patterns of multiple isotype expression associated with HCL are quite different to those of B-LL because all Ig chains can be detected by simple immunofluorescence and do not rely on the more sensitive methods sometimes needed for B-LL. Again unusual for B-LL, hairy cells often express more IgD than IgM sometimes at quite high levels of staining. Predominantly IgG- or IgA-bearing clones are also not unusual for HCL. In all these cases the unequivocal light chain isotype restriction favors that the different Ig classes are endogenously synthesized. To support this

notion, monotypic Ig, and IgG in particular, are occasionally reported as detectable cytoplasmic molecules in hairy cells (Cawley *et al.*, 1979).

C. Biosynthesis of Immunoglobulin by Hairy Cells

The apparent association of HCL with a serum paraprotein may be quite high and mutual clonal origin for the hairy cells and M component would provide good evidence for the Ig secretory capacity of the pathogenic cells in this disease. This notion is, however, compromised by a number of factors which may equally pertain to the consideration of these features in other neoplastic entities. In 1977 Golde *et al.* described a patient having hairy cells with a serum IgM component. Moreover, the establishment of a cell line synthesizing the macroglobulin appeared to provide formal proof for the capacity of hairy cells to synthesize and secrete immunoglobulin molecules. However, following publication, the question was raised whether this case was actually HCL, even though the tartrate-resistant phosphatase marker was noted (Katayama, 1977). More, recently, Catovsky *et al.* (1981) reported three cases where myelomatosis and HCL occurred together. As well as demonstrating the typical clinical features of multiple myeloma, in two of the cases the Ig isotype of the hairy cells differed from that of the paraprotein implying that the plasma cell disorder and the hairy cell proliferation were clonally unrelated. Another case has been described where an IgAκ M component was detected in the patient's serum, while the cells failed to synthesize this isotype *in vitro* but instead showed evidence for the production of IgAλ (Newell *et al.*, 1982). However, in rare examples the neoplastic cells in HCL and a paraproteinemia may share a mutal origin, and this may be particularly manifested in IgG-producing clones (Cawley *et al.*, 1979).

Toward the end of the 1970s we had the opportunity to study several clinically and morphologically well-defined cases of HCL for the capacity of the neoplastic cells to synthesize immunoglobulin molecules *in vitro*. All six cases demonstrated this characteristic with the essential feature of light chain isotype restriction exhibited (Gordon and Smith, 1978). The biosynthetic patterns of these cells, confirmed in later studies, had features both in common and at variance to those associated typically with B-LL. Reflecting the differences, HCL populations tend to commit more of their overall protein synthesis to Ig production reflected in metabolic labeling experiments by a large amount of radioactivity associated with Ig heavy chains. Furthermore, in contrast to nearly all B-LL cases studied so far, peaks of labeled material often appear on SDS gels with a mobility significantly faster than that of μ chains and in positions compatible with δ chains. In three of our six cases, labeled δ chain peaks were clearly distinguishable (an example is shown in Fig. 8). While the mobility of this material was

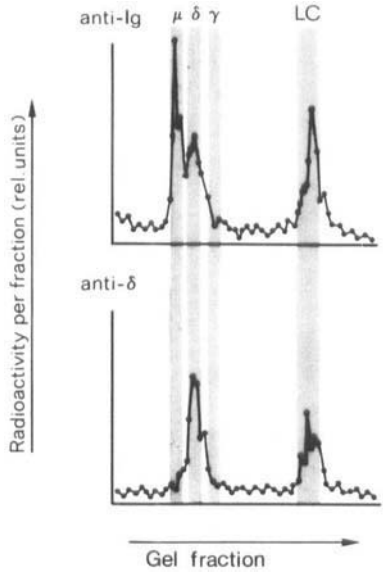

FIG. 8. SDS gel analysis of labeled IgD synthesized by hairy cells; details as for Fig. 6, but with material precipitated from lysates of metabolically labeled hairy cells. (Top) Precipitated with polyspecific anti-Ig; (bottom) precipitated with antibodies specific for IgD heavy chains.

similar to that of reduced myeloma IgD heavy chains, we are unclear whether it represented the membrane or the secreted from of the δ gene product. While the latter is appreciably smaller than the former, the level of glycosylation modifies the mobility of the chains on SDS gels and presents a complex profile when analyzed by this method. The high level of membrane IgD expression already mentioned and the lack of appreciable secretion of this material would suggest that it is the membrane form which is predominantly represented. It will be of considerable interest to determine whether the apparent light chain isotype-restricted IgD secretion noted for the B-LL is operative in HCL and also to probe which form of the messenger is predominantly transcribed in these cells.

Although in some of our cases (Gordon and Smith, 1978, and unpublished observations) the hairy cells clearly made and expressed IgG as their primary isotype, the capacity of hairy cells bearing predominantly IgM and/or IgD to actively synthesize this product is not as clear. We have occasionally noted γ chain-related peaks on SDS gel analysis of labeled material from M- and D-expressing hairy cells but could not definitively ascribe a neoplastic origin to this isotype. Nevertheless, the multiple isotype expression often associated with HCL suggests that these populations would make particularly suitable

models for probing the molecular features of this phenotypic event. Pertinent questions include whether all isotypes are actively translated simultaneously, whether they arise from separate long-lived messengers, whether multiisotype primary transcripts are differentially processed, and whether some clones may be initiating the events leading to *CH* gene deletions. We have noted one particularly interesting case regarding some of these features. On initial examination, the hairy cell population expressed membrane-bound IgM strongly while actively secreting IgG of identical light chain specificity. Reexamination of the leukemic clone several months later revealed that IgM was no longer expressed and IgG was the only detectable isotype synthesized. While such apparent phenotypic switches *in vivo* may relate to the preferential emergence of dominating subclones, the possibility that some cases of HCL represent stages of maturation arrest reflecting *CH* switching phenomena remains intriguing.

The shared biosynthetic feature of the pathogenic cells of HCL and B-LL that is most readily apparent is their ability to manufacture and process surplus light chains. While the ability to detect labeled heavy chains in the culture supernatants of metabolically labeled hairy cells is more frequent than for B-LL, the predominant secreted product is still Ig light chains. This feature was noted in all six of our reported cases and has been confirmed by other more recent studies by ourselves and others (Gordon and Smith, 1978; Rieber *et al.*, 1979, and unpublished observations). While the biosynthetic light chain excess may be less in HCL than in B-LL, this appears to be almost entirely due to the higher rate of synthesis of combined Ig molecules from the former. The actual amounts of free light chain secreted seem to be very similar in the two disorders. A study on a case of HCL at the ultrastructural level suggested that free light chain production in these cells may be particularly concentrated in the cisternae of the perinuclear space. Within the rough endoplasmic reticulum and Golgi apparatus, the heavy and light chains were distributed more evenly (Newell *et al.*, 1982). Similar compartmentalization of heavy and light chain production has been noted in B-LL; we shall return to these ultrastructural aspects in the final section.

While there now seems little reason to doubt the B cell nature of the malignant hairy cell, its precise phenotypic character continues to perplex the immunologist. While we still have to reconcile its apparent monocytoid features to its immunoglobulin synthesizing capacity, there is additional evidence implicating T cell features among the phenotypic profile of the hairy cell (Cawley *et al.*, 1979). So in spite of considerable study at the level of Ig expression, we are still left with unresolved questions on the cell of origin in this disorder. While we may find that the proliferating clone represents a chimaera of multiple lineages, possibly resulting from the abnormal

development of a transformed lymphocyte–monocyte progenitor, we cannot exclude the possibility that the phagocytic and "T cell" features of the hairy cell are transiently expressed by normal B lymphocytes at discrete stages of differentiation and activation.

VII. The Processing of Immunoglobulins by Cells of Non-Hodgkin Lymphoma

A. Introduction

While essentially all neoplasms covered in this review could be encompassed within the umbrella of the malignant lymphoma, this section deals specifically with those cases which occur predominantly as tumor involvements of the lymph nodes and where the predominating clonotypes probably reflect the different functional cell types to be found within these tissues. Even restricting our discussion to these cases involves a group of neoplasms comprising an enormously diverse range of clinical, histologic, morphologic, and immunologic subtypes. Recent attempts to unify some or all of these parameters have produced an equally diverse range of classification schemes for non-Hodgkin lymphoma (NHL). One of the most widely adopted classifications, particularly in the United States, is that proposed by Rappaport in 1966 which relies entirely on descriptive morphology to describe the cell types involved. While this scheme may presently satisfy the clinician, it fails to take account of the recently accumulated knowledge on lymphoreticular development and functional compartmentalization of this system. During the 1970s, various efforts were made at attempting a nomenclature for NHL which was rooted in functional rather than descriptive criteria (Gerard-Marchant *et al.*, 1974; Lukes and Collins, 1974; Lennert, 1978).

The first point to emerge from an immunologic approach was that the overwhelming majority of NHL (>90%) represented neoplastic proliferations of B lymphocytes (Levy *et al.*, 1977; Aisenberg *et al.*, 1980). T cell lymphomas were comparatively rare while the bulk of the so-called histiocytic lymphomas were found to be neoplastic counterparts of transformed B cells (Morris and Davey, 1975). The most widely adopted schemes to emerge from this approach have come from the Kiel group (Lennert, 1978) and from Lukes and Collins (1974). Both systems share a common conceptual approach and strive toward relating tumors to their cell of origin among the functional and morphologic types represented in reactive nodes. The disparity between the two is due to differences in terminology but also in the precise sequence of events proposed for cells of the germinal centers. Common to both schemes is the concept that small lymphocytic cells arise out-

side the follicular centers and are probably equivalent to, or close to, the lymph node counterpart of the leukemic cells of B-LL. Encounter of these cells with antigen drives them to varying levels of cellular transformation, some of these processes occurring within follicular centers. Large blastoid cells emerging from these sites give rise to more small lymphocytes (memory cells) and to plasma cells, although the latter of these cell types may also arise independently of follicular processes. Morphologically, the Kiel and the Lukes and Collins nomenclature distinguish between cells of follicular center reaction as either centrocytes and centoblasts, or small and large, cleaved and noncleaved cells, respectively. If we strive for unification by using the terminology of the Kiel group, centroblasts give rise to centrocytes. Lukes and Collins, however, favor a developmental sequence whereby antigen stimulation first produces the centrocytes, followed by centroblasts, which in turn leave the follicular centers as immunoblasts. It is with this morphologic framework in mind that we consider the capacity of the cells represented to handle Ig molecules *in vitro*.

The lack of uniform concensus in the histopathological diagnosis of NHL renders strict comparison between studies employing different terminologies difficult and cumbersome. While not intending to endorse any single scheme, for the sake of clarity the terminology of the Kiel classification will be adopted in this discussion, which concentrates on the immunologic distinctions between the different subtypes of NHL, particularly those of follicular center cell (FCC) origin.

B. Phenotypic Expression of Immunoglobulin in NHL

There have been two approaches to the capacity of cells of NHL to express products of the immunoglobulin genes. One of these has already been widely adopted for the study of leukemic cells: the staining of individual viable cells in suspension by antibody conjugates. In addition to this approach, NHL lends itself to the examination of neoplastic cells *in situ*. The advantage here is that the tumor clone can be examined in direct relation to its tissue environment where the conservation of the architecture may provide additional information to both clinician and researcher. Here, in contrast to the examination of single cells where fluorescein-labeled antibodies are preferred, the conjugate of choice for the immunologic study of tissue sections has been antibodies coupled to peroxidase (Taylor and Burns, 1974; Isaacson *et al.*, 1980). The prime disadvantage of this approach has been its relative insensitivity coupled to high background levels of staining. Nevertheless, many of these technical problems may soon be overcome, and the functional examination of tumor processes *in situ* may provide valuable insights to the

pathology of these disorders (Clark and Keren, 1982). In this section, however, we concentrate our discussion on information gleaned from the study of viable cell suspensions.

Whether employing fluorescein-labeled antibodies on single cells or peroxidase conjugates on tissue sections, it is quite clear that the overwhelming majority of the NHL represent proliferations of monoclonal B cells. In some cases, morphologic and phenotypic restrictions are similar to those of the typical B-LL, while among some NHL, wider intraclonal heterogeneity may be evident from both histologic and immunologic criteria. The small lymphocytic lymphomas of the non-FCC type tend to reveal features corresponding to the B-LL. Visual assessments on the intensity of SmIg expression by NHL cells confer with this notion by demonstrating a generally weak level of staining for these cell types (Aisenberg and Wilkes, 1976). A more recent study employing flow cytometry to make these judgments more objective revealed that these cell types carry only some 25% of the SmIg detected on NHL cells of the FCC type (Godal *et al.*, 1981). Our own studies confirm the relatively high levels of Ig expression on NHL of the follicular centers by comparison with weak expression of the small non-FCC lymphoma cells (Hannam-Harris *et al.*, 1982). The presence of cytoplasmic immunoglobulins in the NHL may again be more a reflection of sensitivity of technique rather than any absolute qualification. However, with the limited number of studies available, we can conclude in general that easily detectable cIg is extremely rare among small lymphocytic non-FCC NHL, occasionally found among FCC lymphomas, but that it may be quite common in cells of immunoblastic lymphomas (Payne *et al.*, 1977; Hannam-Harris *et al.*, 1982; van der Valk *et al.*, 1983). The latter of the categories appears to carry both surface and cytoplasmic Igs and may relate to an intermediate cell type developing toward Ig-secreting plasma cells. More detailed studies are required before we can comment on differences in the detection of cIg and intensity of SmIg between the centrocytic and centroblastic predominant subtypes of the FCC lymphomas.

The frequency of expression of the *CH* genes may differ between the different histologic groups of NHL, although the precise details are unclear and can be contradictory between different studies. While some consider IgD expression to be a common feature of the FCC lymphomas (Hopper, 1974; Leech *et al.*, 1975), we and others find patterns of expression which are more compatible with a loss of IgD during follicular center processes (Godal *et al.*, 1981; Hannam-Harris *et al.*, 1982). In our recent studies we have noted that, in contrast to the high frequency of IgD among small lymphocytic lymphomas, centrocytic and centroblastic lymphomas often failed to stain for this isotype. The heterogeneity in IgD expression on these lymphomas suggested to Godal and colleagues (1981) that this reflected a

loss of the isotype occurring on B cell maturation within germinal centers. Other studies support this notion with the suggestion that the first phenotypic alteration required for the entry of antigen-primed cells into the germinal centers may be the loss of IgD (Kraal *et al.*, 1982). Variation in IgD expression by FCC lymphomas might, alternatively, relate to optional developmental pathways in the formation of memory cells depending on precise microenvironmental factors.

We and others find IgM to be the predominant isotype expressed among all histologic types of NHL. Within the FCC lymphomas, however, there is a relatively high frequency of IgG expression. It seems reasonable to consider this isotype as an intrinsic membrane constituent for these lymphomas because the staining observed is usually strong and clearly monotypic with regard to light chain. It is also common for the IgG to be the only Ig isotype detectable in these cases. While Godal *et al.* (1981) found that IgG-expressing FCC lymphoma carried only about 20% of the Ig expressed by IgM-bearing clones of the same histologic group, we have failed to notice readily appreciable differences by visual assessments, even though the trained eye is fully capable of discriminating between such disparate levels of expression (Hannam-Harris *et al.*, 1982). IgG has also been reported as the predominant isotype in cases of immunoblastic lymphoma, although the most common phenotypic pattern within this group may again be that of isolated IgM expression (van der Valk *et al.*, 1983).

C. Biosynthesis of Immunoglobulin by NHL Cells

There is a relative paucity of literature concerning the capacity of NHL cells to synthesize actively and secrete their Ig products in shortterm cultures, thus I rely heavily on studies by myself and colleagues for elucidation of these areas. In our initial report of the secretion of free Ig light chains by B-LL cells we also noted that tumor clones from small lymphocytic non-FCC lymphoma shared the property of excreting large excesses of light chain over heavy chain in shortterm cultures of the freshly explanted cells (Gordon *et al.*, 1978). In striking contrast, FCC types revealed apparently balanced synthesis and secretion of the constitutive Ig chains. We have now confirmed these findings in a larger series and additionally noted differences in this property between FCC lymphomas which were centrocytic or centroblastic predominant (Hannam-Harris *et al.*, 1982). Centroblastic-predominant cases revealed virtually balanced production of the Ig chains, whereas those which were centrocytic predominant demonstrated biosynthetic heterogeneity, being similar to the small lymphocytic type or to the centroblastic type or revealing patterns intermediate to both. Additional differences in the biosynthetic capacities of the various histologic groupings

included their level of commitment to the synthesis of immunoglobulins and their capacity to secrete the material *in vitro*. Thus for the centroblastic predominant cases, Ig production often constituted a large proportion of the total protein synthesis, and Ig molecules comprising both heavy and light chains were actively exported. This contrasted with low rate Ig production of the small lymphocytic lymphomas, while centrocytic predominating cases again revealed patterns intermediate of the two. In our recent attempts to quantify the amounts of free light chains secreted by neoplastic clones, we have noted that even in the presence of high rate Ig export by lymphoma cells of centrocytic–centroblastic types, the actual quantities of free light chain detected are relatively invariant by comparison with either B-LL or other lymphoma subgroups. Therefore we now interpret the "balanced" heavy and light chain production noted for centroblastic predominant NHL as simply reflecting the large amounts of whole Ig secretion evident, rather than a lack of surplus light chain production.

In our biosynthetic studies the detection of labeled immunoglobulin heavy chain classes in shortterm cultures of NHL cells corresponded well with the isotypes detected at the cell surfaces. This included those cases expressing IgG and confirmed an endogenous origin for this product. In one case, while only IgM was detected at the cell surfaces, there was evidence for the dual synthesis of IgM and IgG by the neoplastic clone suggesting that this population had undergone, or was in the process of undergoing, *CH* gene switching (Gordon and Smith, 1980). The relatively high incidence of IgG expression among NHL of FCC type is consistent with these sites being major areas involved in the switch processes. Alternatively, the differential expression of the Ig isotypes may represent B cell development from diverging pathways of already switched cells. This can be conceptualized most simply as reflecting the emergence of clones within germinal centers from precursors representing primary and memory processes, respectively. It is clear that the divergent phenotypic groups represented by NHL should provide the potential for valuable insights into the molecular processes of Ig gene expression at stages following the encounter of resting cells with antigen.

D. Isolated Heavy Chain Expression at Lymphoma Cell Surfaces

The aim of this review is toward a broad synthesis of concepts on B cell neoplasia in terms of their capacity to express and process the products of the Ig genes, and thus it has not dwelled on the aberrant patterns sometimes associated with these disorders. Nevertheless, phenotypic profiles occasionally emerge which may challenge our basic concepts gathered from the concensus model, and rather than being simply dismissed as a consequence

of neoplasia, these should be examined in close detail with the potential of providing fresh insights into the more representative processes. In this section we detail a single case of NHL whose handling of Ig molecules fits these criteria.

This case, first reported in 1981 (Gordon *et al.*, 1981), was apparently unique among the clones to be found in NHL by the expression at the neoplastic cell surfaces of μ heavy chains without detectable Ig light chains. Since our report, Godal and colleagues (1981) have described a case where they found γ chains without light chains at the tumor cell surfaces. Contrasting with the reported occasional sparse expression of isolated heavy chains on some of the pre-B ALL subclones, our case of lymphoma expressed these molecules in amounts readily detectable by simple immunofluorescence. The lack of light chain association was confirmed by the reactivity of the lymphoma cells with antibodies specific for isolated μ chains and by the lack of light chain-associated peaks on SDS gels of both surface materials labeled by lactoperoxidase-catalyzed iodination and biosynthetically labeled intracellular Ig. In addition, these procedures revealed that the surface μ chains existed without covalent linkage between the molecules.

In a follow-up study a few months later, we noted that, while none of the patients' peripheral blood cells reacted with the antibody specific for the isolated μ heavy chains, approximately half of the mononuclear cells stained for surface γ chains. An inconsequential number were positive for either κ or λ light chains. The γ chains failed to elute from the cell surfaces at 37°C and were associated with cells of lymphoid morphology which did not adhere to plastic surfaces. Biosynthetic studies revealed that, above a background of normal polyclonal Ig production, this patient's peripheral blood cells were capable of synthesizing and secreting Ig-related material which migrated on SDS gels with the mobility of γ chains (see Fig. 9). Not only could this material be precipitated with antibodies specific for γ chains, but the precipitation was blocked in the presence of myeloma IgG. Investigation of the patient's bone marrow cells failed to reveal any SmIg, although biosynthetic labeling again clearly demonstrated both the active synthesis and, for this subpopulation, a particularly high rate secretion of γ chains which were clearly not associated with either of the light chain isotypes. These observations prompted us to reexamine the patient's original biopsy material, aliquots of which had been stored in frozen cell suspensions. Precipitation by specific antibodies, again confirmed by blocking with myeloma proteins, revealed that the patient's lymph node cells synthesized γ chains without apparent linkage to any light chain in addition to producing free μ chains. Despite a level of synthesis approximately 75% of that of μ chains, we could not detect γ chain expression either at the cell surfaces or in the cytoplasm by immunofluorescent techniques. The lymph node cells failed to secrete

the labeled γ chains over 24 hr. A second lymph node biopsy performed more than a year later revealed a population where the tumor cells were now expressing a phenotype of isolated γ chains at the cell surfaces with no μ or light chains evident. The peripheral blood mononuclear cells again bore the isolated γ chain phenotype. A hypothetical scheme on the longitudinal and compartmental distribution of this patient's immunologic profile is depicted in Fig. 9. Although there is no cytogenetic or idiotypic proof for a mutual clonal origin to the μ-expressing populations, the finding that both heavy chains were cosynthesized by the original biopsy cells strongly argues in favor of this possibility.

The questions posed by this and the other recently reported case are numerous. Most importantly, it is unlikely that these clones reflect pre-B analogs from both the relatively high levels of heavy chain expression noted and the follicular center cell classification in both cases. The capacity of cells

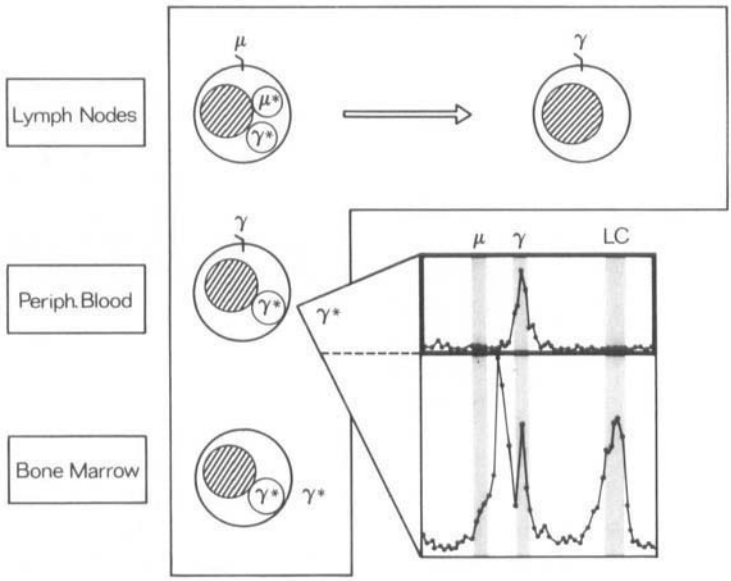

FIG. 9. A schematic representation of the appearance of isolated heavy chain expression in a unique case of lymphoma. Patient's lymph node cells when first examined revealed isolated μ heavy chains at their surfaces (Gordon *et al.*, 1980). The patterns of surface expression and labeled (*) material subsequently found are indicated (see text). The SDS gel profiles of reduced secreted labeled material from the patient's peripheral blood cells are shown to illustrate the secretion of isolated γ chains. (Top) Precipitation of supernatant material (first "cleared" by anti-light chain precipitation) with antibodies specific for IgG heavy chains revealing the presence of endogenously synthesized γ chains. (Bottom) Supernatant material precipitated with antibodies specific for light chain-related material revealing a "background" synthesis of normal Ig.

to handle heavy chains without light chain intervention is clearly exceptional for cells at the post pre-B level; we will return to the molecular significance of this finding in the last section of this review. Studies on the Ig gene rearrangements in such cases are clearly warranted. It would be extremely pertinent to determine whether rearrangements had occurred at the light chain loci. Two possibilities are either that these clones had undergone perturbations early in development which impinged on their ability to carry out light chain gene rearrangements or that, at the other extreme, rearrangements on all light chain alleles had occurred but none were productive. Alternatively, an effective light chain rearrangement may have occurred but errors either at the translational or transcriptional level interfered with its correct phenotypic expression. Similarly, the apparent capacity to undergo *CH* switching in the absence of light chain expression in our case is also intriguing. However, we noted in Section III this possibility at the pre-B level for some cases of ALL and their murine analogs where it may reflect differential RNA splicing mechanisms. There exists no conceptual requirement for the phenotypic expression of light chains to initiate these processes. The finding of γ chain secretion by the clone also implies that light chain intervention need not be a prerequisite for the switch in the differential processing of *CH* genes from primarily membrane molecules to the secretory form. Alternatively, the initiation of these events may have occurred at times when the proliferating cells were synthesizing light chains and the loss of this capacity may have been related to secondary phenomena. Further detailed study of such rare cases is warranted, particularly at the molecular level, from which valuable insights into these different processes might be gleaned.

VIII. Induced Changes in the Phenotypic Expression of Immunoglobulin by Neoplastic Cells

A. Introduction

In the preceding sections we have explored the capacity of freshly isolated tumor clones to express immunoglobulins and have seen that for most neoplasms, the dominant subclones reveal a restricted heterogeneity of cell type wherein lies a large part of their inherent utility as models for probing the mechanisms involved in this and related phenomena. The utility of these clones could be greatly extended if discrete shifts in the stage of maturation of the dominant populations could be achieved at will. With an increased understanding of the molecules and receptors required to induce these changes, this possibility may soon be realized. At present, however, most

studies of this type rely on a "shot-gun" approach where exposure of individual clones to batteries of known B cell activators may, in a few cases, hit upon the right combination to effect the appropriate level of cellular differentiation and/or activation. Indeed, the capacity of clones to respond to external signals could be considered as an integral feature of their phenotypic profile relating to their readiness to receive such stimuli. A lack of response to a given activator may thus not denote functional refractiveness per se, but rather it may reflect another facet of the stage of maturational arrest of the malignant clones. Again, chronic type B lymphocytic leukemia has been the most widely studied model for this functional activity, although other neoplasms are now beginning to receive increased attention.

B. Response of Neoplastic B Cells to Mitogens

A variety of mitogens, often from plant or bacterial origin, have been used in attempts to induce changes in neoplastic B cells *in vitro*. The alterations of the desired cell status fall into two primary modes: activation and differentiation. Activation is commonly assessed by thymidine incorporation and is considered a prelude to proliferation. Differentiation is commonly sought by alterations in the phenotypic expression of immunoglobulin by the reacting clones. While a few researchers show that a number of mitogens appear capable of inducing cell proliferation in B-LL clones (Gahrton and Robert, 1982), most reports conclude that the proliferative response is generally poor (Whiteside *et al.*, 1977; Godal and Funderud, 1982). In contrast, more consistent success is achieved in attempts to induce differentiation. A recent report from Guglielmi *et al.* (1982a) describes the capacity of B-LL populations to respond to phytohemagglutinin (PHA), pokeweed mitogen (PWM), and *Norcadia* mitogen by the appearance of cIg in the leukemic cells. For most cases this was accompanied by a morphologic shift to lymphoblastoid cells although occasionally cells with plasmacytoid features emerged. Others have reported similar changes occurring both with these and other mitogens such as staphlococci areus (Bloem *et al.*, 1982). Bloem and colleagues (1982) noted that, in addition to an accumulation of cIg, mitogens also induced a preferential loss of IgD from the neoplastic cell surfaces. There is evidence to suggest that the accumulated Ig is often released from the cells following mitogen stimulation. Robert (1979) noted the appearance of PFC in B-LL populations following their exposure to a variety of polyclonal activators, while Johnstone *et al.* (1982) quantitated the amounts released by radioimmunoassay. In both studies, a neoplastic cell origin for the secreted material was strongly suggested from the light chain restriction noted. These findings somewhat contradict the studies of others who suggest that only in those cases where there is already evidence for differentiation capacity (as wit-

nessed by the presence of a related M component) can these changes be induced *in vitro*. Fu *et al.* (1979) described two such clear cases of immunocytoma where a combination of PWM and allogeneic T cell factors were capable of perturbing the dominant subclones to express the features of differentiated B cells. A particularly important finding to emerge from this study was the observation that these changes could be induced in one of the cases by the simple addition of allogeneic T cell factors. This implies that the maturation block apparent on the dominant subclone *in vivo* may have resulted from inappropriate helper activity rather than from an intrinsic disturbance to the neoplastic cells per se.

In contrast to the relative ease by which *in vitro* induction of IgM accumulation and secretion can be achieved, the frequency with which B-LL cells switch their expression of *CH* genes in response to external stimuli appears to be extremely low. In the series studied by Guglielmi *et al.* (1982a) one case was shown to exhibit this capacity, but only a single case has been studied in any particular detail for this event. This case was another clear immunocytoma in which the patient had a serum IgM paraprotein (Saiki *et al.*, 1980). Exposure of the leukemic cells to PWM and allogeneic T cell factors not only amplified the level of IgM secretion but also induced the production of secretory IgG. Here, the mutual clonal origin of the two products was unequivocally demonstrated by their reactivity with idiotype-specific antibodies. The reasons why B-LL populations generally fail to respond to external stimuli by isotype switches could be diverse and multifactorial. One explanation we have already considered for resting populations is that most B-LL clones may be irrevocably channeled into a single route of B cell development and are thus already comitted to the generation of plasma cells of a given isotype. Alternatively, the frequency of isotype switching among the normal compartments represented by B-LL may be similarly low so that the leukemic cells are simply reflecting normal physiology. However, it is very likely that the signals delivered to B-LL clones *in vitro* are simply inadequate to induce the changes required for isotype switching. The germinal centers may be intimately involved in these processes and may present factors to the stimulated B cells which are lacking from mixtures of mitogens and T cell "soups." The possibility cannot be excluded for those cases where apparent changes in *CH* gene expression can be induced that these clones have already received the appropriate signals required for the switch but are simply blocked at a stage prior to the translation or transcription of the switched gene. In this situation, the mitogen could be envisaged as simply lifting the maturation block. Clearly, the monoclonal populations of B-LL should prove to be model populations for probing both the factors required to induce these changes and the underlying molecular processes involved.

Another mitogen extensively examined for its effect on normal B cells and

increasingly being studied with neoplastic populations is anti-Ig. Its reported activities on normal cells are both diverse and complex depending on the cellular subsets investigated, the presence of Fc fragments of antibody, the cooperation of accessory cells and factors, and whether the antibody is immobilized or free in solution (Parker *et al.*, 1980; Henriksen *et al.*, 1980; Fothergill *et al.*, 1982; DeFranco *et al.*, 1982). We have found that typical B-LL populations without a related serum M component have reacted to anti-Ig, with or without T helper factors, either by no response or by a sharp decrease in Ig production (unpublished observations). The latter of these effects may relate to the process of tolerogenesis mimicking the premature encounter of undeveloped B lymphocytes with their corresponding antigen. The case of immunocytoma reported by the Japanese group also appeared to respond by increased IgM and IgG production to anti-Ig as well as PWM (Yoshizaki *et al.*, 1982). In this study they demonstrated that anti-Ig by itself was incapable of inducing the appropriate changes, but with the presence of IL2, the neoplastic cells proliferated. Additional supplementation with T cell replacing factor (TRF) induced the differentiation of the clone to high rate secretion and apparent isotype switching. It is quite likely that the role of anti-Ig in these processes is to induce the appearance of acceptors at the cell surfaces for the appropriate growth and maturation factors. There is some evidence that cross-linking of the different surface Ig isotypes on neoplastic cells may deliver different signals for activation. Ruud *et al.* (1983) have recently described a case of FCC lymphoma where the cells responded to anti-δ chain and phorbol ester by proliferation, while anti-μ with the tumor promoting agent induced both proliferation and differentiation. More studies of this type may delineate compartments of B cells identified by their differential responsiveness to external stimuli which may, in turn, reflect their readiness to receive and respond appropriately to these influences.

There has been some discrepancy in the reported effect of mitogen stimulation on the production of free light chains by B-LL populations. In their early studies, Maino *et al.* (1977) found that PHA amplified the secretion of free light chains from these cells. A later report described the effect of PWM on two B-LL populations as selectively amplifying the production of heavy chains so that Ig secreted by the stimulated clones appeared to have a molar equivalence of heavy and light chains (Hannam-Harris and Smith, 1981a). Confirmation of the neoplastic cell origin in these two cases came from the precipitation of Ig with antibodies specific for the clonal idiotypes. These workers have recently described the ultrastructural changes accompanying the apparent shift from surplus to balanced light chain production following mitogen stimulation of B-LL cells (Newell *et al.*, 1983). Immunolocalization of the Ig product revealed that, in contrast to uncultured cells in which only light chains were found staining heavily in the perinuclear spaces and the

rough endoplasmic reticulum, mitogen-stimulated cells showed this distribution pattern for heavy chains as well as for light chains. In a few cases studied by ourselves where exposure of typical B-LL populations to PWM and T helper factors resulted in induction or amplification of IgM secretion, we noted that the level of free light chain secretion remained remarkably static so that only the apparent light chain excess was much reduced (Gordon *et al.*, 1983a, and unpublished observations). These considerations are discussed in the next section.

C. Phorbol Ester-Induced Changes in Neoplastic B Cells

The phorbol esters are tumor-promoting substances derived from croton oil, and their capacity to induce changes in a bewildering variety of cell lineages is both diverse and extremely potent. In some systems these changes may reflect differentiation-related perturbations, while in others they are more consistent with alterations expected from dedifferentiation (Diamond *et al.*, 1980). In 1980 Tötterman *et al.* described the effects of one of the most potent phorbol esters, 12-*O*-tetradecanoylphorbol-13-acetate (TPA), on certain phenotypic features of B-LL populations. This and later reports from the same group (Tötterman *et al.*, 1981a,b) established that, almost without exception, B-LL clones respond by changes in Ig expression consistent with B cell maturation. This was exemplified by the appearance or increase in cIg and a loss of Ig from the neoplastic cell surfaces which was preferentially IgD. While they demonstrated by metabolic labeling an increase in the production of both the Ig light and heavy chain, they failed to show secretion of the product either by ELISA or PFC assays. Following the report of a single case of B-LL where TPA induced IgM secretion (Okamura *et al.*, 1982), we described a large series where an amplification or induction of extracellular IgM production was found to be a common feature (Gordon *et al.*, 1984). We noted that those clones with the most immature characteristics responded the most dramatically to the phorbol ester, although this was in large part because all clones appeared to achieve a similar stage of differentiation following TPA treatment. Our findings confirmed those of Tötterman and colleagues (1980, 1981a) by demonstrating, in parallel with the induced immunoglobulin secretion, the accumulation of cIg and a reduction in SmIg, preferentially of the IgD class. In spite of the high rate of IgM secretion usually induced, we failed to notice any decrease in the output of free light chains which tended to remain at a fairly constant level following exposure of the cells to TPA. Figure 10 illustrates, for a representative case of B-LL, the changes in the phenotypic expression of Ig occurring over several days in response to TPA. A recent study by Cossman *et al.* (1983) has confirmed our observations and additionally shown that the appearance of

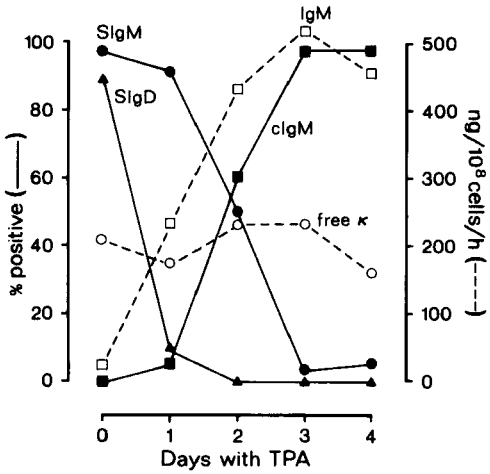

FIG. 10. Response of B lymphocytic leukemia cells to phorbol ester. Cells at 5 × 10^6/ml from a typical case (surface IgMDκ positive) of chronic B lymphocytic leukemia were cultured for the indicated times with the phorbol ester TPA at 10 ng/ml. Changes occurring in immunoglobulin expression and secretion are indicated. Viability remained at >95% while the phenotypic disposition of nontreated cells stayed essentially unchanged.

secretory IgM is related to a specific increase in the intracellular level of mRNA for the secretory form. We have also found that the dramatic increase registered for IgM output by ELISA corresponded to an increase in the number of IgM PFC in the cultures (Gordon, Tskuda, Mellstedt, and Klein, unpublished observations). These observations and a consistent lack of isotype switching following TPA treatment confirm our notion that the developmental options open to B-LL populations may be restricted, at least as reflected by *in vitro* stimulation with TPA and other activators. This system thus appears particularly useful for investigating the molecular principles underlying the phenotypic event of switching from a predominantly membrane bound to secretory mode for the product of restricted *CH* gene regions. Whether accessory factors in combination with phorbol esters would be capable of inducing the differential readout of the *CH* gene cluster is unclear, but our attempts to date with allogeneic T cell factors have proved unsuccessful (unpublished observations).

Phorbol ester by itself seems incapable of producing profound phenotypic changes in some of the histologic groups of NHL. Godal *et al.* (1982) have reported that TPA can amplify the proliferative response of NHL cells to anti-Ig, and in some subtypes this may also lead to increases in the levels of cIg (Godal and Funderud, 1982). In the few cases of FCC lymphomas we

have studied, we concur with the notion that these cell types are generally refractory to *in vitro* differentiation by TPA alone. We have also been unsuccessful in amplifying Ig secretion from hairy cells by exposing them to TPA. These findings strongly argue for compartmentalization of neoplastic B cells by virtue of their responsiveness. This may reflect the possibility that the nonresponding clones are developing along divergent pathways to those typically represented by B-LL or that they are at stages of maturation arrest which either do not require the signal supplied by TPA for further differentiation or where the receptors for this molecule (or the machinery to respond to it) may have been lost. Early cells of the B lineage seem particularly susceptible to the differentiation-inducing effects of phorbol esters as exemplified, not only by the B-LL, but also by some recent studies on ALL populations. We have already discussed the interesting example where Cossman *et al.* (1982) demonstrated that the addition of TPA resulted in a case of ALL functionally expressing the Ig genes which in the unstimulated form were phenotypically silent (see Section III).

D. Interaction of Epstein–Barr Virus with Neoplastic B Cells

In B cell neoplasia, EBV appears to be pathogenically associated only with cells of Burkitt lymphoma. Attempts to obtain immortalized lines of neoplastic cells from other B cell tumors by infection with EBV *in vitro* are almost universally unsuccessful. More often than not, collections of lines representing transformed normal residual B cells are obtained (see for example, Gordon *et al.*, 1977b). This may reflect either an inherent refractiveness of the neoplastic cells to incorporate the EBV genome or a selective growth advantage of the transformed normal cell over the malignant population. Occasional reports on EBV-transformed neoplastic cell lines point to the target clones being atypical in their phenotypic characteristics. Indeed, it seems that those B-LL with particularly mature features are the most capable of yielding EBV-genome carrying lines (Fu *et al.*, 1979). This is highlighted in a study by Rickinson and colleagues (1982) who demonstrated a relatively high success rate for cells of the prolymphocytic type and from Waldenström's macroglobulinemia, whereas the more typical B-LL rarely gave lines (Finerty *et al.*, 1982). In their only reported example of the latter, the cells were derived from a case expressing IgG as its primary isotype whose expression was preserved in the transformed line. In most cases a neoplastic origin for the cell has been indicated by light chain restriction occasionally coupled to isoenzyme markers (Karande *et al.*, 1980). Fu *et al.* (1979) were able to conclusively demonstrate a neoplastic derivation of the line by its reactivity with antibodies raised against and rendered specific for the idiotypes carried by the M component present in both cases. Our own attempts to establish B-

LL lines by EBV infection confirm the findings of others in that the only successful case was a clear immunocytoma with a related M component (unpublished observations). The cells secreted IgMλ prior to infection and continued to secrete this isotype exclusively following EBV transformation. The secretion of surplus light chains continued once the cells had been established in culture even though the morphology and other phenotypic markers of the cells had shifted markedly.

In contrast to the extreme difficulty in establishing continually growing lines, minor B-LL subclones appear to incorporate the EBV genome more frequently. Nearly all B-LL populations probably express receptors for EBV so that entry of the virus is at least theoretically possible (Finerty *et al.*, 1982; Wells, 1982). The possibility that minor subclones in B-LL populations could come under the influence of EBV was first suggested by the studies of Robert (1979) who demonstrated an increase in the number of light chain-restricted PFCs in B-LL clones exposed to virus. The number of PFCs were low, which is consistent with the more recent study from Rickinson's group who found that in all populations studied, a variable number of EBNA positive cells could be detected shortly after addition of virus. Subsequent kinetics were variable, in some cases with the positive cells rapidly disappearing, while in others they were more persistent but still did not yield lines. In recent unpublished studies we have noted in approximately half of the cases of typical B-LL investigated an EBV-specific induced rise in IgM secretion measured by both ELISA and PFC assay. Ig was restricted to the light chain of the neoplastic cell surface, and we confirmed that the few percentage of EBNA-positive cells which arose carried the same light chain and heavy chain isotype as the secreted product. Other phenotypic markers, including some defined by monoclonal antibodies, differed on the EBNA-positive cells by comparison with the dominant subclone. This suggests either that only cells at these discrete stages of differentiation arrest were infectable or that infection caused a shift in the maturation stage of these cells. It is unclear what the mechanisms are that control the outgrowth of the EBV-infected tumor cells, but the desirability of obtaining immortalized lines for studying the molecular events associated with Ig expression by neoplastic B cells warrants further investigation of the problems involved.

IX. Toward a Unifying Concept of the B Cell Neoplasia

A. Introduction

It may be too presumptive at this stage to attempt a single all-embracing scheme in an effort to unify the B cell compartments as jointly represented by successive stages of normal development and by the different phenotypes reflected in B cell leukemia and lymphoma. Clearly, however, great strides

are being made in these areas aided by the fine dissection of the B-cell system with selected batteries of monoclonal antibodies and careful probing of the cellular and molecular processes involved in the functional expression of the CH gene locus. The aim of the following section is to approach a synthesis of current knowledge on the capacity of neoplastic B lymphocytes to express the immunoglobulin genes and to handle the phenotypic product, particularly in relation to normal B cell processes, its relevance to the malignant state, and its possible clinical utility. The following section explores to what extent we can rationalize such an approach based on the model employed.

B. Limitations of the Model

A cytogenetic study of the lymphoid neoplasia requires cells in mitosis. Because the mitotic rate can be low, particularly in chronic type leukemia, proper study has necessitated attempts to induce proliferation by mitogen stimulation. With increasing success in this approach, it has been revealed that chromosomal aberrations can occur with high frequency in chronic lymphocytic leukemia with patterns not entirely random, such as a high incidence of trisomy 12 in B-LL (Gahrton and Robert, 1982). A recent study of a large number of NHL cases revealed chromosomal abnormalities in every case where mitotic figures were obtained. The predominating type of aberration correlated well with the histologic subtype of lymphoma (Yunis *et al.*, 1982). We have already discussed the possibility of an interrelationship between specific chromosomal abnormalities and morphologic and functional cell type using the example of Burkitt lymphoma. When considering B cell neoplasia as a model for examining normal B cell events, the important question is to what extent, if any, the genetic aberrations impinge on the processes under investigation. While in the case of BL there may be a direct relationship between the genetic translocation and the phenotypic expression of the Ig genes, it is possible that even in those cases where the Ig genes are not directly involved, the chromosomal aberration may interfere with some of the machinery (possibly enzymatic) which is critical for the correct processing of the Ig genes and their products. Examples that we have encountered which could constitute such a defect include those cases of B-LL with "unusual" cytoplasmic Ig inclusions and the rare, apparently aberrant expression of heavy chains without light chains at lymphoma cell surfaces. Even for these cases, however, we have no formal proof that totally aberrant processes are represented or that they have arisen secondary to the oncogenic event. On the contrary, phenotypic processes related to the expression of Ig genes appear to be remarkably conserved across histologic or pathologic categories seemingly relating more to functional subtype than any expected feature of malignancy.

The often apparently "atypical" aspects of the lymphoid neoplasia in regard to their phenotypic expression of immunoglobulins may be a reflection of their intrinsic nature of expanding and preserving a phenotypic profile, which might be represented in normal physiology at only very low frequency. This may relate to minor subsets of poorly represented B cell compartments, a reflection of highly transient events in normal development or a combination of the two. Some justification of these concepts has been provided in a study where it was shown that the phenotype represented by typical B-LL populations, and apparently absent among adult or cord blood cells, could be found at low frequency among normal tonsil populations (Caligaris-Cappio *et al.*, 1982). This capacity to represent "single moments frozen in time" of perhaps rare subsets reflects the basic utility of B cell leukemia and lymphoma in examining the complexity of the B cell spectrum. It is this feature of "frozen" maturation states which may be the manifestation of the chromosomal aberrations intrinsic to the proliferating clones. This apparent block could evolve from a number of quite distinct causes, but the observation that specific chromosomal abnormalities are related to different histologic groupings could help to explain the various levels of restricted differentiation capacity in this group of diseases.

The measurement of bulk properties of neoplastic populations and subsequent extrapolation of the observations to normal equivalents representing the dominating subclone relies heavily for its validity on minimal deviation from the concept of restricted stages of maturation arrest in the proliferating clones. We have seen, however, that in many cases the concept of frozen differentiation should be regarded only as relative and not as absolute. The apparent ability of minor subclones to sneak through the differentiation barrier seemingly imposed on the dominating populations could profoundly alter both our concepts of the intrinsic features of the B cell malignancies and our interpretation of the capacity of these cell types to handle Ig.

C. Target Cells and Maturation Blocks

The extent to which the dominant phenotype represents the full intraclonal spectrum of a given tumor is essential to our full understanding of the B cell malignancies. For example, the proliferating subclones or the target cell for the malignant transformation may express phenotypic profiles quite removed from the bulk of the clonal population examined. While these questions are central to all oncologic systems, they warrant particular scrutiny within the context of B cell neoplasia where powerful molecular tools are becoming available to attempt a rational approach.

The key questions to consider relate to (1) the phenotypic disposition of the target cell for the oncogenic "hit," (2) the differentiation potential of the

oncogenic target, (3) the characteristics of dividing subclones, and (4) the intrinsic and systemic controls on the maturation and regulation of the proliferating cells. These individual facets are probably highly interrelated so that none should be considered isolated from the others. However, while we have come a long way in identifying the phenotypic profiles of the dominating subclones, we are only just beginning to probe the detailed properties of these minor, but clinically more relevant, subpopulations.

An essential feature of the B cell malignancies in this regard is the presence of a unique clonal marker with which to probe minor subpopulations of the tumors. This is represented by the idiotypic structures encoded within the V regions of the Ig chains and formed in large part by the light–heavy chain union. The utility of this approach has been highlighted by using myeloma in which antibodies raised against the M component and rendered specific for the idiotypes have been used to identify small B lymphocytes expressing the clonal marker (Mellstedt *et al.*, 1982). In an elegant experiment from Cooper's group (Mayumi *et al.*, 1982), a library of monoclonal antibodies were generated against IgA expressed on the circulating lymphocytes in a case of chronic type B-LL. Some of these antibodies reacted exclusively with those determinants expressed on the heavy chain V region providing clonal probes for the very early members of the B cell series. They succeeded in demonstrating in this one case that among the bone marrow cells there was a higher than expected incidence of pre-B cells expressing the heavy chain idiotype of the malignant population. In addition, their phenotypic profiles were similar to some of the ALL populations already mentioned, with evidence for a switch occurring at the pre-B level in the order of $\mu \rightarrow \gamma \rightarrow \alpha$. A similar study on multiple myeloma further points to the possibility that pre-B clonal members may exist in neoplasms where the dominating subclones are clearly of a more mature phenotype (Kubagawa *et al.*, 1979). These experiments, however, in no way provide proof that the pre-B stage represents the target cell of transformation; they merely demonstrate that this is the most primitive identifiable cell of the malignant clone when utilizing an idiotypic marker.

Potentially, the lineage could be traced back even further by probing for specific *VH* gene rearrangements occurring before the functional expression of the phenotypic product. That the oncogenic target may be more primitive than a pre-B cell is hinted at (but not proven) by the occasional joint manifestation of a chronic B-lymphocytic leukemia with polycythemia vera (PCV) (Jacobsen *et al.*, 1982). PCV represents a malignant proliferation of a very early stem cell whose differentiated members include erythrocytes, granulocytes, and thrombocytes. In the mouse it has been shown that in addition to these lineages, a common stem cell also gives rise to members of the lymphoid series (Abramsen *et al.*, 1977). The possibility thus exists that, in

those cases clinically occurring as PCV with B-LL, the latter simply reflects differentiation of the common malignant stem cell along the B cell option. A similar possibility may exist for hairy cell leukemia (Mufti *et al.*, 1982), although in neither disorder has any evidence yet been supplied either at the cytogenetic or phenotypic level for such an association. It is also unclear whether for all cases of B-LL or myeloma the oncogenic hit arises at a stage equivalent to the pre-B cell or earlier, and it may be dangerous at present to extrapolate from a few isolated reports.

Nevertheless, it is an intriguing proposition that in clinical and functional entities as apparently diverse as chronic B-LL and multiple myeloma a common or closely related target cell of transformation may be involved. If we assume that this possibility exists, then the more relevant question remaining is what determines the stage of maturation arrest reached by the malignant clone, as it is this feature which may determine the clinical attributes of the disease. We have already broached the possibility that this facet may be intimately involved with the specific chromosomal alterations. One possible mechanism could be the failure or abnormal expression of acceptors for maturation factors, although the ability in many cases to induce differentiation *in vitro* would tend to argue against this. In fact, the lymphocytic neoplasms appear to display no unique tumor-associated phenotypic characteristics, and it has been suggested that the restricted differentiation capacity *in vivo* reflects a subtle uncoupling of the normal processes which relate proliferation to maturation (Greaves, 1982). The processes leading to these perturbations may be multifactorial as illustrated by the presence of the EBV genome and specific chromosomal translocations among African Burkitt lymphoma. Such a combination of events may serve to endow the transformed populations with certain growth advantages which allow them to emerge as uncontrolled proliferations. We have already seen that, in some cases, the level of maturation arrest of emerging clones may relate more to systemic host defects rather than to intrinsic clonal aberrations (Section VIII). The finding that chronic B-LL may occasionally progress *in vivo* to a "histiocytic" type of lymphoma (Long and Aisenberg, 1975) further adds weight to the concept that even *in vivo* differentiation blocks at discrete levels may be relative and by no means intransmutable. The possibility that B-LL may rarely progress to myeloma also exists (Buchi *et al.*, 1982).

We have seen that in many cases of B-LL, minor populations apparently escape the controls dictating the maturation state of the dominating subclone. This is exemplified by those cases of immunocytoma where the leukemic clones are associated with an overt serum M component. This in turn distinguishes these cases from Waldenström's macroglobulinemia (WM) by the dominating leukemic population having more features of small lymphocytic cells rather than the sometimes predominating plasmacytoid features of

WM. The Kiel group (Lennert, 1978) prefers an abandonment of the latter as a separate lymphoma entity partly based on the finding that the histologic picture of WM can be manifested in the absence of a 19S M component. Thus there seems to be some justification in considering the B cell neoplasia spectrum as a continuum of differentiating malignant cell types rather than as discrete proliferations of limited functional entities. Figure 11 attempts a schematic representation of these underlying principles, although the details of the processes should be considered as highly speculative and are open to considerable debate.

D. Surplus Light Chain Production: A Vital Phenotype of B Lymphocytes?

The observation that B-LL (Gordon *et al.*, 1978), hairy cells (Gordon and Smith, 1978), and certain histologic lymphoma subgroups (Hannam-Harris *et al.*, 1982) synthesize and secrete a large molar excess of light chain over heavy chain led us to suggest that this phenotype was a property of primarily immature B lymphocytes. Evidence to support this notion came from studies of lymphocytes from patients with certain immunodeficiencies of their B cell system (Gordon *et al.*, 1978) and from investigations on normal B lymphocytes early in ontogeny and associated with adult subsets (Hannam-Harris and Smith, 1981b). The findings together pointed to the surplus light chain production observed in neoplasms of "immature" B lymphocytes being an essentially different phenomenon to that seen among the plasma cell disorders where myeloma associated with Bence–Jones proteins occurs at high frequency and probably represents secondary chromosomal alterations. Free light chains can indeed be readily detected in the serum and urine of healthy individuals, arising from de novo production, and are thus a normal synthetic product of the B cell system (Stevenson, 1962; Sölling. 1975).

The association of a large secreted light chain excess with early cells of the B lineage seemed difficult to reconcile with the known order of Ig gene rearrangements during B cell development, although it could have been argued that B cell subsets had the option of asynchronously expressing either Ig chain first during their emergence, with a preference for light chains being reflected in the cell types represented by B-LL. A unifying concept finally emerged from our detailed studies on rare cases of B-LL which displayed phenotypic characteristics consistent with a transition stage from a small nondividing pre-B cell to an Ig-expressing primary B cell (Gordon *et al.*, 1983a). As noted by Paige and colleagues (1977) using the pre-B murine lymphoma line, the onset of light chain production in this B-LL subgroup was intimately associated with the expression of stable membrane immu-

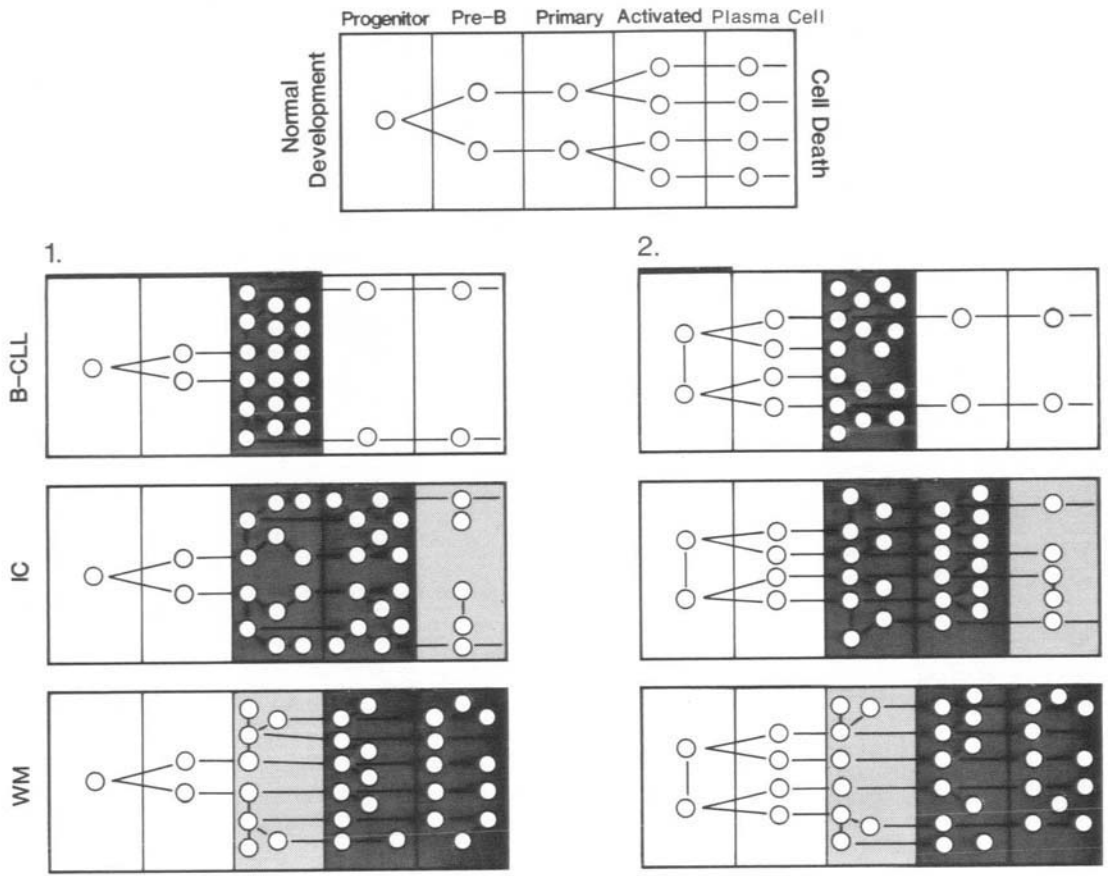
Progenitor
Pre-B
Primary
Activated
Plasma Cell
Normal Development
Cell Death
1.
2.
B-CLL
IC
WM

FIG. 11.

noglobulins. For all clones examined, where even a minority of cells expressed barely detectable levels of membrane IgM, the turnover of free light chains into the extracellular environment could be detected. The single case we have had the opportunity to examine, in which no immunoglobulins were detected at the neoplastic cell surfaces, involved a predominant subclone where the cells contained cytoplasmic μ chains but where no secretion of light chains were detected. The induction of light chain synthesis in this clone led to the emergence of surface IgMκ molecules and the secretion of IgMκ along with free κ chains. Reexamination of this patient's leukemic population several months later revealed a phenotype almost identical to that achieved upon *in vitro* differentiation of the original clone with phorbol ester. Another pertinent finding from our recent studies was that, while the secreted light chain excess could vary considerably between B-LL populations of diverse phenotypes, the actual amounts of free light chains exported from these populations were remarkably invariant. We have now seen that this observation can be extended to clones induced to differentiate *in vitro*, to leukemic hairy cells, and to lymphoma populations of diverse histologic type.

From our accumulated observations, we now feel that there is a convincing case for secreted free light chains being an essential feature of B lymphocytes at all stages of development beyond the pre-B level. The appearance of this phenotype may occur simultaneously with, or shortly after, a productive rearrangement on one of the light chain loci.

It is difficult with our present knowledge to assign a role to the secreted light chain which by itself does not specify the antigen-binding or idiotypic

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FIG. 11. Target cells and maturation blocks in B cell malignancy. This model proposes two possible routes to B cell malignancy, here exemplified by idealized cases of B-CLL, immunocytoma (IC), and Waldenström's macroglobulinemia (WM). (1) This scenario envisages the transforming event (indicated by the heavy line) occurring at any stage up to that represented by the predominant subclone of the disease. Clonal expansion is not realized until a stage where the transformation uncouples the usual controls on differentiation and proliferation leading to a bulk of cells being "trapped" within one or more of a limited number of maturation compartments. (2) This scenario postulates a progenitor cell (pre-B or earlier) as the invariable oncogenic target which itself manifests unregulated proliferation but can progress to limited maturation states where further aberrations which may be intrinsic (chromosomal) or systemic (e.g., inadequate cooperative signals) block normal development leading to an accumulation of malignant cells at those stages. The examples given show different clinical manifestations of the B cell malignancies as reflecting the properties of the compartments in which the dominating subclones are trapped. The maturation options in the case of B-CLL are strictly limited, although occasional cells may sneak through the block but are not usually apparent due to cell death. In the case of IC the clone has more flexibility in its development potential, with a few cells reaching terminal differentiation, while in the example of WM, it is these more differentiated stages where the largest accumulation of neoplastic cells occurs.

characteristics of the emerging cell. We currently favor the notion that its appearance is simply a by-product, reflecting the necessity of the heavy chain-producing cell to ensure that a surplus intracellular light chain pool is maintained. This, in turn, probably reflects the essential role of light chains in processing and handling Ig molecules in their transport and excretion from the cell. For some time it has been appreciated that the heavy and light Ig chains are synthesized on separate membrane-bound ribosomes within the Ig-producing cell (Uhr, 1970). Some recent studies on the intracellular localization of the composite chains suggest that light chain synthesis, particularly in immature cells, may be located primarily in the perinuclear space and that free light chains sequested in the cisterna may then be transported to sites of heavy chain synthesis within the endoplasmic reticulum (Newell *et al.*, 1983). There are several lines of evidence to suggest that free light chains are required to mediate the release of the relatively insoluble heavy chains from their polyribosomes (Askonas and Williamson, 1967; Vassailli *et al.*, 1971; Valle *et al.*, 1981). The precise mode of assembly of H2L2 molecules may then depend on the Ig isotype being manufactured (Nisonoff *et al.*, 1975). Some *in vitro* translation studies suggest that the ratio of light chain to heavy chain may be important in controlling these mechanisms with a light chain excess inhibiting the formation and precipitation of large Ig aggregates (Beychock, 1979). Following their formation, assembled Ig molecules may be transported and secreted from the cell via different pathways to those utilized by the free light chains which appear to be processed primarily by the Golgi apparatus (Newell *et al.*, 1983).

The essential role of light chains in Ig secretion is strongly suggested from observations on myeloma mutants. We have already mentioned the high incidence of light chain secretion from myeloma cells, which in many cases, appears to reflect a loss in the capacity to produce heavy chains. In contrast to spontaneous loss of heavy chain synthesis, myeloma mutants which have lost the capacity to produce light chains, but continue to synthesize heavy chains, are extremely rare (Köhler, 1980). In nearly all cases the heavy chain is either not secreted or shows some deletions in its basic structure (Sonenshein *et al.*, 1978; Franklin *et al.*, 1979). The secretion of the mutant heavy chains can, however, be rescued by fusion of the cells with myeloma variants producing only light chains.

In other recent studies, Köhler and colleagues (1982) isolated a set of IgM plasma cell mutants, some of which were characterized by deletions in the constant regions of the μ chains. Their results indicated that the secretion of HL-containing units could be effected despite significant deletions in any of the four Cμ domains. This suggested that the essential property of secretion lay in the light chain. Others have noted that gross alterations in IgM or IgG structure do not appear to interfere with the secretory process, although the

requirement for the light chain is maintained (Dinnick *et al.*, 1980). The exceptions appear to be those cases where deletions involve the VH domains (Seligmann *et al.*, 1979) leading to the suggestion that for normal heavy chains the variable regions contain structures which, unless covered by light chains, may hinder the secretory processes (Köhler *et al.*, 1982).

The "rule" that apparently normal heavy chains are toxic for B cells unless linked to light chains is seemingly transgressed by pre-B cells and hybridomas obtained from them (Burrows *et al.*, 1979). This apparent discrepancy has been explained in part by the fact that pre-B cells and their neoplastic analogs simply contain too little heavy chain to warrant a problem for the cell, although this argument could not be applied to the high rate heavy chain-producing hybridomas. However, some recent observations have pointed to the possibility that the heavy chains in these cell types may in fact not be "free" but rather are present in association with a binding protein (BIP) (Wabl and Steinberg, 1982). The postulated role for this BIP is to maintain the solubility of the newly translated μ chains until the cell has undergone an effective light chain rearrangement. It has been suggested that emerging B cells make only enough BIP to combine with the heavy chain product of one rearranged allele so that, if both were successfully rearranged, the cell would be eliminated by heavy chain toxicity. It is possible that those extremely rare neoplastic populations expressing heavy chains without light chains at their cell surfaces represent an inappropriate processing of the BIP protein. These and the more typical monoclonal populations of ALL and B-LL should provide ideal models for the detailed examination of the essential features of light chain expression at the levels of genetic rearrangements and posttranscriptional processes, respectively.

E. An Attempt at an Integrated Model

A model is presented in Fig. 12 which attempts to unify our current knowledge on the events involved in immunoglobulin processing at discrete levels of B cell development. It is by necessity simplistic, failing to account for the precise sequence of Ig isotype switches or the origin of IgD- or IgE-secreting cells of which there is scant knowledge at present. Nor does it attempt a description of the complex interplay of the B cell compartments with modulating factors and cells of other arms of the immune system; these concepts are best left to other reviews at a future time. Also, notable by their absence are antigens other than Ig which are associated with the B cell series. These are omitted primarily for clarity although their precise sequence of appearance is still poorly understood in comparison with the phenotypic expression of Ig.

To reiterate and summarize, integral to the model are two sets of gene

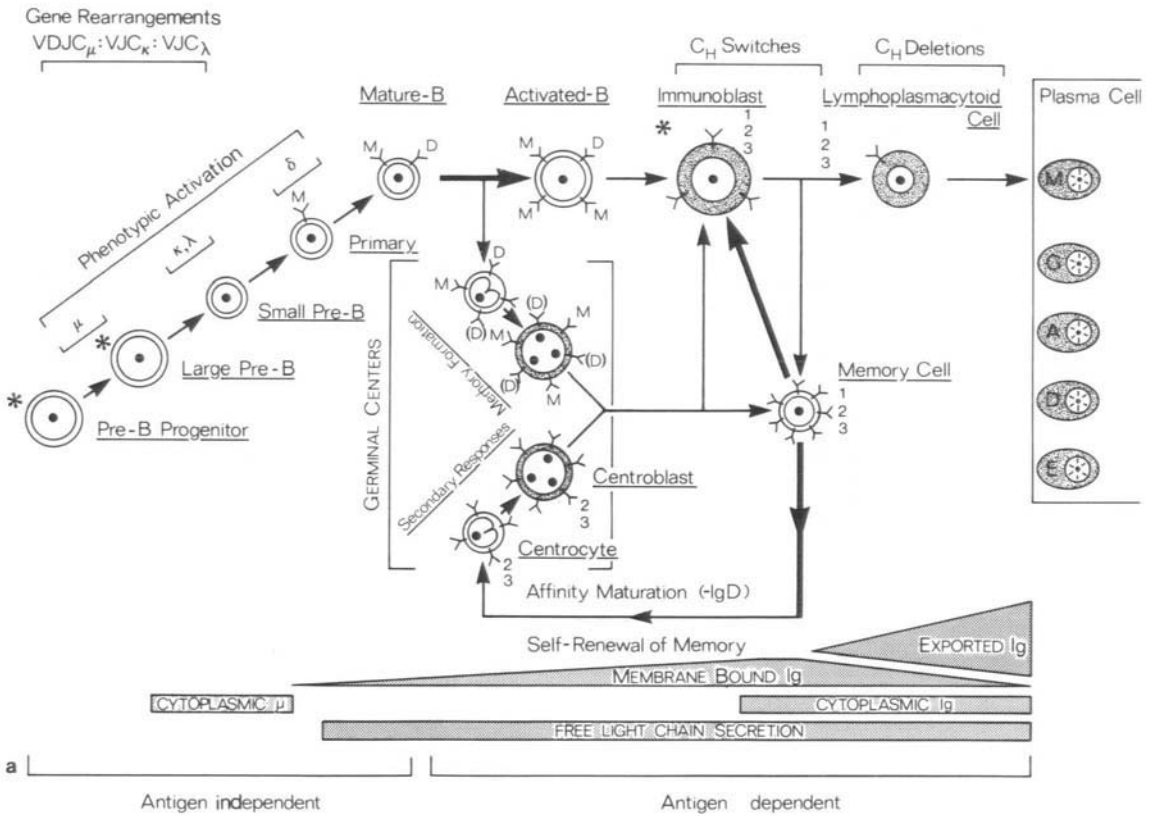

FIG. 12a (see legend p. 140).

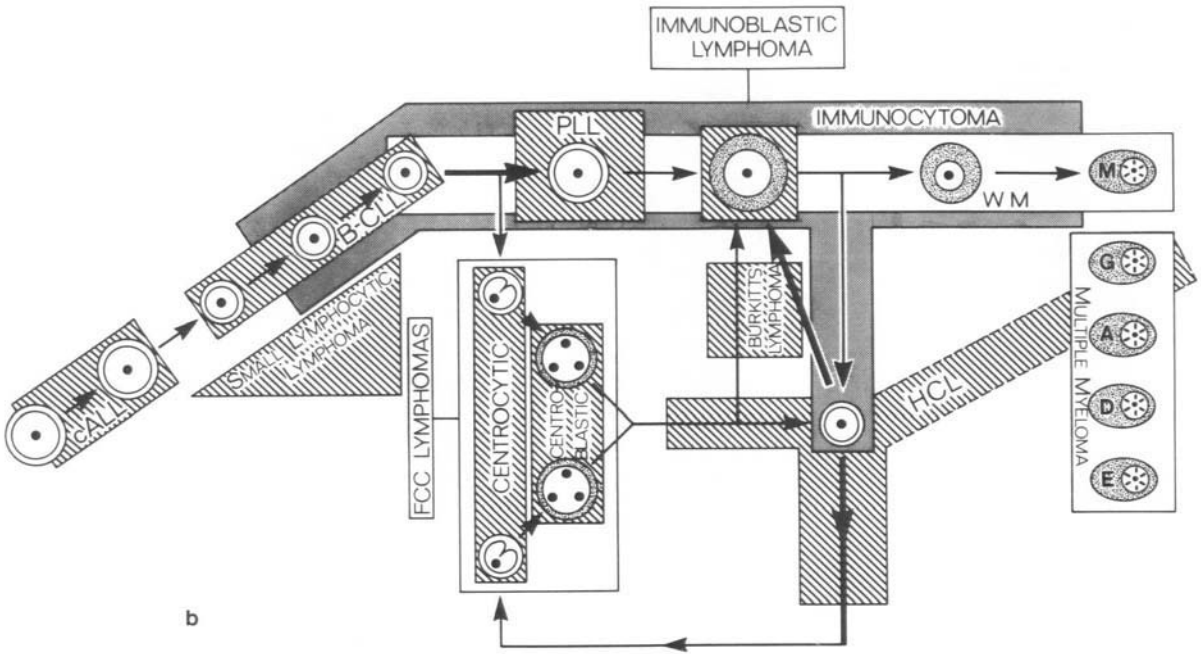

FIG. 12b (see legend p. 140).

rearrangements occurring at different levels of B cell development: those early on, serving to bring the separate genetic elements in the germ line together to form functional Ig genes, and those which occur later in response to antigen stimulation and which switch the expression on the *CH* gene

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FIG. 12. (a) A morphologic scheme of B cell development with special reference to the phenotypic expression of the immunoglobulin genes. The essential features of this scheme are described in Section II. Basic morphologic types are indicated highlighting nuclear and cytoplasmic characteristics while an asterisk denotes the prime proliferative stages. Events occurring on the immunoglobulin gene loci and the predominant modes of phenotypic expression at discrete stages are also included. Two primary routes of development are envisaged following encounter of immunocompetent B cells with antigen (indicated by heavy arrows) and the appropriate cooperative signals. The first of these is independent of germinal centers and leads directly to the formation of IgM plasma cells via an immunoblast and a lymphoplasmacytoid stage with IgM as the predominant isotype expressed. Memory cells appearing in a primary encounter with antigen evolve from germinal centers, possibly via an immunoblast stage, and may present a complex phenotypic profile depending on diverse elements such as the quality of the priming signal, the microenvironment of the developing cell, and the precise interplay with regulating factors. Three main types of memory cells can be identified resulting in the following sequences. (1) Cells arising from germinal centers bearing predominantly IgM (Mp) on subsequent antigen-triggering directly elaborate a plasma cell response via the immunoblast stage which may maintain the phenotypic expression of IgM or may undergo *CH* switching to IgG. (2) Memory cells which are Dp are probably included in a subpopulation which, besides eliciting an IgG plasma cell response, are involved in self-renewal and switching to a phenotype of Gp. These processes may also involve affinity maturation of the clones with the renewed memory cells on further triggering developing directly to a high affinity IgG-producing plasma cell. Whether the self-renewal processes require passage through germinal centers is unclear but this possibility is indicated. (3) Repeated stimulation of IgG-bearing clones may result in successive proliferative cycles with cells switching to downstream specified C_γ isotypes. Other Ig classes (i.e., IgA, IgD, and IgE) might also arise by sequential vectorial switching on the *CH* cluster or, alternatively, arise predominantly from direct switching from Mp cells to the specified gene sequences due to interplay with microenvironmental factors. (b) An integrated model for B cell neoplasia. The morphologic framework of Fig. 12a is taken and the approximate positions of the normal equivalents of the disease entities discussed are indicated. The intention is not to suggest the full interclonal spectra nor to identify the minor subclones but rather to provide an integrated scheme for the bulk populations of the phenotypically most representative cases of the clinical entities. Common ALL is thus envisaged as a malignancy of B cell progenitors which includes the pre-B, cell while in B-CLL, the dominating subclones represent the early stages of true B lymphocytes. Prolymphocytic leukemia (PLL) is clearly more mature than the common B-CLL phenotype and may reflect a recently activated B cell. While some hairy cell clones may mirror similar stages, the majority of HCL cases might be more related to memory cell processes with some displaying differentiated features toward plasma cell equivalents. Waldenström's macroglobulinemia (WM) and immunocytoma (IC) represent pleomorphic malignancies, which should be considered as overlapping clinical and immunologic entities but with the plasmacytic element generally more dominant in WM. Some IC may represent memory cell stages akin to those reflected by the hairy cell phenotype. Those neoplasms considered separately as NHL are integrated into the scheme with relative ease from their morphologic attributes, although the placing of Burkitt cells is a matter of conjecture. Multiple myeloma belongs to the plasma cell compartments.

cluster. The immunoglobulins manufactured in the cell switch also from a predominantly membrane form to a secreted form as they terminally differentiate, while a separate pathway, represented by the memory cells, preserves the transcription of the membrane form. The memory pathway has the capacity for self-renewal and affinity maturation, both processes being associated with the differential expression of the Ig isotypes. The generation of memory appears to be intimately involved with reactions in germinal centers, with their anatomical location (and presumably the microenvironment provided) determining the isotype to be specified. Both plasma cells and memory cells may develop in response to antigen by a number of separate pathways which either may relate to the precommitment of separate B cell compartments or may depend on the precise interaction with other cell types or their mediators. Surplus light chains are manufactured almost immediately from the turn on of expression of a functional light chain gene, and their essential nature is subsequently reflected at all stages of B cell development by their overspill as free chains to the extracellular milieu.

Figure 12b represents an approximation in attempting to ascribe the dominant clonotypes of the various B cell neoplasia to the stages of normal B cell development outlined. This approach is limited primarily by occasional wide intraclonal heterogeneity, by diverse interclonal disparity within single disease entities, or simply by a lack of sufficient information on the capacity of a particular malignant cell type to handle Ig.

As seen in Section III, there is now little doubt in ascribing common ALL as a disease of pre-B cells or their immediate progenitors based primarily on the hierarchy of Ig gene rearrangements observed in the proliferating cells. Occasionally clones phenotypically express a functional μ heavy chain gene in this disease, but in general, phenotypic expression of the Ig gene loci remains silent. There also exists the possibility that rare cases of Burkitt lymphoma can be placed at similar stages of differentiation arrest, although in the overwhelming majority, both the heavy and light chain genes are functionally expressed. The predominant populations of true B-CLL appear to express, almost without exception, functional heavy and light chain genes. In rare cases, the prime phenotype may be consistent with the small nondividing pre-B cell which has just commenced, or is about to commence, the expression of light chain. Most cases, however, can be quite confidently placed at stages representing early Ig-expressing cells which have either just integrated IgM molecules into their membrane or have additionally transcribed and expressed the δ gene segment. Those clones associated with a serum M component and/or predominantly IgG expression in most cases do not represent B-CLL but instead represents immunocytoma, in which a wider degree of intraclonal pleomorphism is generally evident. The former profile clearly represents a more differentiated cell type than is usually found

in B-LL and probably one developing along the equivalent of a primary response. Those atypical cases where IgG expression predominates are more likely to represent members of the memory cell compartments. However, except for these clearly identifiable instances, the predominant subclone of histologically classified immunocytoma may often appear identical to the immature B cell phenotype represented by typical B-CLL. In contrast, the PLL variant is readily distinguished by a high expression of SmIg suggesting that the predominant cell in this disorder is more mature than that found in true B-CLL. Waldenström's macroglobulinemia incorporates some of these cell types into the malignant clone but is distinguished by a larger proportion of cells of plasmacytoid morphology and a 19S M component. The multiple myeloma are firmly rooted in the plasma cell lineage where the dominant malignant subclones may have evolved primarily from memory cell precursors of diverse lineages.

Those cases of NHL considered separately from overt leukemia reflect a diverse spectrum of B cell differentiation and compartmentalization. We feel that the small lymphocytic type non-FCC lymphoma closely resembles the predominant clonotypes of B-CLL exhibiting light chain as their exclusive or major secreted Ig product and expressing the membrane form of IgM, sometimes with evidence for simultaneous IgD expression, presumably reflects differential RNA splicing mechanisms. Following antigen-driven stimulation of these cells' normal equivalents, plasma cells may be formed directly or memory cells may be generated with the involvement of the germinal centers, with the FCC lymphomas representing transition stages in these processes. Some memory cells appear to turn off IgD expression which is represented by the absence or variable expression of IgD among FCC lymphomas. For reasons outlined in Section VII we prefer a sequence of FCC development in which the analogs of the centrocytic lymphoma appear before those of the centroblastic type. Some of the FCC lymphomas exhibit phenotypic patterns consistent with cells having undergone *CH* switching, and such clones may reflect renewing memory cells. The actual process of *CH* switching may occur at a stage possibly represented by the immunoblastic lymphoma. These cell types may reflect a mitotic stage which gives rise to memory cell and plasma cell progeny and in which the genetic decisions on the CH locus are made. There is evidence from some systems that cell mitosis be a prerequisite for these events.

We have already discussed the possibility of a normal counterpart for BL cells (Section IV) which in some cases may be placed at the stage of emergence of an antigen-stimulated cell from the germinal center, possibly the immediate precursor of the immunoblast. Some BL, particularly as represented in ALL with Burkitt cells, may reflect these cell types having emerged from a memory response by the expression of dominant isotypes

other than IgM and IgD. We also feel that there is some justification in associating the Ig phenotype of HCL with memory cell processes, at least at stages more mature than those represented by B-CLL. The level of SmIg expression by hairy cells is usually more like that associated with PLL than typical B-CLL, while the predominant isotypes (sometimes IgD but also IgG or IgA) hint at memory cell equivalents. In studies of normal mouse spleen memory cells, IgD is a predominant isotype of some memory cell subsets (Zan-Bar *et al.*, 1978), whereas for the putative route of memory cell generation represented by FCC lymphoma, this process appears to be associated with a loss of IgD. The HCL are characterized by a gross infiltration of the tumor cells in the spleen, and it is tempting to speculate that the strong IgD expression often noted with this disorder reflects those memory cell processes identified from studies with the mouse. These notions are in keeping with the suggestion that anatomical location of germinal center formation may be crucial in determining isotype commitments. The occasional association of a related M component of the IgG class with HCL suggests that these cases may be the "immunocytoma equivalents" of this disease but with the differentiating clone reflecting a memory cell origin rather than a primary cell origin.

One clear conclusion to emerge from this review is that within the context of just one parameter—Ig expression—the functional compartments represented by even "single" disease entities in B cell neoplasia are amazingly diverse. It is probably far from adequate to attempt a synthesis by a simple two-dimensional representation, although some divergence of primary pathways is indicated. It is hoped, however, that even though simplistic, such an integration of our current concepts may serve to stimulate and perhaps provoke further consideration of the heterogeneity of B cell leukemia and lymphoma in relation to their normal counterparts.

F. Some Clinical Implications

The recent findings on the capacity of neoplastic B cell clones to express their Ig genes may have some clinical utility in potential diagnostic, prognostic, and even therapeutic application. In this section those of major significance are briefly summarized.

We have already seen how modern classification schemes for non-Hodgkins lymphoma evolved almost simultaneously with the probing of the separate functional types making up the lymphoreticular compartment. These schemes are not simply developed for the benefit of the immunologist but are an attempt to satisfy the needs of the clinician who requires some indication of the subsequent clinical course of their patient and the level and quality of therapy that may be called for. There appears to be distinct trends

in the prognostic factors between lymphoma and acute leukemia of B, T, and so-called null types (Bloomfield *et al.*, 1979; Coccia *et al.*, 1976; Brouet *et al.*, 1976) and some suggestion that the precise patterns of Ig expression by chronic type B leukemia may point to separate clinical courses (Hamblin and Hough, 1977; Jayaswal *et al.*, 1977). The patterns of Ig gene rearrangements of common ALL now allow a firmer basis for the functional classification of this important group of malignancies, and in the same way, precise probing of chronic leukemia and lymphoma for their phenotypic expression of Ig may extend current schemes for grouping these disorders. Perhaps more importantly, identification of the tumor stem cells and the degree of differentiation potential exhibited by the malignant clones may prove to be particularly significant in attempting a prognostic rationale, and here again, Ig expression, especially at the specific level of idiotypes, may be of prime benefit.

The secreted phenotypic products of the Ig gene loci have already proved essential in the diagnosis and monitoring of the plasma cell malignancies. Except in those few cases of immunocytoma and HCL, this approach is thwarted in its application to other neoplasms of the B cell system by a lack of appreciable excretion of the clonal product. Nevertheless, while most cases of B-LL and NHL appear to synthesize Ig primarily for integration into the cell membrane, a low level of Ig secretion can be detected in the majority of cases. This secreted product can be found in the serum of B-LL patients by the use of antiidiotype antibodies (Stevenson *et al.*, 1980). Even for cases which may be completely "nonsecretory," antiidiotypes may be prepared by the rescue of secretion from the tumor cells by fusion with myeloma partners or by simple enzymatic digestion of the monoclonal surface Ig (Levy and Dilley, 1978; Hough *et al.*, 1976). Such antibodies may then be used, for example, as probes for detecting residual cells following therapeutic strategies. Where antiidiotypes are not available, more sensitive methods may allow the identification and quantitation of serum idiotype in the nonexporting or low-rate exporting neoplasms. Perhaps the most promising of these for routine diagnostic purposes is that of isoelectric focusing where clonal products can be discretely resolved by virtue of their isoelectric point. However, caution should be used in automatically adopting this type of approach to chronic B leukemia or to NHL, because the secretory capacity of the clonal proliferations may depend on multifactorial processes, including the degree of escape from the maturation blockade at any given time. Therapeutic strategies could be envisaged as selectively eradicating certain subclones in these disorders so that the resultant levels of circulating idiotype may give an inaccurate assessment of the efficacy of a given regime. Perhaps more accurate in providing estimates of tumor load, with the added advantage of being almost universally applicable, will be the monitoring of

monoclonal light chain output in these patients. There have already been some attempts in this direction, with detection relying on discrepancies in the normal ratios of κ and λ light chains (Pierson *et al.*, 1980; Sölling *et al.*, 1982). Other methods, such as isoelectric focusing, may provide more critical assessment of clonal products. As free light chains are rapidly removed from circulation, urine provides a more appropriate source for monitoring the clonal light chain output.

Finally, we turn to the possibility of treating the nonexporting B cell neoplasms by antibodies targeted against the surface idiotypes of the malignant cells. These clonal markers are essentially tumor specific, and antibodies raised against them, either in isolation or as "magic bullets" delivering cytotoxic agents specifically to the tumor cells, may assist in the eradication of the neoplastic population from the host. Work by Stevenson's group has produced some encouraging results using this approach (Hamblin *et al.*, 1980a, and unpublished observations), and in one case reported in the literature, a complete cure appears to have been found (Miller *et al.*, 1982). For such an approach it is of utmost importance to identify the clonal proliferating target, because the strategy would be severely compromised if a non-Ig expressing pre-B or earlier cell type was involved. It is also important to assess the capacity of the individual clones to handle Ig molecules in optimizing the therapeutic procedure. The Ig export noted *in vitro* from many B-LL cases provides a problematic antigen barrier for antiidiotype *in vivo*. However, this can be substantially reduced by the prior precaution of plasmapheresis (Hamblin *et al.*, 1980b); following this, we have found that most circulating tumor cells appear to be coated by infused antiidiotype. While most of an Ig idiotype is formed by the precise arrangement of a heavy–light chain pairing, antibodies directed toward light chain idiotypes appear capable of reacting with the combined molecule. The possibility that free light chains harvested from the urine of patients with B-LL and NHL could be utilized for the generation of tumor-specific antibodies is currently being examined (F. Stevenson, personal communication). The capacity to almost consistently induce *in vitro* high rate Ig excretion by B-LL populations with phorbol esters adds yet another dimension to this approach in which the previously arduous procedure of harvesting immunogen, either by surface digestions or in cell fusions, is essentially overcome.

G. Prospectives

Predictive assessments of rapidly advancing areas can prove futile due either to the narrow approach of the specialist or to a restricted procognitive capacity from simple extrapolation of current awareness. The best we can hope for is some intuitive guesses in these areas. The discipline of molecular

biology is expanding at a dramatic pace, and the phenotypic profiles currently so actively pursued by the immunologist may soon be replaced, or at least complemented, by detailed genotypic profiles. Which proves to be the most reliable indicator of clinical activity remains to be seen, but any tool which can supplement our present incomplete view must be openly welcomed and its application to the study of B cell neoplasia actively pursued.

At a more obvious level, the Ig phenotype of a neoplastic clone should be considered as only a portion (although probably the most important facet) of a phenotypic "identification kit" of individual tumors. Increasing attention is being focused on supplementing the Ig profile by fine dissection of the clonotypes with the aid of libraries of monoclonal antibodies against selected B cell associated antigens. The complexity of B cell types being revealed by

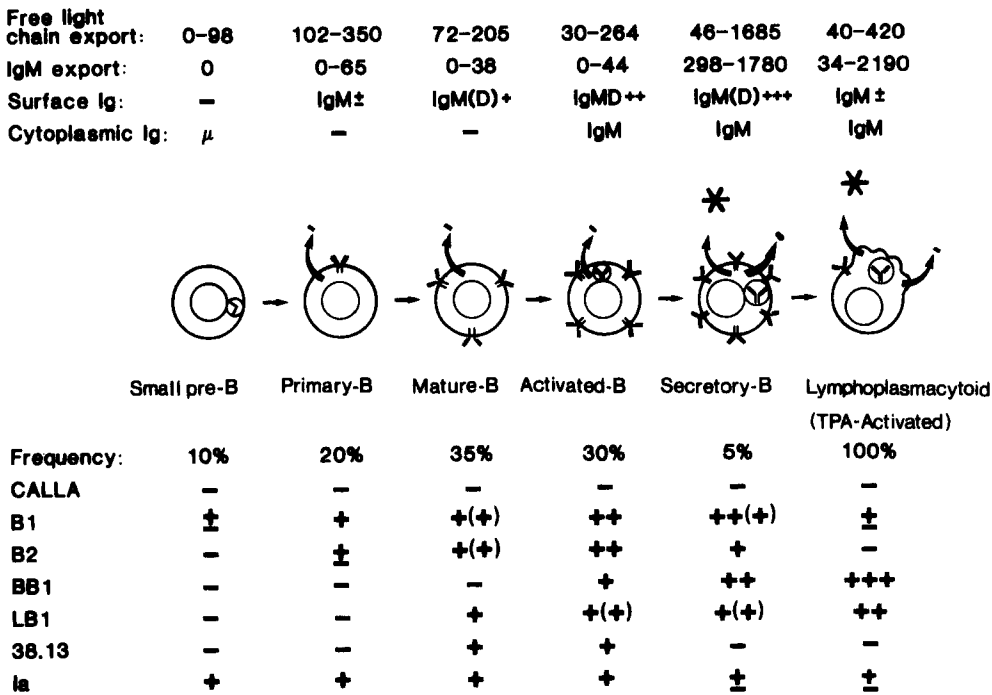

FIG. 13. A schematic representation of phenotypes in chronic B lymphocytic leukemia revealed by multiparameter analysis. From a study of 48 cases of B-LL, five main (but not discrete) phenotypic groups have been identified, and their predominant immunologic characteristics are illustrated along with those of TPA-stimulated clones. The secretion of IgM (*) and free light chains (—) is represented as ng/10^8 cells/hr; the reactivities of the populations with antibodies are graded on a scale of very weak (±) to strong (+ + +). Parentheses indicate a minor contribution to the total phenotype. The antibodies used have been described previously (Gordon *et al.*, 1983a,b).

such an approach has already been discussed by Funderud *et al.* (1983) for NHL. Perhaps more surprisingly, however, we have noted similar complexities for B-LL when studied by a multifactorial analysis. Figure 13 represents a summary of our present accumulated data in regards to B-LL and serves to illustrate the fundamentals of such an approach. Multiparameter analyses of this type may also serve to extend our information on the normal processes of Ig handling at discrete levels of B cell development. We can thus envisage a scenario where an interesting, and perhaps rare, Ig processing event is noted in an expanded neoplastic clone (e.g., the crystalline inclusions of Ig mentioned previously) and then its phenotypic identification kit built and used to identify the functional equivalents among normal tissues. Isolation of these populations by a combination of monoclonal antibodies and fluorescent-activated cell sorting could then allow for the verification of the neoplastic mode of Ig handling in the normal analogs. These considerations can be envisaged as centering on Ig expression at the levels of gene rearrangement, transcription, RNA splicing, and posttranslational aspects, including light and heavy chain compartmentalization, modification of secreted and membrane forms, and the export of light chain surpluses.

While the fundamental interplay between basic science and disease process is not unique to B cell neoplasia, the study of immunoglobulin expression by the clonal proliferations represented has played an essential role in extending our concepts on the cellular handling of macromolecules in general, to an extent where the considerations transcend those of a single disease spectrum or physiologic event. Whatever our field of specialization, it should be with some eagerness that we await the future and its probably very rapid developments in the study of the B lymphocyte malignancies.

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MOUSE MAMMARY TUMOR VIRUS: TRANSCRIPTIONAL CONTROL AND INVOLVEMENT IN TUMORIGENESIS

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I. Introduction

Mouse mammary tumor virus (MMTV) is an RNA-containing tumor virus which causes mammary cancer in mice. MMTV has a life cycle similar to that of other characterized retroviruses, yet MMTV is not completely analogous to other viruses. Some of its unique features have attracted considerable attention in the past few years. First, MMTV transcription is regulated by glucocorticoid hormones. The combination of isolation of MMTV proviruses by gene cloning followed by their transfer into cultured cells has allowed major insights into the mechanism of steroid hormone action. Second,

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MMTV belongs to the group of retroviruses which do not carry an oncogene. Recent results suggest that the transformation of mammary gland cells is related to the integration site of the MMTV viral DNA in the host genome. Thus, MMTV most likely transforms cells via insertional mutagenesis.

In this review we will concentrate our discussion on these two aspects of MMTV. We have also devoted a section to GR mice. This strain has a high mammary tumor incidence, and the genetic transmission of a virus was first described in GR mice. Extensive studies using these mice have added much to our knowledge of the biology of MMTV.

II. Background

A. Transmission of MMTV

In strains of mice with a high mammary tumor incidence MMTV particles are found in the milk of lactating females (Bittner, 1936). The virus is congenitally passed on to the suckling offspring and infects the mammary gland cells where it replicates and leads to high titers of virus in the milk and eventually causes mammary tumors. Exogenously acquired proviral copies can be detected in the mammary tumor DNA of these mice and are instrumental in tumor development. Mice freed of exogenous virus by foster nursing have a very low incidence of mammary tumors (Bentvelzen and Hilgers, 1980).

MMTV can also be genetically transmitted from parent to offspring. Proviral DNA is present in the germ line and somatic cell DNA of the majority of mouse strains (Varmus *et al.*, 1972; Michalides and Schlom, 1975). Mice which are not infected by milk-borne virus, thus carrying only endogenous MMTV proviruses, generally do not develop mammary tumors early in life. There are, however, two cases where an endogenous provirus has been implicated in mammary tumor formation. These are proviruses present in the GR strain of mice associated with the *Mtv*-2 locus (Bentvelzen and Daams, 1969) and in the DBAf and C3Hf mouse strains associated with the *Mtv-1* locus (van Nie and Verstraeten, 1975) (see Section III). It is not clear why most of the endogenous proviruses are inactive, but some of the mechanisms which may control their expression will be discussed in Section IV.

B. Expression of MMTV in Cultured Cells

The availability of cultured cells which synthesize MMTV-RNA and virus particles has facilitated the study of MMTV expression. MMTV-producing

cell lines have been developed from virus-induced mammary tumor cells and by exogenous virus infection of *in vitro* cultured cells (reviewed in Varmus *et al.*, 1979; Michalides and Nusse, 1981).

The life cycle of MMTV is similar to that of other retroviruses (Varmus, 1982). Following virus infection the RNA genome is reverse transcribed into a double stranded viral DNA which integrates into the host genome. The integrated proviral genome has a characteristic structure which is outlined in Fig. 1. The structural genes are flanked by long terminal repeats (LTR). Viral RNA synthesis begins in the U5 region of the 5′ LTR and ends in the U3 region of the 3′ LTR. The majority of MMTV RNA molecules detected in virus infected cells have sedimentation coefficients of 35 S and 24 S (Groner *et al.*, 1979; Robertson and Varmus, 1979; Sen *et al.*, 1979; Dudley and Varmus, 1981). The 35 S molecule is the primary transcript of the proviral gene and encodes the viral gag and pol proteins (Nusse *et al.*, 1978; Dahl and Dickson, 1979; Robertson and Varmus, 1979; Sen *et al.*, 1979). The 24 S RNA, of approximately 3.8 kb, is spliced from the 35 S mRNA and codes for the viral envelope proteins (Sen *et al.*, 1979; Arthur *et al.*, 1982). MMTV does not contain an oncogene. The mechanism by which it transforms mammary gland cells seems to be indirect and related to the retroviral lifecycle. Some implications will be discussed in Section VI.

Early studies using MMTV-infected cell cultures showed that virus production was highest when cells were grown in medium containing glucocorticoids (McGrath, 1971; Fine *et al.*, 1974). In the variety of cell lines which have been studied virus production is stimulated 10- to 20-fold when dexamethasone, a synthetic glucocorticoid, is added to the culture medium (reviewed in Varmus *et al.*, 1979). The enhancement of MMTV expression by glucocorticoids has been shown to be a primary response to the hormone. This made the MMTV proviral gene an attractive model system to describe how steroid hormones control gene expression (Groner *et al.*, 1983). Experiments related to this topic will be discussed in Section IV.

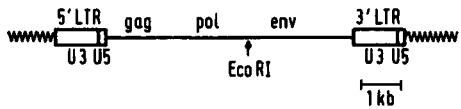

FIG. 1. Schematic diagram of MMTV proviral DNA. The provirus comprises approximately 10 kb and encodes the viral group-specific antigens (gag), reverse transcriptase (pol), and envelope glucoproteins (env). Viral RNA synthesis begins in the U5 region of the 5′ LTR and terminates in the U3 region of the 3′ LTR. Most MMTV proviral variants contain one *Eco*RI restriction enzyme site close to the center of the genome. This site is also indicated in the figure.

III. MMTV Expression in GR Mice

A. Tumor Induction Loci for MMTV in GR Mice

Since MMTV can be transmitted both genetically and by congenital infection, endogenous proviruses are present in the DNA of germ line and somatic cells of mice. They segregate as stable Mendelian traits. Endogenous MMTV proviral genes have been found in all inbred mouse strains tested (Varmus *et al.*, 1972; Michalides and Schlom, 1975), but they are absent or only heterozygously present in feral mice (Cohen and Varmus, 1979; Callahan *et al.*, 1982). The inbred strains of mice examined vary with respect to the number of MMTV proviral genes in their germ line DNA and the proviral location in the cellular genome. These findings have led to the idea that genetically transmitted MMTV proviruses are the result of infrequent insertions of infectious MMTV DNA into the germ line during evolution of the species (Cohen and Varmus, 1979). Callahan *et al.* (1982) found that both MMTV-negative mice and feral mice contain MMTV-related sequences, called MMTV-β sequences. These sequences can be visualized in hybridization experiments using relaxed conditions. MMTV-β sequences are ubiquitously present in the genus Mus and lack variation in their pattern of restriction enzyme DNA fragments. MMTV-β may therefore be regarded as the evolutionary progenitor of the infectious MMTV present in inbred strains of mice, which is now called MMTV-α.

Whether MMTV-β is involved in the development of mammary tumors is yet unknown. The exogenously, milk transmitted MMTV-α is a causative agent in mammary tumor development, and two endogenous MMTV-α proviruses have been implicated in mammary tumorigenesis (Michalides *et al.*, 1981a). The endogenous MMTV proviruses have been classified by Traina *et al.* (1981) using the characteristic *Eco*RI fragments of mouse DNA which contain MMTV-DNA sequences. *Eco*RI cleaves at only one site within most of the nondefective MMTV genomes (see Fig. 1). Thus, cellular DNA fragments contain one-half of the MMTV proviral DNA and a cellular DNA sequence up to the first *Eco*RI recognition site in the flanking DNA. This yields well-defined *Eco*RI fragments composed of MMTV and cellular DNA which are characteristic for proviral copies integrated at a specific cellular genomic location. Analysis of recombinant inbred strains yielded 15 loci containing MMTV proviral DNA information called Mtv loci (Traina *et al.*, 1981). Two of these loci, *Mtv-1* and *Mtv-2*, are directly related to mammary tumor development (Michalides *et al.*, 1981a).

The *Mtv-1* locus is responsible for expression of moderate levels of MMTV and for late appearing mammary tumors in the genetically related C3Hf and DBAf mouse strains (Van Nie and Verstraeten, 1975). A particular endoge-

nous MMTV provirus was mapped in the *Mtv-1* locus by relating the presence of MMTV particles in the milk of individual mice of the backcross Mtv-1(−/−) × Mtv-1(−/+) with the segregation of MMTV-specific restriction fragments in the DNA of these animals (Michalides *et al.*, 1981a). C3Hf and DBAf have five endogenous MMTV proviruses. One proviral gene, characterized by *Eco*RI fragments of 5.9 and 4.2 kb is associated with the *Mtv-1* locus. Similarly, one of the five endogenous MMTV proviruses of GR is present in the *Mtv-2* locus. This dominant locus controls expression of high levels of MMTV in the milk and the occurrence of pregnancy-dependent mammary tumors in GR mice (Van Nie *et al.*, 1977). Backcross studies identified the MMTV provirus characterized by *Eco*RI fragments of 11.0 and 6.9 kb as part of the *Mtv-2* locus. Two congenic strains have been bred. One is identical to GR but lacks the *Mtv-2* locus and is called GR-Mtv-2$^-$ (Van Nie and de Moes, 1977). One has acquired this locus and is called 020-Mtv-2$^+$ (Van Nie, 1983). The lack or the acquisition of the *Mtv-2* locus in both congenic strains corresponds with, respectively, the loss or gain of the 11.0 and 6.9 kb *Eco*RI MMTV-DNA fragments (Michalides *et al.*, 1981a). These findings indicate that expression of two endogenous MMTVs is related to mammary tumor formation. The *Mtv-1* and *Mtv-2* genes are located on chromosome 7 and 18, respectively (Verstraeten and Van Nie, 1978; Verstraeten *et al.*, 1981).

Gr-Mtv-2$^-$ females do not express MMTV particles in their milk and do not develop mammary cancer. Hyperplastic alveolar nodules, which are intermediate stages between normal mammary glands and tumor tissue, are very rare in the congenic GR-Mtv-2$^-$ but appear early and frequently in GR mice (Van Nie, 1981). The early expression of the *Mtv-2* associated MMTV provirus most likely causes the early appearance of hyperplastic nodules in GR.

Although Gr-Mtv-2$^-$ females do not express MMTV particles in their milk, there is a low expression of the internal MMTV protein p27 in the milk and mammary glands of lactating Gr-Mtv-2$^-$ females (Michalides *et al.*, 1978b; Nusse *et al.*, 1980a). Strikingly, the MMTV gp52 envelope protein, MMTV gp52 is not expressed. The induction locus *Mtv-3* for this MMTV p27 expression in Gr-Mtv-2$^-$ females has been linked with Es-3 on chromosome 11 (Nusse *et al.*, 1980b). Lactating mammary glands of Gr-Mtv-2$^-$ mice contain a genome length 35 S and a 24 S MMTV RNA (Michalides, unpublished data) identical to the mRNA species found in MMTV-producing cells. The lack of envelope glucoproteins (env) in lactating mammary glands of GR-Mtv-2$^-$ suggests that the MMTV provirus associated with *Mtv-3* carries a mutation. Two out of five endogenous MMTV proviruses of GR are associated with *Mtv* induction loci. It is unknown which MMTV provirus is linked with *Mtv-3*. Since expression of MMTV p27, characteristic for *Mtv-3*

in Gr-Mtv-2$^-$, segregates as one genetic trait, it is unlikely that the remaining MMTV proviruses of GR express MMTV. Transfection studies indicate that one of the remaining MMTV proviruses, unit II (Traina *et al.*, 1981), can become expressed upon transfection (see Section IV,A). This suggests that the location of the endogenous MMTV proviruses within the cellular genome is crucial to their expression. Endogenous MMTV proviral genes appear to be differentially regulated *in vivo*. Their full expression, as is the case with the *Mtv*-2-associated MMTV provirus, may lead to mammary tumor development.

B. Hormone-Dependent and Hormone-Independent Mammary Tumors in GR Mice

The high incidence of mammary cancer in breeding GR female mice is due to the expression of the MMTV provirus associated with the *Mtv*-2 locus (Bentvelzen, 1968; Mühlbock and Bentvelzen, 1968; Van Nie *et al.*, 1977; Michalides *et al.*, 1978b, 1981a). Breeding females of GR develop pregnancy-dependent mammary tumors which appear under the hormonal influence of pregnancy and regress upon parturition (Van Nie and Dux, 1971). The pregnancy-dependent tumors in GR are like the plaque type tumors described by Foulds (1954) and are composed of ductal and alveolarlike elements. The occurrence and growth of pregnancy-dependent mammary tumors require the hormones prolactin, progesterone, and estrone. Ovariectomized animals develop mammary tumors only with a combined treatment of progesterone and estrone (Van Nie and Dux, 1971). The requirement for prolactin was shown by the reduction of pregnancy-dependent mammary tumors following treatment of GR females with the prolactin suppressing drug 2-bromo-α-ergocryptine (Welsh *et al.*, 1979). Hormone-dependent mammary tumors appear in 100% of ovariectomized GR mice treated with progesterone and estrone. This combined treatment is mimicked by treatment with the steroid compound 17α-ethynyl-19-nortestosterone (ANT) (Van Nie and Hilgers, 1976). The early stages of the mammary tumors induced in ovariectomized GR mice with a combined treatment of progesterone and estrone or with ANT are morphologically similar to the pregnancy-dependent mammary tumors. The later stages of the hormonally induced mammary tumors are rather homogeneous and are classified as adenocarcinomas type B. The tumor incidence in virgin GR mice is as high as in breeders. The tumors appear later at the average age of 15 months and are in the majority adenocarcinomas (Van Nie, 1981). Their growth is not influenced by steroid hormones.

Genetic studies which indicate that one gene, *Mtv*-2, controls mammary tumor development in GR mice took only pregnancy-dependent or ANT-

induced mammary tumors into account (Bentvelzen, 1968; Van Nie *et al.*, 1977). The *Mtv-2* gene most likely also controls the appearance of hormone-independent mammary tumors in GR. In GR-Mtv-2$^-$ mice only a few females develop adenoacanthomas later than 18 months, whereas mammary tumors appear in all breeding and virgin GR mice long before that age. Adenoacanthomas also appear late in life in other low mammary cancer strains of mice and are different from the adenocarcinomas and plaque type tumors in GR. GR-Mtv-2$^-$ females do not develop hyperplastic nodules, which are regarded as precursor stages of mammary tumors (Van Nie, 1981). The *Mtv-2* gene, therefore, is involved in the induction of hyperplastic nodules, pregnancy dependent, and hormone-independent and -dependent mammary tumors in the GR mouse strain.

Since *Mtv-2* controls the appearance of hormone-dependent as well as hormone-independent mammary tumors in GR, it is highly unlikely that the hormone dependency of the tumor will be an intrinsic property of the *Mtv-2* gene or of the MMTV provirus associated with it. The hormone dependency of the GR plaque type tumor may be determined by the type of cell transformed by the virus and/or by the tropism and early spread of the virus. The MMTV variant of GR belongs to the group of plaque-inducing MMTVs (Bentvelzen and Daams, 1969). Compared with the MMTV variant of C3H strain, MMTV (GR) is more potent in promoting pregnancy-responsive mammary tumors, but some of the MTV (C3H) induced mammary tumors are also hormone responsive (Van Nie, 1981). Nothing is yet known about the target preference of the various MMTV variants. A target preference might exist but might not be exclusive because MMTV (GR) induces pregnancy-responsive mammary tumors as well as adenocarcinomas in other strains of mice.

Expression of the MMTV provirus associated with the *Mtv-2* gene is required for mammary tumor development in GR mice. One may ask whether expression of this endogenous MMTV provirus itself is sufficient for mammary tumor development or whether reintegration of MMTV-DNA is a prerequisite. Both hormone-dependent and -independent GR mammary tumors contain only a few extra MMTV-DNA copies when compared to liver DNA. Most of them are present at submolar concentrations, suggesting that not all tumor cells contain the extra MMTV proviral copies (Cohen and Varmus, 1980; Michalides *et al.*, 1981b). Using restriction enzymes which specifically detect the *Mtv-2*-associated MMTV provirus, Fanning *et al.* (1980) showed that mammary glands of GR contain additional copies of the *Mtv-2*-associated MMTV provirus. Premalignant outgrowths and tumor cells of GR contained an average of three additional *Mtv-2*-associated MMTV proviruses.

In addition, it has been found that mammary tumors of GR mice consist of

heterogeneous cell populations. This was described by Macinnes *et al.* (1981) and Michalides *et al.* (1982a) who characterized the exogenously acquired proviral copies in serially transplanted GR mammary tumors. Hormone-induced mammary tumors of GR convert, following serial transplantation, to autonomous, hormone-independent cells. Extra MMTV-DNA copies which were present in the primary tumor and in the initial hormone-dependent tumor passages, disappeared from the hormone-independent passages of a particular tumor line (Michalides *et al.*, 1982a). This is explained by assuming that the original tumor contains both hormone-dependent and hormone-independent cells. The hormone-dependent cells and the exogenously integrated MMTV-specific restriction fragments associated with them disappear from the tumor upon serial transplantation.

Other studies also indicate that GR mammary tumors are heterogeneous in composition. Various histologic patterns are seen in hormone-responsive and hormone-independent GR mammary tumors (Sluyser *et al.*, 1980; Percy *et al.*, 1980), and hormone-induced mammary tumors show a heterogeneous population of estrogen receptor positive and negative cells (Sluyser, 1981). Therefore, GR mammary tumor cells may be able to undergo differentiation into more than one cell type. They may represent independently differentiated cells with common ancestry. At each step of this differentiation a new integration of MMTV-DNA may result in a subpopulation of the final tumor mass with a characteristic extra MMTV provirus.

Hyperplastic nodules are the earliest stages of transformation in the mammary gland. They appear earlier in GR mice than in other strains of mice including high mammary cancer strains bearing a milk transmitted MMTV (Van Nie, 1981). The very early expression of the endogenous MMTV provirus associated with the *Mtv-2* gene increases the likelihood that GR mammary gland cells will become infected with MMTV at early stages. In other strains of mice bearing milk-transmitted exogenous MMTV, a gradually increasing proportion of mammary gland cells becomes infected with MMTV, as is apparent from the increasing load of MMTV in the milk (Verstraeten *et al.*, 1975). If random integration of MMTV viral DNA in the vicinity of a cellular oncogene leads to mammary tumor formation (see Section VI,B), the probability that such an integration takes place at early stages is much greater in mammary glands of GR than in mice of other strains. The early expression of MMTV (GR) and a certain tropism of MMTV (GR) for particular cell types may cause a transformation of the type(s) of GR mammary gland cells that retain the capability to differentiate after transformation. The tumors could, therefore, still be derived from one initially transformed mammary gland cell. The various forms of mammary gland neoplasms in GR, from hyperplastic nodules and pregnancy-responsive mammary tumors to hormone-dependent and -independent mammary tumors, may represent

a range of succeeding transformation events in which integration of MMTV-DNA may play an essential role.

C. Spontaneous T Cell Leukemia in GR May Be Induced by MMTV

MMTV is a slowly transforming retrovirus and lacks a transforming viral oncogene in its genome. It may exert its transforming ability by activating cellular oncogenes upon insertion of proviral DNA into the cellular DNA of the host cell. Therefore, it is possible that MMTV induces other types of tumors, provided that it can enter the cell and activate a cellular oncogene. Such a situation may exist in T cell leukemia in GR males. About 10% of the GR males develop leukemia at about 400 days of age (Haisma *et al.*, 1982; Hilkens *et al.*, 1980). Males of the congenic strain GR-Mtv-2$^-$, which lack the *Mtv*-2 locus and the associated MMTV provirus (see Section III,A), do not develop leukemia. Other cancer incidences, such as lung cancer, remain the same in GR and GR-Mtv-2$^-$ mice. Most of these leukemias are of T cell origin with phenotypes ranging from immature to more mature T cells, whereas only a few B cell leukemias have been found. These T cell leukemias contain large amounts of MMTV proteins but produce no virus particles. This is probably due to an impaired phosphorylation of the precursor gag protein of MMTV (Nusse *et al.*, 1979). The T cell leukemias contain extra MMTV proviral DNA in addition to the endogenous MMTV proviruses present in all cells (Michalides *et al.*, 1982b). The extra MMTV proviral DNA information is present in the primary thymomas and encompasses at least two different endogenous MMTV proviruses out of the five present in GR. The T cell leukemias of GR do not show any amplification nor expression of type C related proviruses, and all show trisomy of chromosome 15, as also found in other T cell leukemias (R. Dofuku, personal communication; Dofuku *et al.*, 1975; Spira *et al.*, 1981).

Rearrangements or altered expression of the cellular oncogenes *ras*, *mos*, *abl*, *fes*, *fos*, *sis*, *rel*, *erb*, *myc*, *myb*, or *src* are being studied in these T cell leukemias. The c-*myc* gene, located on chromosome 15, is of particular interest since *myc* is involved in a characteristic translocation in B cell plasmacytomas (Shen-Ong *et al.*, 1982). The clonal origin of the T cell leukemias of GR males, their late appearance, and their association with extra MMTV proviruses are all consistent with a model in which an (as yet) unknown cellular oncogene is activated by insertion of MMTV proviruses in the cellular DNA of a precursor T cell causing subsequent leukemogenesis.

Replication of MMTV in lymphocytes is uncommon, but expression of endogenous MMTV proviruses has been found in various B and T cell leukemias (Racevskis and Sarkar, 1982; Mermod *et al.*, 1983). The cellular recep-

tor gene for MMTV has been mapped to chromosome 16 (Hilkens *et al.*, 1983). The GR T cell leukemias do contain receptors for MMTV which provide an entry for infectious virus. The early expression of the *Mtv*-2-associated MMTV provirus in target cells of GR and reintegration in the cellular genome at crucial sites may provide the initial step in mammary tumorigenesis and also in the development of T cell leukemias of GR.

IV. Control of MMTV Expression

A. Glucocorticoid Hormone-Controlled Transcription

The regulation of MMTV expression provides an excellent experimental system for the study of steroid hormone action. Mouse mammary tumor cell lines as well as heterologous *in vitro* infected epithelial cells produce MMTV (Parks *et al.*, 1974; Fine *et al.*, 1974; Dickson *et al.*, 1974; Vaidya *et al.*, 1976; Ringold *et al.*, 1977a). In all cases virus production is stimulated 10- to 20-fold when the cells are grown in the presence of dexamethasone, a synthetic glucocorticoid (Varmus *et al.*, 1979). The stimulation of viral RNA synthesis is rapid, independent of simultaneous protein synthesis, and appears to be caused by an increase in the rate of transcription of the MMTV provirus (Young *et al.*, 1977; Ringold *et al.*, 1977b; Ucker *et al.*, 1981). These results suggest that glucocorticoids exert their effect directly at the level of viral RNA transcription.

1. Transfection of MMTV Proviral DNA

Molecular clones of MMTV proviral DNA have been isolated and used to study the effects of steroid hormones on viral RNA expression. Various cloned MMTV proviral genes have been introduced into cultured mouse fibroblasts (Hynes *et al.*, 1981a,b; Buetti and Diggelmann, 1981; Diggelmann *et al.*, 1982; Hynes and Groner, 1982) or into mink epithelial cells (Owen and Diggelmann, 1983) by a DNA-mediated gene transfer technique. Individual cell clones were isolated and analyzed for the acquisition of transfected DNA, MMTV-RNA transcription, and the effect of glucocorticoids on MMTV expression. In each case cells grown in dexamethasone contain 5- to 10-fold more MMTV-specific RNA, an increase which corresponds quite well with the stimulation seen in different MMTV-producing cell lines (Varmus *et al.*, 1979). These experiments suggest that the DNA sequence which confers hormone sensitivity on MMTV transcription is contained within the proviral genome. The recipient cells provide the components which allow the transcription of the transfected MMTV provirus to be influenced by dexamethasone. Other genes which are known to be regulated by glucocor-

ticoids *in vivo* have been cloned and reintroduced into *in vitro* cultured cells. The rat α_2-globulin gene (Kurtz, 1981) and the human (Robins *et al.*, 1982) and rat (Doehmer *et al.*, 1982) growth hormone genes retain their glucocorticoid sensitivity following their transfection.

2. *Transfection of MMTV-Containing Chimeric Genes*

The MMTV proviral DNA which contains the hormone responsive sequence is 9–10 kb in length and codes for several viral structural proteins. To identify the hormone regulatory region of the provirus, chimeric genes containing portions of the MMTV genome linked to indicator genes have been constructed. The hormone inducibility of the various chimeric genes has been tested following their transfection into cultured cells. Viral RNA transcription initiates in the long terminal repeats (LTR) region of MMTV. The DNA sequence of the LTR contains signals important for the transcription of RNA polymerase II dependent genes (Majors and Varmus, 1981; Klemenz *et al.*, 1981; Donehower *et al.*, 1981; Fasel *et al.*, 1982; Kennedy *et al.*, 1982), and it has been assumed that the LTR also harbors signals involved in the hormonal response. This assumption has been proven since chimeric genes in which the MMTV LTR has been combined with the *ras* gene of Harvey sarcoma virus (Huang *et al.*, 1981), the mouse dihydrofolate reductase gene (Lee *et al.*, 1981), and the thymidine kinase gene of herpes simplex virus (Groner *et al.*, 1982a) (Fig. 2A) each respond positively to glucocorticoid hormones. Therefore, the DNA of the LTR is sufficient to trigger the hormonal response, and no other viral functions are required for this process.

The MMTV-LTR contains 1328 nucleotides, and viral RNA transcription begins 134 nucleotides from its 3′ end. A schematic drawing of the MMTV-LTR is shown in Fig. 2B. Two approaches have been taken to define the region of the MMTV-LTR which confers glucocorticoid sensitivity. First, a provirus lacking the initial 516 nucleotides of the 5′ LTR was constructed and reintroduced into cultured cells. The mutated provirus containing the viral RNA initiation site plus 678 bp rather than 1194 bp of upstream flanking sequences responded positively to glucocorticoid hormones (Ponta *et al.*, 1983). Therefore, an intact 5′ LTR is not required for inducibility of the transcription of viral genomic RNA. Since an intact 3′ LTR was present in this deletion molecule, a second approach was taken to unequivocally define the DNA requirements for hormone induction. The chimeric LTR *TK* gene described in Fig. 2A was used for this purpose. Sequences located 5′ to the LTR RNA initiation site were deleted in a stepwise fashion. Each deletion mutant was transfected into cultured mouse cells, and the hormonal responsiveness of LTR-initiated transcripts was monitored (Hynes *et al.* 1983). Sequences from the 5′ region of the LTR could be deleted up to 202 nu-

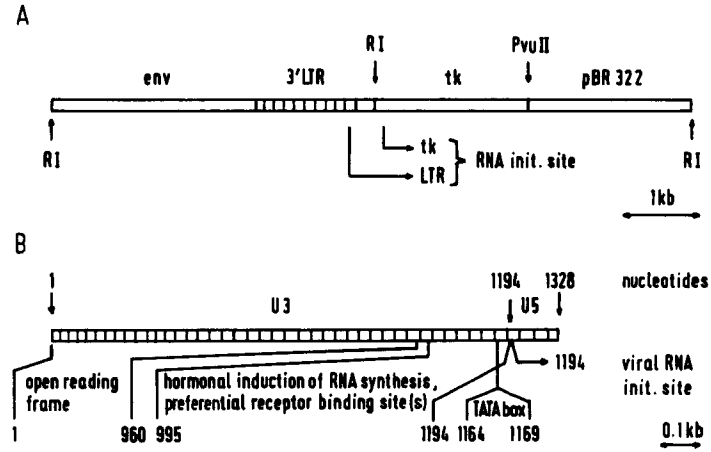

FIG. 2. (A) Schematic diagram of a chimeric MMTV-LTR *TK* gene. A 4.2 kb fragment of a cloned exogenously integrated proviral gene copy present in a GR mammary tumor cell line, which comprises the envelope region, the 3′ LTR, and 200 nucleotides of mouse DNA flanking the proviral DNA at its 3′ end, was fused to the *TK* gene of HSV. The *TK* gene was cleaved at the *Eco*RI which precedes the RNA initiation site by 77 nucleotides. The subfragment of pBR 322 extending from the *Pvu*II site to the *Eco*RI site and comprising the origin for plasmid replication and the ampicillin resistance region was used to propagate the chimeric gene. (B) Schematic representation of the MMTV-LTR. The MMTV-LTR comprises 1328 nucleotides. Its 5′ end is designated 1 and its 3′ end is designated 1328. The polarity refers to the direction of viral RNA transcription. The U3 region of the LTR contains a long open reading frame (orf) extending from the 5′ end (nucleotide 1) to position 960. The region between nucleotide 995 and the RNA initiation site at position 1194 has been shown to confer hormonal inducibility on LTR-initiated transcripts. The same region preferentially binds glucocorticoid hormone *in vitro*. A TATA box, a nucleotide sequence which precedes the RNA initiation site and which is part of most RNA polymerase II transcribed gene promoters, is found at positions 1164 to 1169.

cleotides of the RNA initiation site without an apparent effect upon the accuracy or efficiency of transcription in the presence of dexamethasone. No LTR-initiated RNA was detected in the absence of hormone. Molecules deleted to within 137 or 50 nucleotides 5′ of the LTR initiation site showed a marked decrease in the amount of transcription, but hormone induction was still observed. LTR-initiated transcripts were not observed when a deletion containing only 37 nucleotides 5′ of the RNA cap site was tested (Hynes *et al.*, 1983). From these results it can be concluded that the 202 nucleotides flanking the LTR RNA initiation site contain the hormone-responsive sequence of DNA.

Following transfection of the MMTV-LTR TK chimeric molecule described in Fig. 2A into cultured cells, dexamethasone-sensitive LTR-initi-

ated transcripts have been detected. Transcripts of the *TK* gene have also been mapped and were shown to initiate at the authentic TK RNA cap site. It has been observed that the TK initiated transcripts also respond to glucocorticoids (Hynes *et al.*, 1983). This suggests that the hormone can work over a distance and affect another promoter in the vicinity of the LTR regulatory region which is normally not responsive to hormonal regulation. In this construction the *TK* gene was located 433 bp downstream from the hormone-sensitive region in the MMTV LTR. It will be interesting to determine over what distance the hormone can exert its effect and if the effect has directionality.

3. *Characterization of the Glucocorticoid Response*

The presence of a sequence within the MMTV proviral DNA which controls the hormonal responsiveness of its expression does not imply a mechanism for hormone action since only the steady-state concentration of LTR-initiated RNA was analyzed (Hynes *et al.*, 1983). Measurements of the rate of specific RNA synthesis in MMTV-infected cells and the rapid response following hormone addition have suggested that there is a direct effect on the gene (Young *et al.*, 1977; Ringold *et al.*, 1977b). In cells transfected with the MMTV-LTR *TK* chimeric gene, no LTR-initiated transcripts can be detected in the absence of hormone. Therefore, it is possible to measure the kinetics of induction of LTR-initiated RNA following dexamethasone addition to the culture medium without having to account for preexisting LTR transcripts. This facilitates measurements of changes in the rate of transcription. The first LTR RNA was detected 7.5 min after hormone addition, and the steady-state level was reached after 2 hr. Identical kinetics of LTR-initiated RNA accumulation were observed when protein synthesis was inhibited by cycloheximide at the same time as dexamethasone addition to the culture medium. These results show that the rate of transcription of transfected DNA is stimulated rapidly upon hormone addition and that no newly synthesized proteins are required for this process (Groner *et al.*, 1983).

Induction of MMTV expression in infected cells is mediated via a specific cytoplasmic glucocorticoid receptor (Young *et al.*, 1975; Ringold, 1979). A glucocorticoid analog, RU486 (Herrmann *et al.*, 1982), which is known to bind specifically to the glucocorticoid receptor has been tested in cells transfected with the MMTV-LTR *TK* chimeric gene. Induction of LTR-initiated RNA synthesis by 10^{-6} *M* dexamethasone was completely suppressed by 10^{-5} *M* RU486 (Groner *et al.*, 1983). These experiments show that the mechanism of induction of MMTV-LTR RNA following dexamethasone addition is probably the same in virus-infected cells containing an exogenously integrated proviral gene and in cells into which an artificial chimeric gene

containing only a subfragment of the MMTV-LTR has been introduced by transfection.

4. *Specific Binding of the Glucocorticoid Receptor Complex to MMTV-DNA*

The model for steroid hormone regulation of gene expression predicts that the hormone receptor complex exerts an effect on its target gene via a specific interaction with the gene in the cell nucleus (Jensen and DeSombre, 1973). Early observations have shown that following the entry of a steroid hormone into a cell it becomes bound to a receptor protein. This complex migrates into the nucleus where it becomes associated with chromosomal DNA (Gorski and Gannon, 1976). It has recently been shown that the glucocorticoid hormone receptor complex can specifically interact with MMTV-DNA *in vitro*. These results suggest that a specific reaction may also occur *in vivo*.

The ability of *in vitro*-formed hormone receptor DNA complexes to bind to nitrocellulose was used to distinguish binding affinities of individual restriction fragments of MMTV proviral DNA to partially purified rat liver receptor protein preparations (Payvar *et al.*, 1981; Govindan *et al.*, 1982; Geisse *et al.*, 1982). Different exogenous and endogenous proviral genes were compared, and a common preferential binding sequence, located in the MMTV-LTR, was found (see Fig. 2B). Delimitation of the preferential binding site(s) to a sequence of a few hundred nucleotides preceding the RNA initiation site was possible (Geisse *et al.*, 1982). However, other DNA restriction fragments, originating from the envelope coding part of the proviral DNA and from the mouse genomic DNA flanking the proviral integration site, were also found to bind the hormone receptor complex, albeit with less preference than the LTR site.

The binding of glucocorticoid receptor complex to DNA immobilized on cellulose and the ability of free DNA to compete in the binding reaction is a second experimental approach which has been used to identify specific binding between MMTV proviral DNA and the glucocorticoid hormone receptor complex (Pfahl, 1982). The strongest competitor DNA, i.e., the DNA with the highest affinity for the hormone receptor complex, is a restriction fragment which contains the promoter region of the MMTV-LTR. The results are consistent with those obtained with the nitrocellulose filter-binding assay.

Other steroid responsive genes have been tested for their ability to bind the hormone receptor complex. The chicken egg white protein genes whose expression is under the control of estrogen and progesterone bind specifically to the progesterone receptor (Compton *et al.*, 1982; Mulvihill *et al.*, 1982).

B. Nonhormonal Influences on MMTV Expression

All inbred strains of mice contain endogenous copies of MMTV proviral DNA (Varmus *et al.*, 1972; Michalides and Schlom, 1975; Cohen and Varmus, 1979). In all but two exceptions the endogenous proviruses do not appear to play a role in mammary tumor formation. Exogenous proviral copies acquired by the mammary gland are transcriptionally active and are involved in mammary tumor formation. The difference in biological activity between the endogenous proviruses present in the mammary gland cell and the exogenously acquired proviral copies suggests that hormonal regulation of proviral transcription is supplemented by other mechanisms which control proviral expression.

1. Proviral Structure

Restriction enzyme analysis and DNA sequencing of MMTV proviral variants have shown that there are differences among the proviruses (Shank *et al.*, 1978; Cohen and Varmus, 1979; Cohen *et al.*, 1979a; Herrlich *et al.*, 1981; Donehower *et al.*, 1981; Fasel *et al.*, 1982; Kennedy *et al.*, 1982; Etkind *et al.*, 1982; Redmond and Dickson, 1983). Although most proviral copies appear similar, small alterations in the DNA sequence could affect transcription, translation, or processing events necessary for virus production.

An endogenous MMTV provirus which was isolated from the GR mouse and transfected into mouse fibroblasts was transcribed in a hormone-dependent fashion. Viral RNAs of 35 S and 24 S were observed in the transfected cells, but no virus particles were detected in the medium (Hynes *et al.*, 1981b). This provirus, termed Unit II (Traina *et al.*, 1981), seems to be transcriptionally inactive in GR mice. This suggests that the isolation and transfection of this provirus has caused it to escape from the normal control mechanisms which inhibit its expression in the GR mammary gland. In addition, the Unit II provirus may be defective and incapable of synthesizing virus. The DNA sequence of its envelope gene differs from that of the envelope gene of the exogenous GR virus (Unit V) (Redmond and Dickson, 1983). In the exogenous variant the open-reading frame of the envelope gene continues 17 amino acids into the 3′ LTR, while in the Unit II provirus the stop signal is approximately 50 bp upstream from the 3′ LTR (G. Knedlitschek and N. Kennedy, personal communication). In addition, immunoprecipitation with antiserum specific for the envelope proteins has revealed that the transfected cells synthesize an envelope precursor which is smaller than expected and which is not processed (B. Salmons and S. Kozma, personal communication).

2. *DNA Methylation*

In addition to the normal bases, the DNA of eukaryotic cells contains the modified base 5-methylcytosine associated with the dinucleotide CpG (Wyatt, 1951; Doskocil and Sorm, 1962). It has been suggested that such a base modification could play a role in the regulation of gene expression (Sager and Kitchin, 1975). It is possible to test a gene for the presence of 5-methylcytosine by digesting with specific restriction enzymes (Waalwijk and Flavell, 1978; Singer *et al.*, 1979). In general it has been observed that transcriptionally active genes are less methylated than their inactive counterparts (Bird and Southern, 1978; Mandel and Chambon, 1979).

MMTV proviruses acquired as a result of exogenous viral infection and present in mammary tumors are less methylated than the endogenous copies (Cohen, 1980; Fanning *et al.*, 1982). In this case hypomethylation and transcription seem to be directly correlated. It has also been observed that the endogenous proviral copies are in some cases hypomethylated. The GR mouse contains an endogenous MMTV provirus, associated with the *Mtv-2* locus, which is responsible for early-appearing hormone-dependent mammary tumors (Van Nie *et al.*, 1977; Michalides *et al.*, 1981a). This provirus is methylated in liver DNA and specifically demethylated in mammary gland and mammary tumor DNA (Groner *et al.*, 1982b). The C3Hf mouse also contains an MMTV provirus which is responsible for late-occurring mammary tumors, the *Mtv-1* locus. This provirus is methylated in liver DNA and mammary gland DNA (W. Günzburg, personal communication) and is hypomethylated in spleen DNA (Etkind and Sarkar, 1983). Thus, two endogenous proviruses which are known to be transcriptionally active are in some cases undermethylated. It is difficult to show that these two phenomena are directly related, since it is not completely certain in which organ the endogenous proviruses are originally transcribed and in which organs viral expression is a consequence of infection and integration. Mammary gland cells are capable of supporting MMTV-RNA transcription and virus production, thus it is reasonable to assume that the *Mtv-2* provirus is active in this organ. Splenic tissue and lymphocytes have been implicated in MMTV infection, and viral antigens have been observed in these cells (Hilgers *et al.*, 1972; Kozma *et al.*, 1980), but no virus particles have been detected. A correlation between hypomethylation of the *Mtv-1* locus and transcriptional activity cannot be drawn at this point. Other endogenous proviruses present in the genome of various strains of mice have been shown to be methylated in liver DNA and hypomethylated in mammary tumor tissue (Breznik and Cohen, 1982; Drohan *et al.*, 1982). These mammary tumors did not arise as a result of MMTV infection. A number of different studies on the role of MMTV in nonviral tumors have concluded that the endogenous proviruses are not

important for this process. While it is sometimes possible to detect MMTV-RNA, the levels are very low and no virus particles have been observed (Dudley *et al.*, 1978; Michalides *et al.*, 1978a, 1979; McGrath *et al.*, 1978; Dusing-Swartz *et al.*, 1979). Therefore, in these cases no direct link can be made between proviral transcription and hypomethylation.

It has been observed that mouse mammary tumor DNA is generally hypomethylated when compared to other organ DNA (Cohen, 1980). Perhaps the hypomethylation of some of the endogenous MMTV proviruses represents a demethylation of a much larger region of the chromosome during mammary tumor formation. Other genes present in this region could become activated during this process but not necessarily the proviral genome.

The genomic location of the MMTV proviruses in different strains of mice has been analyzed and found to vary among the strains (Cohen and Varmus, 1979; Morris *et al.*, 1979; Hynes *et al.*, 1980; Groner and Hynes, 1980; Traina *et al.*, 1981). Mice containing no endogenous copies of MMTV have also been found (Cohen *et al.*, 1982; Callahan *et al.*, 1982). These results suggest that the MMTV proviruses most likely arose as individual integration events in the germ line cells of the mouse. The methylation of individual proviruses present in one strain of mice but absent in another has been studied. It has been shown that the provirus takes on the methylation characteristics of the region of the genome into which it is integrated. A region may be fully methylated in one organ but demethylated in another, and the proviral copy shows the same methylation pattern (Günzburg and Groner, 1983). These results suggest that hypomethylation may be a structural feature of an active region of the chromosome but that it might not be a sufficient signal for the transcription of each gene in such a region. Therefore, methylation may play a role in determining the transcriptional activity of MMTV proviruses, but it is not a sufficient prerequisite.

V. The Long Terminal Repeat of MMTV

Retroviruses, including MMTV, replicate via a DNA intermediate. Unintegrated viral DNA as well as the integrated provirus are flanked by long terminal repeats (LTR) which arise during reverse transcription of viral RNA (Hsu *et al.*, 1978). The LTRs are important for viral DNA integration, RNA transcription, and the reverse transcription process.

A. Characteristics of the MMTV-LTR

The LTR of MMTV is particularly interesting because it confers glucocorticoid sensitivity on MMTV proviral transcription. The DNA sequence of the LTR of different endogenous and exogenous MMTV proviruses has been

determined (Donehower *et al.*, 1981,1983; Majors and Varmus, 1981; Fasel *et al.*, 1982; Kennedy *et al.*, 1982). The LTR encompasses 1328 nucleotides, and the sequences of the various LTRs are similar but not identical. Viral RNA synthesis begins in the 5′ LTR. A Goldberg–Hogness TATAAA sequence (Goldberg, 1979; Breathnach and Chambon, 1981) important for the initiation of RNA polymerase II transcripts is found about 25 nucleotides upstream from the viral RNA cap site. As discussed in Section IV, the 202 nucleotides preceding the LTR cap site are involved in the glucocorticoid response. The DNA sequence preceding two other glucocorticoid sensitive genes, rat growth hormone and human propiomelanocortin, has been compared to the MMTV-LTR. A similarity in a 21-bp sequence located approximately 440 nucleotides upstream from the different RNA cap sites has been detected (Cochet *et al.*, 1982). At least for MMTV the deletion of this region does not inhibit glucocorticoid-sensitive expression (Hynes *et al.*, 1983). Located 105 bp upstream from the MMTV-LTR cap site is a 19-bp sequence which shows 75% homology to the consensus sequence in the chicken egg white protein genes which is recognized by the progesterone receptor (Mulvihill *et al.*, 1982). The significance of this similarity is unknown, but it is interesting that progesterone competitively inhibits the action of glucocorticoid hormones by binding to the glucocorticoid receptor (Rousseau *et al.*, 1972).

B. Protein Coding Potential of the MMTV-LTR

The sequence analysis of the LTR has revealed that 960 nucleotides of the U3 region are in an open reading frame and thus capable of coding for a protein with a molecular weight of 36,000 (see Fig. 2B). This makes MMTV an exception among the retroviruses, since LTR sequences of the other retroviruses generally contain multiple terminator codons in all three reading frames (Temin, 1981). The proteins encoded in this open reading frame have been expressed *in vitro* either from virion RNA or from the cRNA transcribed from a LTR cloned into a bacterial plasmid (Dickson and Peters, 1981; Dickson *et al.*, 1981; Sen *et al.*, 1981; Peters *et al.*, 1982). This protein, which has been termed orf (for open reading frame), has not yet been detected in cells. But the conservation of the orf sequence in all of the LTRs tested (Peters *et al.*, 1982) suggests that the orf protein exists and is biologically relevant. Based upon the predicted amino acid sequence of the orf protein, a peptide has been synthesized (Fasel *et al.*, 1982) and used to raise antisera. These antisera immunoprecipitate the iodinated peptide and the *in vitro* synthesized orf protein and are being used to search *in vivo* for the putative orf protein (H. Diggelmann, personal communication).

An mRNA which could code for the orf protein has been detected in

mammary glands and mammary tumors of MMTV infected mice and in BALB/c preneoplastic and neoplastic lesions of nonviral etiology (Wheeler *et al.*, 1983; van Ooyen *et al.*, 1983). The size of the RNA is approximately 1.7 kb. Its anatomy suggests that it arises by splicing of the transcriptional leader sequence of MMTV located in the 5′ LTR onto the U3 region of the 3′ LTR. Another possibility is that it is transcribed from an endogenous provirus which contains a deletion. It is interesting to note that BALB/c mice which contain the highest amounts of this transcript (van Ooyen *et al.*, 1983; Wheeler *et al.*, 1983) have a defective endogenous provirus (Unit I) (Cohen *et al.*, 1979a; Groner *et al.*, 1980) which appears to contain only LTR sequences (Wheeler *et al.*, 1983). The Unit I provirus is specifically hypomethylated in some BALB/c mammary tumors (Breznik and Cohen, 1982) suggesting that in some instances it may be transcribed. The orf mRNA is also found in MMTV-infected mammary glands and tumors in mice lacking the Unit I provirus (van Ooyen *et al.*, 1983). Thus, it can also transcribed from full-length viral genomes.

The function of the orf protein remains speculative. It could have a role in the virus life cycle or in the mammary tumorigenesis process. It is not involved in glucocorticoid-sensitive transcription of the MMTV provirus since deletion mutants which lack the entire open reading frame of the LTR are still capable of responding to dexamethasone (Hynes *et al.*, 1983).

VI. Mechanism of MMTV Tumorigenesis

Strains of mice which carry milk-borne MMTV virus particles have a high incidence of mammary cancer. The exogenous virus infects the mammary gland cells of the offspring, and the viral RNA is reverse transcribed into viral DNA which integrates into the genomic DNA of the cell where it is active in the production of new virus particles. The proviral genome is important for mammary tumor formation since mice which are not exogenously infected have a much lower mammary tumor incidence. But host genetic factors as well as the hormonal status of the animal also play a role in the tumorigenesis process (Bentvelzen and Hilgers, 1980).

A. Tumorigenesis via Promoter Insertion

The genome of MMTV does not carry a transforming oncogene. In this sense it is related to avian leukosis virus (ALV) and murine leukemia virus whose genomes also contain only the information for viral structural proteins. How do these viruses cause tumors? Some hints as to how they do this can be taken from two of their characteristics. First, there is a long latency period between virus infection and tumor formation. Second, the tumors

produced by these viruses are clonal or semiclonal in origin. These results suggest that, although many cells of the target organ are infected, only a few of these cells become transformed and grow out into a tumor. Since viral DNA seems to integrate at random into the host genome (Hughes *et al.*, 1978; Ringold *et al.*, 1979), it appears that a particular host integration site has to be hit to promote the process of tumorigenesis. This hypothesis has been documented in the case of ALV and reticuloendotheliosis virus-induced bursal lymphomas. In there tumors proviral DNA has been found to be integrated in a specific genomic site (Payne *et al.*, 1981; Neel *et al.*, 1981; Fung *et al.*, 1981; Noori-Daloii *et al.*, 1981) adjacent to a cellular oncogene—the c-*myc* locus. Transcripts initiating in the ALV proviral LTR and continuing into the c-*myc* have also been detected (Hayward *et al.*, 1981), and it is assumed that the enhanced c-*myc* expression leads to neoplastic transformation. Not all of the analyzed tumor DNAs contained the ALV LTR and the c-*myc* sequences in the same transcriptional orientation (Payne *et al.*, 1982). Thus, it is possible that the LTR not only promotes transcription from its initiation site into downstream sequences but also contains sequences which enhance the transcription of genes in its vicinity.

B. MMTV Proviral Integration Site in Mammary Tumors

Mammary tumors arising from exogenous MMTV infection contain high levels of MMTV-RNA (Varmus *et al.*, 1973). Exogenous proviruses have been detected in the tumor DNA by liquid hybridization (Michalides *et al.*, 1976; Morris *et al.*, 1977), and more recently, individual copies have been visualized (Cohen *et al.*, 1979b; Groner and Hynes, 1980; Cohen and Varmus, 1980; Fanning *et al.*, 1980; Michalides *et al.*, 1981b; Morris *et al.*, 1982; Altrock *et al.*, 1982; Etkind and Sarkar, 1983) using the DNA filter transfer technique (Southern, 1975). When this method is employed and the DNA digested with a restriction enzyme which cuts into the provirus frequently, fragments of DNA containing only proviral sequences are preferentially detected. When an enzyme which cuts the proviral genome rarely is used, the proviral DNA plus its surrounding sequences (i.e., its integration site) will be visualized. It is possible to detect exogenous proviruses in MMTV-infected mammary gland DNA using only the first type of enzyme (Cohen *et al.*, 1979b). In mammary tumors it is possible to detect exogenous proviruses using both types of enzymes. These results show that the mammary gland is composed of many different cells containing exogenous proviruses in different locations while the tumor appears to be clonal. It is composed of the descendents of one or a few infected cells whose exogenous proviruses can be directly visualized.

The fact that the mammary gland contains many new proviral copies but

that only a few of the infected cells became transformed and gave rise to the tumor suggests that the proviral integration site in the genomic DNA plays some role in the process of tumorigenesis. By comparing the sizes of the MMTV-restriction enzyme fragments in different mammary tumors, it has not been possible in most cases to see a specific genomic integration site. Nusse and Varmus (1982) have taken a different approach to show that there is a domain of the mouse genome which appears to be important for the tumorigenesis process. A molecular clone containing an exogenous MMTV provirus genomic DNA junction fragment was isolated from mammary tumor DNA. The cloned cellular DNA, which represents a viral DNA integration site (hence the name *int-1*), was used to analyze a number of other mammary tumors. In 18 out of 26 tumors tested the *int-1* locus, which spans 35 kb of DNA, was found to be occupied by an exogenous MMTV provirus. A portion of the *int-1* DNA is transcribed into a 2.6 kb RNA in at least some of these tumors and has not been detected in normal mammary glands. These findings suggest that the *int-1* locus may contain a gene whose activation plays a role in the mammary tumorigenesis process. The *int-1* domain has been tested for the presence of cellular homologs of retroviral transforming genes (*c-onc*), but no homology has been found with any of the 12 viral oncogenes tested (Nusse and Varmus, 1982).

The *int-1* locus has been identified in the C3H strain of mice which carry milk-transmitted MMTV. A similar type analysis has been carried out with BR6 mice infected with the MMTV virus of RIII mice. The BR6 strain was originally derived from a cross between C57BL and RIII mice, and females have a high incidence of mammary tumors. A total of 17 out of 40 tumors examined contain an MMTV provirus integrated in the same region of the genome. But it is interesting that this region does not cross-hybridize with the *int-1* locus (Peters *et al.*, 1983) and is referred to as *int-2*. It is possible that the mouse genome contains a few chromosomal sites where MMTV integration may trigger the tumorigenesis process. This possibility would also explain why not all the C3H mammary tumors tested contained a provirus in the *int-1* locus (Nusse and Varmus, 1982).

Foster nursing of C3H mice on virus-free mothers results in mice with a low incidence of late-occurring mammary umors (Bentvelzen, 1974). The tumors in these C3Hf mice result from the expression of the endogenous provirus present in the *Mtv-1* locus (Van Nie and Verstraeten, 1975; Michalides *et al.*, 1981a). C3Hf mammary tumors contain additional copies of MMTV DNA (Cohen and Varmus, 1980). A recent comparison of the genomic location of the tumor-specific copies in 12 different mammary tumors shows that in at least 50% the integration appears to be at the same site (Etkind and Sarkar, 1983). It will be interesting to compare this site to the *int-1* and *int-2* loci.

The integration of Moloney murine leukemia virus (MoMuLV) in virally induced thymic lymphomas in the rat has also been investigated. Tschichlis *et al.* (1983) have shown that in 5 out of 16 tumors the same region of the genome has served as a substrate for MoMuLV viral DNA integration. The authors mention that another DNA region serves as a common integration site in a few other tumors. These results correlate closely with those obtained for MMTV viral DNA integration in mammary tumors and suggest that a common mechanism, different from the promoter insertion mechanism observed in avian bursal lymphomas, may be active.

C. MMTV Activation of Cellular Genes

Nusse and Varmus (1982) have shown that a 2.6-kb poly(A)$^+$ RNA transcribed from the *int-1* locus is found in some virally induced mammary tumors. This suggests that viral integration may activate a cellular gene which is responsible for mammary tumorigenesis. But this process will probably differ from that described for ALV-induced lymphomas. In many of these lymphomas a chimeric LTR cellular *myc* RNA has been detected (Hayward *et al.*, 1981). The 2.6-kb RNA from the *int-1* locus does not contain MMTV-LTR sequences. Therefore, an LTR-initiated transcript into a cellular gene is probably not important in the mammary tumor formation process. It is possible that the presence of the provirus in the *int-1* region disrupts the normal control and allows inappropriate activation of a cellular gene leading to tumor formation.

In transfected cells the MMTV-LTR affects the expression of another gene (Hynes *et al.*, 1983). When the chimeric LTR *TK* molecule (Fig. 2A) was transfected into mouse L cells, transcripts initiating in the TK responded to dexamethasone (discussed in Section IV). This response was due to the hormone-sensitive region present in the LTR. In this case there was a basal level of TK RNA synthesis which was elevated approximately fivefold in the presence of hormones. It will be interesting to see if following its transfection, the provirus can activate a gene located in its vicinity.

Transforming genes have been detected by DNA-mediated gene transfer of tumor DNA into cultured NIH/3T3 cells (Weinberg, 1982). Transfection of DNA isolated from C3H mammary tumors into NIH/3T3 cells resulted in the formation of foci of transformed cells (Lane *et al.*, 1981). The cells in these foci do not contain tumor-specific MMTV proviruses; therefore they probably do not contain the *int-1* locus which is closely linked to tumor-specific proviruses. These results indicate that the gene product of the *int-1* locus affected another gene in the mammary gland cell. The transfection of this second altered gene into the NIH/3T3 cells caused their transformation. The viral DNA integration into the *int-1* locus would appear to be the

primary event in the viral-induced tumorigenesis process since transfection of DNA isolated from MMTV-infected mammary glands did not lead to transformation of the NIH/3T3 cells (Lane *et al.*, 1981).

The mammary tumor-specific transforming gene detected by transfection into NIH/3T3 cells appears to be homologous whether the DNA is isolated from an MMTV-induced mouse mammary tumor or from MCF-7 cells, a human mammary tumor cell line (Soule *et al.*, 1973). Before transfecting, the DNA was digested with different restriction enzymes. The ability to transform the NIH/3T3 cells was lost following digestion of the DNA with some enzymes. The DNA from MCF-7 cells and from MMTV-induced tumors displayed the same pattern of sensitivity to different enzymes (Lane *et al.*, 1981).

The mammary tumor gene which is capable of transforming the NIH/3T3 cells has not been fully characterized, but an antigen which appears to be associated with the gene has been described (Becker *et al.*, 1982). Sera from mice bearing MMTV-induced mammary tumors were capable of precipitating an 86,000-dalton glycoprotein from NIH/3T3 cell transformed by MCF-7 cell DNA. NIH/3T3 cells transformed by other means did not contain this protein, thus the gp86 is a candidate for a mammary tumor-specific protein. However, extracts of the original mouse mammary carcinomas or MCF-7 cells did not appear to contain gp86; only the NIH/3T3 cells transformed by the DNAs contain this protein. The isolation of this mammary tumor-specific transforming gene should help to clarify this matter.

D. Chromosomal Abnormalities in Mammary Tumors

Specific chromosomal abnormalities are associated with certain human and animal tumors (Klein, 1981). It is postulated that abnormalities may result in cellular transformation by increasing the expression of a normal cellular product. Double minute chromosomes have been observed in human breast carcinoma cells (Barker, 1982), and trisomy of chromosome 13 has been described for virally induced mammary tumor cells from GR and C3H mice (Dofuku *et al.*, 1979) and in chemically induced tumors of BALB/c mice (Dofuku and Matsuzawa, 1983). Trisomy of chromosome 13 is likely derived from endomitosis and loss of one chromosome 13. The endogenous MMTV proviruses which are active in virus production and subsequent mammary carcinoma, the *Mtv-1* and *Mtv -2* loci, are not located on chromosome 13 but on chromosomes 7 and 18, respectively (Van Nie and Verstraeten, 1975; Van Nie *et al.*, 1977). However, other genes important for the replication of mammary cells could be located on chromosome 13. As discussed previously, the integration of exogenous proviral DNA in a particular genomic site seems to be important for tumor formation. It is likely that

the changes invoked by this integration have other effects on the cell, one of which could lead to this trisomy.

VII. Conclusions

Hormonal regulation of gene expression has been studied in a variety of systems. Advances in our understanding of the mechanism of hormonal regulation are dependent upon the availability of hormone-responsive cell culture systems and molecular clones of hormonally regulated genes. The proviral DNA of MMTV was the first gene in which the close association of a regulatory DNA sequence with the regulated gene was demonstrated. Additional studies with *in vitro* manipulated MMTV-DNA containing molecules have allowed us to define a region of the MMTV-LTR, 202 nucleotides upstream of the RNA initiation site, which regulates hormonal inducibility. This sequence shows a preferential affinity for the glucocorticoid receptor *in vitro*. Since a functional glucocorticoid receptor is required for induction in the transfected cells and since this induction is rapid and protein synthesis independent, it is reasonable to assume that there is a direct interaction of the receptor with the regulated gene. This interaction results in an increase in the rate of transcription. The glucocorticoid receptor assumes the role of a transcription factor and seems to focus correct initiation of RNA in the MMTV-LTR.

Although the regulatory features governing MMTV transcription have been studied in detail, the mechanism of MMTV-induced mammary tumorigenesis is less understood. It is likely that the viral DNA integration site in mammary gland cell DNA is important in this process. Following integration the proviral DNA could affect the activity of a cellular gene. The integration domains, the *int-1* and *int-2* loci, appear to contain cellular gene(s) whose expression is enhanced following viral DNA integration.

How can the activity of a gene be affected over a distance? A few examples of this type of control have been described. The hormonal induction in the chimeric LTR *TK* gene is not restricted to the RNA that is initiated in the LTR but extends to the initiation site of the *TK* gene 431 nucleotides to the 3′ side. This observation shows that the MMTV proviral DNA in combination with the glucocorticoid hormone receptor complex can affect the transcription of a neighboring gene. In addition, transcriptional enhancing sequences which exert their effect over a distance of several hundred nucleotides have been found in SV40 (Banerji *et al.*, 1981), polyoma (De Villiers and Schaffner, 1981), and Moloney murine leukemia viruses (Laimins *et al.*, 1982). Recent results suggest that these elements show cell specificity suggesting that they interact with host-specific elements (Laimins *et al.*, 1982). It has not been reported that MMTV contains such a transcriptional en-

hancer element, but the possibility that it has a region of DNA which interacts with a mammary gland factor to promote the transcription of a neighboring gene is still open.

Thus, several possible molecular mechanisms involving proviral insertional mutagenesis and subsequent mammary tumorigenesis can be envisaged. The experiments leading to a further molecular understanding will be aimed at the identification of the genes activated by MMTV integration and a characterization of their function.

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DOMINANT SUSCEPTIBILITY TO CANCER IN MAN

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I. Introduction

Much attention has been focused recently on the role of environmental factors in the etiology of cancer, and there is no doubt that these factors are very important. However, there is a great deal of variation within the human population, and a true understanding of the role of environmental factors will only be achieved when this individual variability in cancer susceptibility has also been defined. Such susceptibility may or may not be genetically determined; there is good evidence for a strong genetic component in a small proportion of cancers and some evidence for a weaker genetic component in many of the common cancers. Often no clear mode of inheritance is

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discernible, and perhaps in these cases the genetic element is multifactorial. In some instances, however, the susceptibility is clearly dominant or recessive. In other cases, while a clear mode of inheritance can be found in some families, it is not possible to say that all families follow the same pattern. The purpose of this chapter is to consider those conditions where, in some families at least, there is a clear-cut pattern of dominant inheritance in the hope that this will throw light on mechanisms of susceptibility to cancer.

A. The Nature of Dominant Inheritance

"Dominance" is a purely relative concept. In an allelic series one particular allele may be dominant to some of the alleles in the series but recessive to others. When we speak of human disorders inherited in a dominant manner we usually mean disorders controlled by a gene which is dominant over the normal (wild type) allele. Dominant genes do not, therefore, form a separate class of genes characterized by a particular mode of action. However, for many human disorders which are inherited in a recessive manner, a biochemical basis for the disorder is understood, while for dominantly inherited abnormalities the biochemical basis is known only in a few cases (e.g., clotting factors). To some extent the concept of dominance will also depend on the means at the disposal of the observer for recognizing a phenotypic effect in the heterozygote. In the hemoglobinopathies, for example, the heterozygotes may be recognized by electrophoresis, and the abnormal allele is therefore codominant, even though the heterozygous individual appears "normal," and thus the gene is recessive at the level of the clinical phenotype.

Nor is the concept of dominance absolute even for a given allelic pair of genes. Examples are known, even in humans, where the expression of a dominant gene is conditional upon the presence or absence of a specific environmental factor [e.g., some forms of porphyria which are only dangerous when the carriers are exposed to barbiturates (Dean, 1956)]. Similarly the genetic environment (or genotypic milieu) may have a profound effect on the expression of a gene. The best known examples come from experimental systems; for example, the dominant factor Tail-short (T-s) in the mouse produces a shortening of the tail in its original genotypic milieu while in other stocks the effect of T-s may be increased or diminished (Morgan, 1950). At the extreme it may, in some stocks, become an embryonic lethal factor. It is not usual to distinguish between the environmental and genetic effects but to refer to these phenomena as *penetrance* (the proportion of heterozygotes in which the gene is expressed) and *expressivity* (the range of severity of the phenotype in those heterozygotes in which there is some

expression of the gene). In some of the conditions to be considered in this article the distinction is, however, of importance.

In the case of those genes controlling a morphogenetic function, the extent of the *potential* expression of the gene will be determined by the stage of embryogenesis at which the function is required; however, in the case of those genes which control a cellular or physiological process in the adult organisms, it will be determined by the distribution of that function within different cells and tissues of the body. For example, the gene for brachydactyly must control a function which is important only in the morphogenesis of the digits while the gene for polyposis coli (see later) must control the functioning of specific cells in the adult.

Since dominant traits do not form a special class, in terms of mode of action, they may be controlled at the chromosomal level by any of the known genetic mechanisms. In experimental systems it is clear that a gene, whether dominant or recessive, may be not only a point mutation but also a deficiency (deletion), a translocation, an inversion, or even a duplication (Hadorn, 1961). Possibly the best example of the latter is the Bar mutant in *Drosophila* where a repeat of six bands on the X chromosome is associated with a reduction in number of eye facets from 780 to about 360 in the heterozygote and to about 70 in the homozygote (Bridges and Brehme, 1944). Both heterozygote and homozygote are fully viable. In this respect Bar is unlike many other dominant traits which are often lethal in the homozygous condition, e.g., Tail-short in the mouse or sickle cell trait in humans.

In considering human conditions with a dominant susceptibility to cancer we could reasonably expect to find point mutations, deletions, inversions, translocations, or even duplications within any one syndrome. The fact that clinical syndromes may be genetically heterogeneous is now widely recognised (e.g., thalassemia, xeroderma pigmentosum).

While the biochemical basis of these diverse syndromes is often not known, it is helpful to consider what the possiblities are. Different loci may be involved in different individuals with the same clinical phenotype but for each critical locus there are three possibilities: (1) a new gene product modified in either structure or function, (2) a reduced amount of the normal gene product or a gene product with the same function but lower efficiency, or (3) absence of any functional gene product due to either total loss of product or to the loss of function. This will be true of any gene, and dominance or recessivity will depend on other factors. If it is assumed that the normal allele is present and active, the net result would depend on whether or not the normal allele can compensate for the loss of product from the mutant allele either by increasing the amount of gene product or because the amount of gene product was not limiting in the first place. If such compensa-

tion occurs, then in the case of (1) any phenotypic expression will be due solely to the structure or function of abnormal gene product. If it does not occur, then expression could be due to a combination of the new structure or function and partial loss of the original gene product. In cases (2) and (3) dosage compensation could result in absence of phenotypic effect and the mutant allele would be recessive. Dominant expression of a mutant allele in these cases would therefore depend on absence of dosage compensation. If the mutation is not in a structural gene but is in a regulatory gene the same arguments apply, although in this case the phenotypic effect will be expressed through the failure of a regulatory sequence to activate or inactivate one or a group of other gene functions in response to the appropriate developmental or physiological signal.

B. Dominant Cancer Susceptibility Factors in Animals Other Than Man

A brief consideration of some of the complexities of the inheritance of cancer susceptibility in animals will help to draw attention to those points which may be important in humans.

The appropriate parallel in wild animals of the human diseases discussed in this article would be the demonstration of individuals or groups in which there is an unusual susceptibility to cancer. Such situations are impossible to identify, but the point is worth brief consideration. Most animals in the wild have a very short life span compared with the potential life span of the individual. In man, cancers are strongly age related, and if this is also true of animal species, most animals in the wild will not live long enough to get cancer. Furthermore any cancer susceptibility gene which frequently leads to cancer prior to the age of reproduction would be severely deleterious. This is particularly true of dominant genes, and one would not expect to find them in wild populations except as new mutations. Recessive gene frequency could, of course, build up if there were any heterozygote advantage. Mutations which lead to cancer later than the normal reproductive age could be advantageous in that older animals would be killed off leaving the ecological niche less densely populated. Under conditions of high population density relative to the availability of food this could be a critical factor for survival. Even in man the age distribution of cancers may not be entirely a matter of the probability of rare events or exposure to environmental agents. It could, at least in part, be due to a genetically determined decrease in the stability of cellular mechanisms which would increase the probability of malignant disease. The dominant cancer susceptibility genes may therefore be only the more obvious of a group of genes which have a major effect on the pattern of occurrence of cancer.

In experimental and domestic animals a wide range of genetically determined cancer susceptibility is recognized and in some cases the mechanisms have been elucidated. While inbred animal stocks were developed partly to facilitate the transplantation of tumors, it was recognized very early that both the spontaneous incidence of neoplasia and the susceptibility to tumor induction by environmental agents varied greatly between inbred strains. Indeed some strains were developed because of their tumor susceptibility (Strong, 1978):

> Shortly after the initiation of the A strain (in 1920) an outcross had been made between one of these albinos and a survivor from Little's dilute browns. Cancer, although infrequent, was known to occur in both ancestral stocks. This hybrid cross was designed to test the idea than an increase in variability ought to increase the incidence of spontaneous tumours, the rationale being that cancer is just one more variable. Continued hybridising in this stock proved the prediction true. Subsequent mating of a cancer-bearing mouse with a normal one produced the disease in the tell-tale 3:1 Mendelian ratio in the F2 generation, a result that became the first laboratory proof that cancer is inherited and as a dominant trait contrary to Maude Sly's conclusion that the trait was recessive. This historic mating was the start of the so well known C3H high tumour subline.

While some of this interstrain variation is due to the presence of oncogenic viruses, much is due to the inheritance of susceptibility factors. A few of the examples where there is some idea of the mechanisms are given below.

The susceptibility of different strains of laboratory mice to the induction of fibrosarcomas by the subcutaneous administration of methylcholanthrene (MC) varies with the extent of inducible aryl hydrocarbon hydroxylase activity (AHH), and this variation is under genetic control (Nebert *et al.*, 1974). It is necessary to specify which strains are being considered before one can determine the mode of inheritance of AHH inducibility. In crosses between C57BL/6N and RJ/F strains the expression of AHH induction by MC is inherited as an autosomal dominant trait. The C57BL/6N strain has high levels of induced specific activity, RJ/F has virtually zero inducible activity, and crosses between the two strains have induced levels exactly like the C57BL/6N parent (Thorgeirsson and Nebert, 1977). However, in other crosses inducibility of AHH activity by MC may be codominant or even recessive. These authors conclude that there may be a complex genetic situation involving a multiple allelic system and at least 2 regulatory loci so that inheritance of inducibility depends not only on the alleles present but also on the genetic background.

These studies suggest one level at which genetically determined susceptibility to cancer may operate, namely the processing of the carcinogenic agent prior to the exposure of the target cells. It is clear however that other

types of host response are also under genetic control. For example, in mice, the reactivity of natural cell-mediated cytotoxicity to the inoculation of tumor cells is clearly under genetic control, and in the case of response to YAC-1 lymphoma cells a high level of natural killer reactivity is inherited in a dominant fashion. A/Sn mice have a low reactivity in this system but C57BL and (A × C57BL)F_1 have a higher reactivity (Petranyi *et al.*, 1975). Again, however, dominance is not always absolute. Glimcher *et al.* (1977) using the RL♂1 lymphoma system found intermediate levels of natural cell-mediated cytotoxicity in F_1 crosses between a parent with a low response (BALB/c) and a parent with a high response (B6). However, this situation is complex since there appear to be interactions between several different loci, some linked to the H2 locus and some not so linked which determine the response (Clark and Harmon, 1980).

Some inbred strain differences in cancer incidence are known to be due to the presence within the mouse genome of DNA sequences representing the provirus of oncogenic RNA retroviruses. The location of these proviruses has been mapped in some mouse strains. For example, the *Akr*-1 locus (which is itself proviral DNA) has been located on chromosome 7 in the AKR/J strain (Rowe *et al.*, 1972). The expression of the products of this locus and the production of virus particles appear to be essential for leukemogenesis. Therefore the presence of the provirus acts as a dominant gene provided it is in an appropriate background (Nayar *et al.*, 1980).

The occurrence of a neoplasm, however, does not depend solely on the presence of integrated provirus. A number of loci are known which control virus expression. The presence of the FV-1^b allele at the FV-1 locus in BALB/c and other inbred strains causes suppression of expression of murine leukemia virus. Thus in AKR × BALB/c crosses the suppression of expression of the ecotropic murine leukemia virus is dependent on the segregation of the FV-1^b gene in the offspring (Lilly, 1972). Suppression, and therefore absence of leukemogenesis, is dominant. This illustrates the difficulty of classifying any "cancer gene" as dominant without first defining the genetic background.

In mouse leukemogenesis the situation is further complicated by an additional level of regulation—namely the control of tumor outgrowth which is dependent on a complex interaction between the H-2 haplotype and the specific strain of virus (Lilly, 1972).

The main points we wish to make from this brief consideration of inheritance of susceptibility to cancer in animals are (1) that there are many different levels in the pathogenesis of cancer at which a genetic component may exert its influence and (2) that the phenotypic expression of a gene will always be modulated by the genetic environment of that gene.

II. Outline Description of Syndromes

There are many syndromes or clinical conditions in which there is dominant inheritance of a susceptibility to cancer or in which such a susceptibility has been suggested (listed by Mulvihill, 1977). It is not possible to review all in detail here, but it is necessary for the following consideration of mechanisms to give a description of a few of these syndromes. Those outlined below are not necessarily the most common but in each example the dominant mode of inheritance is clear cut in at least a proportion of the cases, and the susceptibility to cancer is beyond dispute. Other dominant syndromes are listed in Table I. In addition to these syndromes, it has been reported that individual neoplasms may have a familial occurrence resembling dominant inheritance, and in some cases many diverse cancers occur in families. This has been called the "cancer family syndrome" (Lynch *et al.*, 1977). While this is undoubtedly an area of considerable interest, it is outside the scope of the present article.

A. Retinoblastoma

1. Introduction and Physical Features

Retinoblastoma is a childhood tumor of the retina, and there is now convincing evidence that the tumor is derived from embryonic neural retina. Undifferentiated regions of the tumor consist of small, round cells having hyperchromatic nuclei and little cytoplasm, which resemble undifferentiated retinoblasts. The cells of differentiated structures in the tumors (the Flexner–Wintersteiner rosettes, Homer–Wright rosettes, and fleurettes) are similar to photoreceptors in their cilia (9 + 0 filament pattern) and in their cell junctions, stacks of membranes, and extracellular matrix (Tso *et al.*, 1970; Tso, 1978). Albert *et al.* (1970) found microtubules in retinoblastoma cells similar to those in photoreceptors, and Radnot (1975) reported a tumor with synaptic lamellae resembling those of the inner plexiform layer. With the exception of other forms of cancer (discussed later) there appear to be no other clinical features associated with the occurrence of retinoblastoma.

The incidence figures in Western Europe, United States, Japan, and Australia range from 1:15,230 live births (Schappert-Kimmijser *et al.*, 1966) to 1:32,793 (Griffith and Sorsby, 1944), and retinoblastoma may be more frequent in Africa, India, South America, and China (Davies, 1973; Pawlak, 1975; Ben Ezra and Chirambo, 1976; Goldberg, 1977; Miller, 1977; Gaitan-Yanguas, 1978). Four studies indicate an increasing incidence of retinoblastoma, not all of which can be explained by improved diagnosis and

TABLE I

ADDITIONAL DOMINANT CONDITIONS WITH CANCER SUSCEPTIBILITY[a]

McKusick (1983) number	Condition	Features	Associated malignant neoplasms
13170	Epidermolysis bullosa dystrophica	Vesicles, bullae, epidermal cysts on extensor surfaces and sites exposed to trauma; bullae and ulcers of mucous membranes	Carcinoma of mucous membranes, basal and squamous cell skin carcinoma
13370	Multiple exostosis	Bony and cartilaginous dysplasia; multiple exostosis	Osteosarcoma, chondrosarcoma
13500	Fibrocystic pulmonary dysplasia	Progressive dyspnea and cyanosis; digital clubbing, pulmonary hypertension, polycythemia and diffuse pulmonary fibrosis	Bronchial carcinoma
14160	Hemochromatosis	Cirrhosis of the liver, diabetes, hypermelanotic pigmentation of the skin, heart failure, elevated serum iron	Hepatocellular carcinoma
14415	Hyperkeratosis lenticularis perstans	Hyperkeratosis of legs, arms, trunk, dorsum of hands and feet; pink and reddish-brown scaly papules	Squamous and basal cell skin carcinoma
14850	Tylosis (keratosis palmaris et plantaris)	Diffuse hyperkeratosis of palms and soles—typically late onset in cases associated with malignancy	Oesophageal carcinoma
15190	Multiple lipomatosis	Late onset lipomas, somtimes of neck and conjunctivae	Skin carcinoma
15340	Lymphedema with distichiasis	Late onset lymphedema, double eyelashes, sometimes neck webbing and photophobia	Lymphangiosarcoma

15535	Megalencephaly	Megalencephaly; mental retardation	Ganglioneuroblastoma
15740	Multiple eruptive milia	Multiple eruptive subepidermal keratin cysts	Carcinoma of colon
15835	Multiple hamartoma syndrome (Cowden's disease)	Multiple hamartous lesions especially of skin mucous membranes, breast, and thyroid but also of colon and intestine	Thyroid carcinoma
16720	Pachyonichia congenita	Thickening of nails, hyperkeratosis of palms and soles, knees and elbows; leukoplakia; hair, teeth, and bone abnormalities	Carcinoma of mucous membranes
16780	Hereditary pancreatitis	Pancreatitis, fever, abdominal pain, elevation of serum amylase	Carcinoma of pancreas
17580	Porokeratosis of Mibelli	Cornoid lamella—a spreading skin lesion with horny edges, linear, unilateral, or diffuse	Squamous cell and (rarely) basal cell skin carcinoma
18160	Scleroatrophy and keratotic dermatosis	Atrophic fibrosis of skin and limbs, hypoplasia of nails, keratodermia of palms and soles	Skin and bowel carcinoma
19110	Tuberous sclerosis (epiloia)	Adenoma sebaceum; white macules, shagreen plaques; neurologic symptoms, mental retardation; sclerotic and nodular lesions of brain, benign tumor of heart, kidney, and lungs	Astrocytoma and other brain tumors
19330	von Hippel–Lindau syndrome	Angiomata of the retina; angiomatosis of cerebellum and less commonly of other organs; hypertension, polycythaemia, pheochromocytoma	Renal cell carcinoma

[a] Only conditions in which dominant susceptibility is clear and in which the susceptibility is to malignant tumors have been included. Some are, however, very rare and depend on a small number of families.

reporting (Tarkkanen and Tuovinen, 1971; Horven, 1974; Goldberg, 1977; Anders, 1978). Some increase would be expected as a consequence of successful therapy and the hereditary nature of the disease in a proportion of retinoblastoma patients.

The two most common forms of treatment are enucleation and irradiation. Early radiation therapy used radium seeds and low voltage X rays, but these have now been replaced by cobalt 60 applicators and megavoltage units (Bedford *et al.*, 1971). Where suitable treatment is available overall survival is now in the order of 80–90% (Barry and Mullaney, 1971; Aherne and Roberts, 1975; Howarth *et al.*, 1980).

2. *Hereditary and Nonhereditary Retinoblastoma*

Predisposition to retinoblastoma is inherited in an autosomal dominant manner (Griffith and Sorsby, 1944) having a penetrance of about 90%. This gene (Rb) may be acquired either as a germinal mutation or by inheritance from a carrier parent who may or may not be affected. The genetics of retinoblastoma have been extensively reviewed by Vogel (1979).

Approximately 10% of all retinoblastoma patients have at least one other member of the family affected (Briard-Guillemot *et al.*, 1974). In addition, retinoblastoma develops in almost 50% of the offspring of bilaterally affected retinoblastoma patients with no previous family history of the disease compared to 5.5% of the children of sporadic unilateral retinoblastoma patients (Vogel, 1979). All bilateral patients can therefore be regarded as Rb gene carriers as can 10–12% of sporadic unilateral retinoblastoma patients. Estimates of the proportion of retinoblastoma patients bilaterally affected vary from 20 to 40% (Devesa, 1975; Horven, 1974). Knudson (1971) estimates the proportion of retinoblastoma patients with hereditary retinoblastoma as 35–45%.

Rb gene carriers tend to develop multiple tumors compared to noncarriers who invariably develop single tumors. The tumors also develop significantly earlier in Rb carriers. The mean age at first diagnosis for bilateral retinoblastoma patients is 10–14 months compared to about 30 months for unilateral patients (Aherne and Roberts, 1975; Knudson, 1971; Pawlak, 1975).

Penetrance of the Rb gene is incomplete. Many reported pedigrees show sibships with two or more affected individuals from unaffected parents; sometimes expression of the gene skips a generation, and other families show multiple occurrence of retinoblastoma among collateral relatives presumably linked by unaffected gene carriers. Many such pedigrees are summarized by Hermann (1977). Macklin (1959, 1960) reported 11 pedigrees in which extensive investigation of a proband's family revealed at least one other affected collateral relative. This applied to 10.5% of patients who

would otherwise have been classified as nonhereditary. Assuming that within each of these families the Rb gene has arisen once, penetrance in these families was estimated at 20–30%.

The expression and expressivity of the Rb gene in a carrier's offspring increases with increased expression in the carrier parent (Matsunaga, 1976, 1978). Of the carrier children of an unaffected carrier 39% were unaffected, compared to 16% of those of a unilaterally affected parent and 2% of those of a bilateral parent. Of the carrier children of a bilaterally affected parent 88% had bilateral retinoblastoma, compared to 64% of those of unilateral parents and 34% of those of unaffected carrier parents. These observations were extended by Bonaiti-Pellie and Briard-Guillemot (1981) who demonstrated heterogeneity in the expression and expressivity of the Rb gene in offspring of unilaterally affected and unaffected Rb gene carriers which they classified into low and high transmitters.

Estimates of the rate of the germinal mutation (μ) involved in hereditary retinoblastoma are of the order of $5–8 \times 10^6$, where $\mu = S/2b$ and S is the number of sporadic cases thought to be caused by germ cell mutations (all sporadic bilateral + 10–15% sporadic unilateral cases) and b is the total live births in the population in the period in which the patients were born.

The mean paternal age of sporadic bilateral retinoblastoma patients is significantly higher than that of the general population, whereas those of the other groups are not (Pellie *et al.*, 1973; Matsunaga, 1965; Czeizel and Gardonyi, 1974).

3. *Other Cancers Associated with Retinoblastoma*

Treatment for bilateral retinoblastoma frequently involves the use of radiation, and second primary neoplasms are seen within the irradiation field (Abramson *et al.*, 1976; Sagerman *et al.*, 1969), although the incidence of such neoplasms has been reduced by reduction in radiation dose and the use of megavoltage equipment (Editoral, *Lancet*, 1971; Soloway, 1966).

The majority (80%) of these radiation-induced neoplasms are osteogenic sarcomas (Francois, 1977) which have a peak incidence 5–6 years after ocular irradiation. Such a peak is not characteristic of childhood or of radiation-induced bone sarcomas (Strong, 1977b).

Second primary neoplasms in retinoblastoma patients also arise outside the field of irradiation or in patients who have not received radiation, although they are relatively rare, being found in 0.8% of all retinoblastoma patients or in 1.9% of bilateral patients (Abramson *et al.*, 1976). The most common of these is osteogenic sarcoma of the long bones in hereditary retinoblastoma patients, although a variety of other cancers have also been reported.

Osteogenic sarcoma is a rare tumor with an incidence in the United States

of 1:117,000 persons. The incidence of osteogenic sarcoma of the femur in retinoblastoma patients is approximately 500 times higher than normal (Abramson *et al.*, 1976), and the tumors develop at an early age.

The occurrence of pinealoblastoma in retinoblastoma patients is of particular interest. Bader *et al.* (1982) reviewed 11 cases of bilateral retinoblastoma with pineal tumors. The average age of the patients at diagnosis of the pineal tumors was 4 years, 10 years younger than average for pinealoblastoma patients. Retinoblastoma in these patients was diagnosed at an average age of 6 months, which is 6 months younger than average. This study also refers to three patients who developed retinoblastoma-like nonpineal brain tumors 2–6 months prior to diagnosis of retinoblastoma.

Neoplasms other than retinoblastoma have been reported in the families of retinoblastoma patients (Gordon, 1974; Stefani, 1976). Chan and Pratt (1977) reported a family with 18 neoplasms in 13 patients over 6 generations which included 2 retinoblastomas. Osteogenic sarcoma has been reported in unaffected relatives of hereditary retinoblastoma patients (Aherne, 1974; Gordon, 1974; Francois, 1977). Bonaiti-Pellie and Briard-Guillemot (1980) found an excess of deaths due to cancer in the grandparents of both hereditary and nonhereditary retinoblastoma patients which suggests that the factor increasing their risk of cancer may not be the Rb gene.

B. Basal Cell Nevus Syndrome

1. Introduction and Physical Features

Basal cell nevus syndrome (BCNS), also known as nevoid basal cell carcinoma syndrome or Gorlin's syndrome, is characterized by the presence of multiple basal cell nevi, multiple systemic anomalies, odontogenic keratocysts, and a predisposition toward basal cell carcinomas and less frequently toward other neoplasms such as medulloblastoma and ovarian fibroma (Rater *et al.*, 1968; Gorlin and Sedano, 1971). Not all features of BCNS are seen in each patient, as expression of the BCNS gene is very variable between individuals.

Reports of families that with hindsight can be recognized as having members with BCNS have appeared as far back as 1894 (Jarisch, 1894) and have continued to appear regularly since then (e.g., Straith, 1939; Binkley and Johnson, 1951; Howell and Caro, 1959; Ward, 1960). It was not until Gorlin and co-workers (Gorlin and Goltz, 1960; Gorlin *et al.*, 1963) tabulated and analyzed all known cases of BCNS that it became established as a discrete clinical entity. The frequency of the gene in the general population has not been estimated but it is rare.

The major feature of the disease is the presence of multiple basal cell nevi which are precancerous lesions that can transform into basal cell carcinomas. The distinction between the precancerous nevi and the carcinomas is vague; the point at which one becomes the other is not well defined.

Another characteristic sign in a number of these patients is the presence of palmar and plantar pitting (Gorlin *et al.*, 1963; Kennedy and Abbott, 1968). These pits represent a localized maturation retardation of basal cells, and it may also be seen on the fingers and toes (Howell and Mehregan, 1970a,b). In a few cases basal cell carcinomas have been seen on the palms of the hands and the soles of the feet and in one or two instances were at the base of a pit (Ward, 1960; Taylor and Wilkins, 1970; Holubar *et al.*, 1970).

Of the other systemic anomalies the most important, because of the distressing effect on patients, are the jaw cysts (odontogenic keratocysts) which occur in between 65 and 75% of BCNS patients (Lorenz and Fuhrmann, 1978). In most cases they initially appear in the first 10 years of life and may be the earliest manifestation of the disease, but in some cases they do not appear until 40 years of age or more (Pollard and New, 1964). Found in both jaws, the cysts vary in size from microscopic to as large as several centimetres in diameter, actually causing displacement of teeth and pathological fractures (Anderson and Cook, 1966; Meerkotter and Shear, 1964). There is a tendency toward transformation of the cysts into ameloblastoma (e.g., Happle, 1973).

The facies is characteristic although not all of the features are present in every case. These features include frontal and temporoparietal bossing, well-developed supraorbital ridges, broad nasal root, hypertelorism, and mild mandibular prognathism (Gorlin and Goltz, 1960; Hermans *et al.*, 1960; Jablonska, 1961; Gorlin and Sedano, 1971).

A variety of different musculoskeletal abnormalities may be found in BCNS patients. Among the most common (about 40% of all patients) is a bifurcated rib, but other abnormalities of the ribs may also be present (McEvoy and Gatzek, 1969; Novak and Bloss, 1976). Other common abnormalities include kyphoscoliosis, spina bifida occulta, and shortened metacarpels.

Ectopic calcification of dura in the parietal region, the falx, and the petroclinoid region, while observed in the normal population, is more extensive and shows a lamellar appearance in BCNS patients giving a characteristic X-ray appearance in these patients (Novak and Bloss, 1976).

A great range of other abnormalities have been described in BCNS patients (Gorlin and Sedano, 1972; Featherstone, 1980). In view of the relatively small number of cases reported, it is easy to conclude that patients with this syndrome have an extraordinarily high incidence of many different developmental anomalies.

2. *Heredity*

Gorlin and Goltz (1960) originally postulated that BCNS is a dominant autosomal trait. This was further confirmed by various family studies (McKelvey *et al.*, 1960; Maddox *et al.*, 1964; Gerber, 1965; Gorlin *et al.*, 1965; Anderson *et al.*, 1967a).

Penetrance is high, although the expression of the gene is variable. This variation, however, is only marked between families (Anderson *et al.*, 1967a; Totten, 1980). Many of the reported cases are isolated cases and may be new mutations, although family studies are often lacking. Linkage analysis with several blood group loci did not indicate close linkage of any of them to the BCNS locus (Anderson, 1968).

3. *Susceptibility to Neoplasia*

Basal cell nevi are precancerous lesions which may transform into basal cell carcinomas (BCC). Histologically the BCC which occur in BCNS patients are essentially indistinguishable from those found in non-BCNS patients. While BCC in normal individuals are generally solitary, occur on sun-exposed areas of the skin, and do not usually appear until later in life, the nevoid BCC occur multiply (about 50–100 average), appear on both sun-exposed and nonexposed skin, and usually appear at puberty and during the second or third decade of life. In rare instances, tumors may even be present in large numbers at birth (Kahn and Gordon, 1967). BCC are not invariably present, and in one large kindred only half of the affected individuals over 20 years of age manifested skin tumors (Gilhuus-Moe *et al.*, 1968). The tumors are 1.0 mm in diameter and larger, they appear on the face, neck, back, thorax, abdomen, and upper limbs, and as many as 1000 separate lesions have been seen on some patients (Howell and Caro, 1959). The larger lesions are usually pigmented and ulceration is common. Favored sites for these carcinomas are the periorbital areas, eyelids, nose, malar region, and upper lip (Gorlin and Sedano, 1971).

It was pointed out by Gorlin that the large number of cases reported in the literature (e.g., Rival *et al.*, 1975) in which several members of a family had multiple dentigerous cysts but had only a few or no basal cell carcinomas could suggest that other genetic factors and/or environmental influences affect the appearance of BCC in any single BCNS patient (Gorlin *et al.*, 1965). For example, there have been several reports of blacks with BCNS (Ellis *et al.*, 1972; Giansanti and Baker, 1974; Repass and Grau, 1974; Ryan and Burkes, 1973; Towns and Lagattuta, 1974); the incidence of BCC is lower in these black BCNS patients than in caucasian BCNS patients. This same pattern is also observed if the normal black and caucasian populations are compared (Fleming *et al.*, 1976). Strong (1977a) agrees that the inherited predisposition to BCC is modified by other genetic factors and by environ-

mental factors. She also suggests that since the distribution of BCC found in the absence of the syndrome (Harris, 1976) is similar to that in BCNS, the same environmental factors are involved, for example, sunlight may be involved in the development of the two types of tumor. Other authors, however, (as noted previously) would not agree that the distribution of carcinomas is similar in the two cases.

Medulloblastoma, which comprises 20–25% of intracranial tumors in children, has been reported in about 20 BCNS patients (e.g., Cook, 1964; Heimler *et al.*, 1976), the tumours usually appearing in the first two years of life (Neblett *et al.*, 1971). Although there are relatively few cases of medulloblastoma and BCNS occurring together, the normal incidence of medulloblastoma as calculated by Gorlin and Sedano (1971) is only 1 per 600,000 people; they conclude that it is unlikely that this is a chance association and that it is rather a manifestation of the same pleiotropic gene. They further suggest that the incidence of medulloblastoma in BCNS may be even higher and that causes of death in supposedly unaffected members of BCNS families should be studied more closely.

The most frequent neoplasms, other than BCC, seen in BCNS patients are ovarian and uterine fibromas and cysts. Out of the 23 female patients with BCNS reviewed by Southwick and Schwartz (1979), 4 had uterine fibromas and 4 had ovarian fibromas. This association has been reported by others and is almost certainly a real manifestation of the syndrome. Similar associations have been noted with a variety of other benign tumors, including 5 patients who had both BCNS and multiple neurofibromatosis (Happle, 1973).

There are several reports of neoplasms in the relatives of patients with BCNS. Happle *et al.* (1971) found a scirrhous carcinoma of the breast in the mother of their patient, and three other neoplasms occurred in the same family. Yunis and Gorlin (1963) found four members of the family of one of their patients to have carcinoma of the breast. Kedem *et al.* (1970) mention bilateral carcinoma of the breast in the sister of their patient. It is difficult to conclude that these cases constitute a significant excess, but it does suggest that a more detailed study of relatives might detect an excess of cancer as has been shown for ataxia–telangiectasia relatives by Swift (1982). Rather than heterozygote susceptibility, it could suggest a cancer prone genetic background.

C. Neurofibromatosis (Von Recklinghausen Disease)

1. Introduction and Physical Features

The main feature of Von Recklinghausen disease is the presence of multiple skin nodules which histologically are neurofibromas and which may un-

dergo malignant change (see below). There are several other manifestations of neurofibromatosis (Harlan and Okazaki, 1972). Café-au-lait spots, which vary in both size and shape, are found on the skin, sometimes in large numbers. Larger nevi may occasionally be present. The presence of tumors on nerve routes can give rise to a variety of neurologic signs; most commonly involvement of the eighth cranial nerve results in deafness, loss of corneal reflex, facial palsy, and cerebellar signs. Pressure against the spinal cord can result in paraplegia, while a high proportion of the patients show mild mental abnormality.

Skeletal changes are common and include scoliosis, both hypertrophy and underdevelopment of bone and congenital pseudoarthritis. Many of these changes, as well as the neurological signs, are a secondary consequence of the occurrence of the tumors rather than primary manifestations of the effects of the neurofibromatosis gene. Similarly, hypertension, pancreatic duct obstruction, intestinal obstruction, and exertional dyspnea all of which are found in these patients are secondary effects due to the occurrence of local tumors.

Two types of tumors of the nerve routes and peripheral nerve are recognized, the Schwannoma and the neurofibroma (Russell and Rubinstein, 1977). Schwannomas are derived from the Schwann cells which sheath peripheral nerves and are thought to derive from the neural crest. They are composed of interwoven bundles of long bipolar spindle cells which may form whorls or pallisades and are rich in reticulin. They may arise on cranial and spinal nerve roots as well as on peripheral nerves and may be solitary or multiple. Multiple Schwannomas are a feature of Von Recklinghausen disease, and they tend to occur particularly on the cranial and spinal nerve roots and on the larger nerve trunks. Neurofibromas are distinguished morphologically by having a scantier cell content and looser texture. The cells may be bi- or tripolar and the bundles of cells are separated by an indefinite matrix rich in both reticulin and collagen. While the name suggests that these tumors are derived from the fibroblasts of neural connective tissue, it is quite probable that these tumors are also derived from Schwann cells. Solitary neurofibromas are rare and they are most commonly found in larger numbers associated with Von Recklinghausen disease at many sites including cranial and spinal nerve roots and ganglia, the major nerve roots of neck, trunk, and limbs, the sympathetic system, and the dermis.

The incidence of the disease in the United States is estimated to be about 1 in 3,000 (Crowe *et al.*, 1956).

2. *Heredity*

Different forms of Von Recklinghausen disease have been suggested; the central form in which principally Schwannomas are found only on cranial and

spinal nerve roots and the larger nerve trunks, and the commoner peripheral form in which multiple neurofibromas appear as disfiguring cutaneous nodules. There is also a visceral form (Hochberg *et al.*, 1974) in which multiple neurofibromas are found in the alimentary tract and mesentery. Bilateral acoustic neuroma without peripheral lesions is probably a separate autosomal dominant disease (Young *et al.*, 1970).

The mode of inheritance is well established as autosomal dominant (McKusick, 1983) for all forms of the disease. It is possible that all the forms are differing expressions of a single gene because the different forms may occur within a single family.

3. *Susceptibility to Neoplasia*

Both Schwannomas and neurofibromas may undergo malignant change to sarcomas. Malignant change in a neurofibroma is less common than in a Schwannoma and is probably always associated with Von Recklinghausen disease (Russell and Rubinstein, 1977). Estimates of the proportion of Von Recklinghausen patients who develop malignant change vary from 3 to 15% (McKusick, 1983), from 15 to 25% (Rosenberg *et al.*, 1982), and up to 30% (Brasfield and das Gupta, 1972). It seems likely that bias in reporting such cases will make the lower figures more realistic.

These patients also appear to have a predisposition toward developing other cancers, but as Hope and Mulvihill (1981) point out the frequency of the disease (about 1 in 3000) would lead one to expect a substantial number of cases of cancer in these patients by chance and therefore reports of cancer in neurofibromatosis must be interpreted with caution.

Other tumors that occur include meningiomas, astrocytomas, optic nerve gliomas, diffuse gliomas of cerebrum, cerebellum, and spinal chord, and ependymomas. Pheochromocytomas have been reported in 5% of Von Recklinghausen patients (Glushien *et al.*, 1953). Other tumors such as malignant melanoma have been reported to be associated with this disease but they probably do not occur in excess (Hope and Mulvihill, 1981).

Neuroblastoma and neurofibromatosis have been reported in the same patient on four occasions. The case of Knudson and Meadows (1976) would not have been diagnosed as neurofibromatosis if the patient had died early in the course of neuroblastoma. Bolande and Towler (1970) investigated a possible association between neurofibromatosis and neuroblastoma based on the observation that regressed (matured) skin neuroblastomas showed an increase in neural connective tissue and loss of ganglion cells such that they resembled neurofibromas. In a histological study of 19 cases of neurofibromatosis, 6 cases were identified as having ganglion or neuroblastic cell elements; 3 of these involved tumor tissue some distance away from normal sites of ganglion cells and were considered unlikely to represent ganglion

cells trapped in tumor tissue. Abnormal Schwann cell proliferation could be related to abnormal neural crest cell migration.

D. Multiple Endocrine Neoplasia Syndromes

Three reasonably well-defined entities all appear to be inherited in an autosomal dominant manner (Brennan, 1982; Schimke, 1977).

Multiple endocrine neoplasia type 1 (MEN 1) involves mainly the parathyroid, pituitary, and pancreatic islet cells. Although isolated instances had been reported previously, it was Wermer (1954) who brought together descriptions of a number of families and suggested a genetic defect (hence, the name Wermer syndrome). Most gene carriers will have hyperparathyroidism, and screening for serum calcium, phosphorus, and parathyroid hormones may reveal as yet unrecognized affected family members. It is characteristic that the tumors may be functional and produce a characteristic syndrome from overproduction of a specific hormone (e.g., islet cell tumors may produce insulin, glucagon, or gastrin). Hormone estimations may aid in diagnosis. This syndrome encompasses a high proportion of cases of Zollinger–Ellison syndrome in which persistent duodenal ulcer is associated with a gastrin producing islet cell tumor. Jackson *et al.* (1977) found that 10 out of 91 patients with parathyroid tumors had other affected family members. Five of these were apparently simple hereditary hyperparathyroidism while three families had MEN 1 and two had MEN 2. They suggest, however, that some of the hyperparathyroid families could possibly belong to MEN 1. From a mechanistic view point it seems likely that these tumors are all derived from the neural crest. This is discussed in more detail by Schimke (1977).

Multiple endocrine neoplasia type 2 is often subdivided into MEN 2A (Sipple syndrome) and 2B depending on the absence or presence of mucosal neuromas (Brennan, 1982). Schimke (1977) and McKusick (1983) both argue that the mucosal neuroma syndrome is sufficiently different from Sipple syndrome to warrant its classification as MEN 3.

MEN 2 (Sipple syndrome) describes a familial association among medullary carcinoma of the thyroid, pheochromocytoma, and less frequently parathyroid adenoma. In practical terms all patients with familial medullary carcinoma of the thyroid are considered examples of MEN 2. Thus, by definition, medullary carcinoma of the thyroid occurs in 100% of MEN 2 patients and is bilateral in 90–100% (Brennan, 1982). The production of calcitonin by these tumors forms the basis of screening done by a provocative test that is necessary because some of these patients will have normal base line levels of calcitonin. Pheochromocytoma occurs in over half of the patients with this syndrome while parathyroid tumors occur in about 20% of

cases. The thyroid tumors are derived from the C-cell component of the thyroid and are therefore of neural crest origin. The neural crest origin of the pheochromocytomas is also clear but there is debate about the possible neural crest origin of parathyroid tumors (Schimke, 1977). In one instance the thyroid tumor and the pheochromocytoma have been shown to have the same clonal G-6PD phenotype (Baylin *et al.*, 1976) suggesting that the basic defect in the cells may have arisen at a very early stage in embryogenesis prior to the migration of the neural crest.

Lips *et al.* (1982) report that in the Netherlands there are 500 patients with MEN 2 in 20–30 kindreds and that 5000 persons at risk in these families should be examined each year for expressions of the syndrome. Extrapolating to the United States they suggest that there could be over 8000 patients in 400 families and 80,000 subjects requiring annual screening. These figures seem unreasonably high and certainly do not fit with the U.K. prevalence of MEN 2. The point does remain, however, that screening in this disorder may be a real possibility.

MEN 3 differs by definition from MEN 2 in that all of the patients have mucosal neuromas and/or intestinal ganglioneuromas. Medullary carcinoma of the thyroid occurs in over 75% of the patients and pheochromocytoma in about 50%, but parathyroid adenomas are rare. These patients may have a variety of other physical abnormalities, particularly a slender (Marfanoid) habitus. Again, it is of interest that all these tumors found in this syndrome are of neural crest origin (Schimke, 1977).

Medullary carcinoma of the thyroid comprises 5–10% of all thyroid carcinomas but there has recently been a marked increase in the number of cases diagnosed in the United States, usually in the younger age groups because of screening for thyrocalcitonin.

It is clear that all three syndromes are inherited in an autosomal dominant manner (McKusick, 1983), and while they do have distinctive features, the boundaries between them may not be clear cut.

E. Dysplastic Nevus Syndrome

It is well recognized that some cases of malignant melanoma are familial. Such patients (about 6% of the total) show a number of features which distinguish them from the nonfamilial cases: they tend to be younger at diagnosis, the disease tends to be less aggressive, and there is an excess of males (Anderson *et al.*, 1967b; Anderson, 1972). It has been recognized that a proportion of such patients can be distinguished by the presence of atypical moles which coexist with the melanomas and sometimes precede them. Several groups recognized this new syndrome at about the same time. Clark *et al.* (1978) suggested the name *B-K mole syndrome* but later refer to it as

the *large atypical nevus syndrome* (LANS) or the *dysplastic nevus syndrome* (Greene *et al.*, 1980; Elder *et al.*, 1980). This latter name seems now to be most widely used (see Editorial, *JAMA*, 1981) but Lynch *et al.* (1978, 1983) describe this syndrome as the familial atypical multiple mole melanoma syndrome (FAMMM). Though there is variability in the expression of this syndrome in different families and even between different patients in the same family, the main features seem clear. The affected individual may have from a few to more than 100 nevi on any part of the body, although they are less common on nonexposed surfaces. The distribution on the body may be similar in different members of the same family. Unlike typical nevi, which are usually symmetrical, uniform in color, and less than 5 mm in diameter, the nevi in this syndrome may be small but they may be over 10 mm in diameter. They are irregular in outline and often show a range of colors in the same nevus from pink through various shades of brown to almost black (Clark *et al.*, 1978).

The histology of these moles or nevi is quite characteristic (Clark *et al.*, 1978). In addition to the nests of melanocytes and individual melanocytes of normal morphology seen in acquired melanocytic nevi, atypical melanocytes are present. These have some of the features of malignant melanocytes, and Clark *et al.* draw a parallel to the abnormal cells of obscure malignant potential seen in cervical dysplasia. The dermal component is uniformly cellular, and unlike acquired nevi, it does not show neurotization.

Malignant progression within individual nevi has been well documented by photographic techniques. In some cases malignant progression can be prevented by prophylactic treatment with topically applied cytotoxic drugs (Bondi *et al.*, 1981).

From the reports of Clark *et al.* (1978) and Lynch *et al.* (1978) the mode of inheritance is clearly autosomal dominant with a high degree of penetrance. However, Lynch *et al.* (1983) describe a remarkable family in which members showed different expression of the gene. Two patients, one of whom was an obligate gene carrier, had malignant melanoma without any evidence of the occurrence of nevi. On the other hand, two patients (ages 30 and 39) had multiple nevi but no malignant melanoma. They also remark on the usually high incidence of other cancers (one patient had carcinomas of the prostate and lung as well as malignant melanoma) and suggest that this may be a feature of the syndrome. There is, however, no consistent pattern of other tumors in these families, and the most common are not embryologically related to melanoma. The possible association between breast carcinoma and sporadic melanoma may also apply to dysplastic nevus syndrome patients but may reflect the importance of hormonal status in the development of these malignancies. Environmental insult, hormonal changes, or alterations in immune status have been suggested to affect the malignant

progression of dysplastic nevi (Reimer *et al.*, 1978). The dominant mode of inheritance was confirmed by Greene (1982) who studied 400 members of 14 families prone to the development of cutaneous malignant melanoma. He reported that over 90% of familial malignant melanoma patients and 40% of their first-degree relatives show dysplastic nevi. Over a period of 5 years 31 new primary cutaneous malignant melanomas developed in these families, all in patients with dysplastic nevi. He estimates that family members with dysplastic nevi are 100 times more likely to develop malignant melanoma than are persons in the general population. However, Lynch's family shows that within some of these families absence of nevi does not necessarily guarantee that the subject is not a gene carrier.

F. Diseases with Polyps of the Gastrointestinal Tract

There are several different syndromes which are characterized by polyps of the gastrointestinal (GI) tract or of one region of it. The classification of these syndromes does present some problems, and these problems may not be purely semantic. Turcot syndrome, having fewer larger polyps and malignant tumors of the central nervous system, may be a rare variant of Gardner syndrome (see below) but a recessive mode of inheritance has also been suggested. The rare juvenile polyposis coli (Veale *et al.*, 1966) has an early age of onset, has hamartomatous polyps which are not usually considered to carry any increased risk of malignancy, and is clearly an autosomal dominant condition. Goodman *et al.* (1979) report a case in which there are juvenile (hamartomatous) and adenomatous polyps and also a carcinoma of the colon. This case is nonfamilial and could possibly be a different entity.

The main problem arises over adult adenomatous polyposis. In most descriptions a clear distinction is made between familial polyposis coli (FPC) [which is also known as adenomatosis of the colon and rectum (ACR) or intestinal polyposis type I] and Gardner syndrome (intestinal polyposis type III), based largely on the distribution of the polyps. In FPC the polyps are confined to the colon and rectum, and there are no extra intestinal symptoms. In Gardner syndrome there may be polyps in other parts of the GI tract and also extraintestinal symptoms. This apparently clear distinction is blurred, however, by the work of Utsunomiya *et al.* (1981) based on their Japanese population. They consider the two diseases together under the name adenomatosis coli. Of their series, 42.8% showed the extraintestinal stigmata of Gardner syndrome. There was no difference between the Gardner cases and the others with respect to sex ratio, average age, or frequency of colo-rectal cancer. However, of cases in the series, 70% had polypoid lesions of the stomach, 100% had polyps of the duodenum, and 70% had polyps of the jejunum. Therefore many of the non-Gardner cases

had extra colonic polyps. Utsunomiya *et al.* make it clear that adenomatous polyps of the entire gastrointestinal tract are found in some members of almost all the families. Thus the gene or genes responsible may be behaving differently in the Japanese population, either because of the different genetic background or because of interations with environmental factors. There is a distinction, even in Japanese, between those with and those without extraintestinal stigmata. H.J.R. Bussey (personal communication) would now agree that in the United Kingdom a high proportion of polyposis patients without extraintestinal lesions will show polyps and malignant neoplasms at sites in the GI tract other than colon and rectum.

Much of the interest in this group of diseases centers aroung the nature and origin of carcinoma of the colon, an area reviewed by Sugarbaker *et al.* 1982). Briefly, there is a close association between adenomatous polyps and colonic cancer; all adenomatous polyps, even single ones, carry a risk of undergoing malignant change. It has further been suggested that all cancers of the colon arise from preexisting adenomatous lesions, and while the evidence is not overwhelming, the data are certainly consistent with that possibility.

In FPC and Gardner syndrome, polyps in the colon and rectum may begin to appear at an early age. There is good agreement between the London series (Bussey, 1975) and the Japanese series (Utsunomiya *et al.*, 1981), with the progression in the Japanese group being slightly slower. About 50% have polyps by late adolescence and 90% by about 25 years. Large bowel cancer is found in 50% by the late twenties and in 90% by about 40 years of age. The number of polyps may be enormous, as much as 10,000 or more, and they may cover the entire surface of the colon and rectum.

Another distinction between the Japanese studies and others is that Utsunomiya *et al.* (1981) only include patients with over 100 adenomas within their definition of adenomatosis coli, whereas other authors agree that variation in expression of the gene means that in some family members only a relatively small number of polyps are present. The probability of malignant change increases with the size of individual polyps and the total number of polyps.

In Gardner syndrome (Gardner, 1962) in addition to the adenomatous polyps of the colon and rectum, polyps in the stomach and small intestine are also a regular feature. As with FPC there is a high probability of malignant change. Gardner's original description notes the occurrence of a number of benign neoplasms such as osteomas and fibromas as well as epidermal cysts. Osteomas and overlying fibromas on the mandible and forehead are characteristic, while sebaceous and epidermoid cysts may occur on the back. Following surgery mesenteric fibromatosis is common.

It is clear that the adenomatous polyp syndromes, both Gardner and FPC,

are inherited as autosomal dominant traits with high penetrance but variable expressivity.

Peutz–Jegher syndrome (also called Peutz–Jegher disease or intestinal polyposis type II) is differentiated from FPC and Gardner syndrome by the presence of melanized spots on the lips, buccal mucosa, and digits. Like the other two disorders it is characterized by polyps of the gastrointestinal tract but in this case the polyps are hamartomatous; they are most common in the jejunum but also occur in the ileum, the duodenum, the stomach, and rarely the colon. The polyps can cause pain, intussusception, obstruction, and bleeding. Since the polyps are hamartomatous, the probability of malignant change is less than in the other two syndromes. Some authors consider that it is not a premalignant condition (Bartholemew *et al.*, 1962) but there are several reports of carcinomas of the duodenum, stomach, and colon in Peutz–Jegher syndrome, and Utsunomiya *et al.* (1975) consider it to be associated with neoplasia. There is also evidence that it is associated with ovarian tumors (Young *et al.*, 1982).

Again there is no doubt that Peutz–Jegher syndrome is inherited as an autosomal dominant trait.

III. Mechanisms of Dominant Cancer Susceptibility

A. Mutation Models

1. The Two Mutation Hypothesis

Knudson (1971) observed that a plot of the fraction of undiagnosed bilateral retinoblastoma patients (S) against time in months (t) fitted a "one hit" curve, $\log S = -t/30$, whereas that for unilateral retinoblastoma patients fitted a two hit curve: $S = -4 \times 10^{-4}t^2$. He therefore suggested that retinoblastoma resulted from two mutations. In hereditary retinoblastoma patients, the first mutation, the Rb gene, would be present in all cells so that a retinal cell would become malignant following one somatic mutation; in nonhereditary retinoblastoma patients, two somatic mutations would have to occur in the same retinal cell before a tumor could develop.

The distribution of unaffected individuals, unilateral retinoblastoma cases, and bilateral retinoblastoma cases among Rb gene carriers suggested that tumor numbers followed a Poisson distribution. The mean number of tumors in an Rb gene carrier was calculated as 3, and this was supported by data from examination of affected eyes (Knudson, 1971). Knudson *et al.* (1975) derived a relationship between the mean number of tumors [$m(t)$] in Rb gene carriers with time. This function increases linearly until about the age of 5 years after which it approaches an asymptote $m(t) = 3$ or 4. This can be

interpreted as a consequence of a constant mutation rate occurring in a susceptible population of cells which varies in size with time, reaching a maximum at the age of 3 years and then declining. The susceptible population would be retinoblasts before they fully differentiated and lost their capacity to divide.

The second (somatic) mutation rate in hereditary retinoblastoma patients was calculated by Knudson (1971) as 0.75×10^{-6} mutation/cell/generation by using the estimated number of tumors per gene carrier and an estimated number of retinoblasts, 2×10^6/retina, which is based on the number of ganglion cells. Assuming that the probability of the second mutation is the same in hereditary and nonhereditary patients, the data are consistent with the probability of the first somatic mutation in nonhereditary patients being the same order of magnitude as the second. This would be expected if these events are random.

Bonaiti-Pellie *et al.* (1976) did not find a good fit of the age incidence data from a larger series of retinoblastoma patients (604) to the expression derived by Knudson (1971). The shape of the curves were similar for unilateral and bilateral cases but the decrease in percentage of cases undiagnosed was faster for bilateral cases. A second-degree regression fitted the curves until about 50 months when a better fit was obtained with a third-degree regression. Hethcote and Knudson (1978) fitted the data of Bonaiti-Pellie *et al.* (1976) to a two mutation model in which the age at incidence was related to the number of cell divisions, the number of undifferentiated retinoblasts, the somatic mutation rates, and the mean number of tumors.

Bonaiti-Pellie *et al.* (1976) found a difference between the age at diagnosis of hereditary unilateral retinoblastoma patients and bilateral retinoblastoma patients and suggested that if Knudson's hypothesis were correct the first tumor should appear at the same time in both groups. However, Hethcote and Knudson (1978) pointed out that hereditary retinoblastoma patients with later age of onset would have a greater probability of being unilateral due to the decreasing number of susceptible cells.

Data of Sughara and Uyama (1975) from four different hospitals did not always fit Knudson's model. They suggested environmental mutagenesis as an explanation for the steeper than expected slope for unilateral retinoblastoma incidence.

If two mutations are necessary for retinoblastoma, it is possible that they could occur at the same locus on homologous chromosomes (Knudson, 1978). Thus expression of the retinoblastoma phenotype at the cellular level would be recessive, although predisposition to retinoblastoma is inherited as a dominant condition. This is compatible with evidence from suppression of malignancy in somatic cell hybrids that malignancy is a recessive phenomenon (Ozer and Jha, 1977). However, it is important to bear in mind that

while in most systems the malignant phenotype is often suppressed in hybrid cells by the presence of normal chromosomes, this is not the case in all cell systems, and that loss of specific chromosomes may be associated with the regaining of the malignant phenotype (Klinger, 1981). There are also hazards in extrapolating from the behavior of hybrids cells derived from established cell lines in immune-deficient mice to human tumors.

Alternatively retinoblastoma could be the result of two dominant mutations at different loci. The sequence in which the mutations occur could be important especially if the mutation frequencies differ or the presence of a mutation in one gene increases the probability of mutation in the other but not vice versa. Such a situation would produce two categories of hereditary retinoblastoma patients with different penetrance and expressivity (Bonaiti-Pellie *et al.* 1976). It is a necessary consequence of this idea that while the first mutation must have some expression in the heterozygotes, it must in itself be insufficient to produce the malignant phenotype. The second mutation could then either provide a second entirely different step or simply provide a suitable genomic environment in which full expression of the first mutation becomes possible.

Knudson's hypothesis can be criticized in that it is assumed that the probability of a malignant cell producing a clinically recognizable tumor is the same in both the hereditary and nonhereditary cases. It does not consider the possibility that since every retinal cell in a predisposed patient would carry the Rb gene, the cellular environment of hereditary and nonhereditary retinoblastomas may be different and hence the probabilities of a malignant cell arising and/or progressing to a tumor may be different due to epigenetic effects. Although a second event must follow the first mutation (since in hereditary retinoblastoma patients only a few out of millions of embryonic retinal cells carrying the first mutation develop into a tumor), the second event is not necessarily a mutation. In a study of 215 bilateral retinoblastoma patients, Matsunaga (1979) observed a high intraclass correlation coefficient (82%) between the age at diagnosis of the first affected eye and the predicted age at which the tumor in the other eye would have been recognized if the diagnoses had been independent. In only seven cases (3.3%) did the interval excede 5 months. It is argued that this would be improbable if the second event were a randomly occurring somatic mutation. However, this depends on whether or not cell divisions in the retina are distributed randomly in time. If cell division proceeds in waves, and given that the mutation frequency and number of cells at risk mean that one mutation is almost inevitable for each round of division, this may not be as improbable as it appears at first sight.

Peto (personal communication) examined the data of Bonaiti-Pellie *et al.* (1976) and 306 cases recorded in the Oxford survey of childhood cancers. He

pointed out that while the two mutation model is a plausible explanation of the genetics of retinoblastoma, the age distribution data would equally well fit any mechanism which increased the incidence in high-risk individuals by a constant factor at each age.

The arguments which have been applied to retinoblastoma with respect to the two mutation hypothesis apply equally to at least some other dominantly inherited neoplasms. A similar pattern of age distribution to that seen in retinoblastoma was observed for hereditary and nonhereditary pheochromocytoma (Knudson and Strong, 1972). A two mutation hypothesis was again proposed, in this case leading to a nonmalignant tumor of differentiated cells. Anderson (1975) has drawn attention to the pattern of age occurrence of basal cell carcinoma in BCNS as compared with basal cell carcinomas of a nonhereditary type. The same pattern as in retinoblastoma is again quite evident. Strong (1977a,b) has also drawn attention to the parallel between BCNS and retinoblastoma with respect to the occurrence of radiation-induced cancers.

2. *The Delayed Mutation Model and Host Resistance*

Genetic models of the mode of action of dominant cancer genes must explain incomplete penetrance. Knudson's two mutation hypothesis suggests that by chance some gene carriers will be unaffected, which would explain occasional skipped generations. However, any model must also explain the fact that, in the case of retinoblastoma, once the tumor has occurred in a family, penetrance is usually high.

The proportion of unaffected, unilaterally affected, and bilaterally affected Rb gene carriers in the data of Bonaiti-Pellie *et al.* (1976) did not fit the Poisson distribution expected by Knudson (1971). This was due to an excess of unaffected gene carriers but is not necessarily incompatible with Knudson's hypothesis since delayed mutation or host resistance may be involved.

Early work with *Drosophila* (Auerbach, 1976) has shown that an induced mutation may not be expressed until several generations have elapsed. It was suggested that a partial structural change could occur giving a labile premutated gene, leading only to a full mutation after further changes had taken place following passage through unaffected carriers. This was termed *delayed mutation*. This phenomenon has been suggested to explain the simultaneous appearance of a "new" dominant mutation in several members of the same family (Knudson, 1971; Hermann, 1977). Delayed mutation to a fully mutated Rb gene could occur in germinal or somatic cells. Such a hypothesis is compatible with Knudson's two mutation model since it concerns the process leading to the first mutation, although Hermann (1977) believed that it was unnecessary to postulate a second mutational event.

Matsunaga (1978) suggested that since delayed mutation involves a labile

premutation which presumably may revert to the normal allele, carriers could be gonadal mosaics. The proportion of affected offspring, unaffected carrier parents, and unilaterally affected parents would therefore vary between sibships depending on the proportion of gonadal cells with the normal allele. Matsunaga examined the segregation ratios in 70 sibships with two or more affected sibs from unaffected parents and 46 sibships with a unilaterally affected parent with at least one affected offspring. At least six of the latter parents had an unaffected carrier parent. Neither set of data indicated that the parents carried a labile premutation in their gonadal cells, and Matsunaga believed there was little need to postulate delayed mutation at the Rb locus.

A host resistance model in which the degree of gene expression is determined by the genetic background of the carrier was proposed by Matsunaga (1978) as an alternative mechanism to explain the variation in distribution of unaffected, unilaterally affected, and bilaterally affected offspring, according to parental phenotype, and also to explain the occurrence of retinoblastoma in collateral relatives. The possibility that this was due to multiple Rb alleles with different penetrance was excluded by the variability of penetrance between sibships in the same family.

The distribution of the three phenotypes in Rb gene carriers fitted a multifactorial model with two thresholds (Reich *et al.*, 1972; Matsunaga, 1978) rather than a Poisson distribution.

The mean age at diagnosis of hereditary unilateral retinoblastoma patients was 21.5 months if the parent was an unaffected Rb gene carrier but was 15.7 months if the parent was unilaterally affected (Matsunaga, 1979), as would be expected if genetic host-resistance factors affected the age of onset. Variation also occurred in age of onset of bilateral retinoblastoma patients. Although these patients represent the most susceptible population, they are not homogeneous in their resistance. Nonhereditary retinoblastoma would arise following somatic mutation possibly in the most susceptible group of the normal population.

The high intraclass correlation between ages at diagnosis of tumors of bilateral retinoblastoma patients also indicates that the age of onset is related to host factors (Matsunaga, 1979). The host-resistance mechanism is probably related to the second event in the development of retinoblastoma which could be an error in differentiation. The heritability (h^2) of host resistance was estimated at about 90% (Matsunaga, 1978), which implies little dominance or environmental effect. Since 13% of carriers of a new Rb mutation inherited from an unaffected parent are unaffected and since the mutation rate is about 5×10^{-6}, Matsunaga (1978) has suggested that these suppressors of malignancy are not necessarily specific to the action of the Rb gene and that they occur throughout the population. Matsunaga (1979) has

showed that the probability of an Rb gene carrier developing osteogenic sarcoma is not related to the retinal phenotype. Thus the host resistance mechanism is tissue specific. Matsunaga (1979) has also suggested a multifactorial threshold model for development of osteogenic sarcoma (h^2 = 70%) and has suggested that radiation-induced osteogenic sarcoma could result from mutation in the suppressor genes, although this assumes that the resistance is on an individual cellular level rather than the result of cellular interaction.

Segregation analysis by Bonaiti-Pellie and Briard-Guillemot (1981) detected two classes, "high and low transmitters," among unaffected and unilaterally affected Rb gene carriers. This was not detected by Matsunaga (1978) since he did not include in his analysis any unilaterally affected probands with at least one affected relative nor did he include unaffected gene carriers with at least one affected relative and at least one affected child; both of these groups have different segregation ratios from unilaterally affected patients with at least one affected child and unaffected carriers with at least two affected children. While this observation could support the delayed mutation hypothesis, analysis of the segregation ratios of high-transmitter unaffected and unilaterally affected Rb carriers differed from that of bilaterally affected carriers, which favored the host-resistance hypothesis.

The pedigree reported by Bundey and Morten (1981) in which the Rb gene was transmitted through at least one and, in one branch, at least two generations prior to expression could be explained by a host-resistance mechanism due to a few genes which segregated independently of the Rb gene. Delayed mutation alone would not explain this pedigree. Similar families were reported by Matsunaga (1976) and Harper (1979).

The mutation mosaic model of Carlson and Desnick (1979) is essentially the same as Knudson's two mutation hypothesis, although it gives more detailed consideration to the proportion of germinal and somatic cells which carry the first mutation and could determine the pattern of transmission and degree of expression of the Rb gene. They explain reduced penetrance in some families by suggesting that there are multiple Rb alleles which can have full penetrance and expressivity (amorphs) or reduced penetrance and expressivity (hypomorphs), a suggestion previously rejected by Matsunaga (1978). Carlson and Desnick also suggest that since late onset unilateral retinoblastoma would be the result of somatic mutation within the cephalic region, such patients would be unlikely to develop distant osteogenic sarcomas compared to bilateral cases in which every somatic cell carries the Rb gene. However, unaffected and unilaterally affected Rb gene transmitters develop osteogenic sarcoma at the expected frequency from their proportion of Rb gene carrier phenotypes. Some form of inherited, tissue-specific, differentiation-linked mechanism is clearly needed to explain this and the si-

multaneous occurrence of bilateral tumors (Matsunaga, 1979, 1980a, 1981) as well as the relationship between penetrance and expressivity of an Rb gene carrier and the penetrance and expressivity of the Rb gene in the carrier's parent. Matsunaga's tissue resistance model fits these criteria.

B. Cytogenetic Models

1. Mutations as Chromosomal Events

Studies on species other than humans indicate that in many cases the gene which controls a specific phenotype may be a chromosomal rearrangement (which may or may not be visible using cytogenetic techniques) rather than a point mutation. Such rearrangements may be expected in a proportion of subjects showing an autosomal dominant predisposition to cancer. Such rearrangements can affect more than one locus, which could explain the variety of features associated with some cases of these syndromes.

A small proportion (5%) of retinoblastoma patients have a cytogenetically demonstrable deletion of chromosome 13. Harnden and Herbert (1982) reviewed 33 patients with retinoblastoma and constitutional abnormalities of chromosome 13. In 21 of these patients breakpoints could be identified. Additional patients with an interstitial chromosome 13q deletion have been reported by Rivera *et al.* (1981) (2 patients), Motegi (1981) (1 patient), Johnson *et al.* (1982) (3 patients), Junien *et al.* (1982) (1 patient), and Michalova *et al.* (1982) (1 patient). Two further chromosome 13 translocations have been reported in retinoblastoma patients: Hida *et al.* (1980) t(X;13)(p22;q12) and Motegi *et al.* (1982) t(13;18)(q14.1;q12.2). Strong *et al.* (1981) reported a family in which retinoblastoma was transmitted through 8 unaffected individuals. Cytogenetic analysis showed that the patients in this family had a 13 deletion (q13.1–q14.3) inherited from unaffected carriers of a balanced insertional translocation to chromosome 3.

Where banding has been done, all patients (26) with a deletion show loss of or break in 13q14. Studies with high-resolution banding have demonstrated small subband deletions within 13q14 (breakpoints 13q14.1 and 13q14.3) in two patients (Yunis and Ramsay, 1978; Johnson *et al.*, 1982). A second patient of Johnson *et al.* had breakpoints at 13(q14.1q21.3), and two patients of Rivera *et al.* (1981) had breakpoints at 13(q13.07q14.3) and 13(q13.03q14.3). These data would suggest the involvement of band 13q14.2 or distal 13q14.1 or proximal 13q14.3 in retinoblastoma.

Noel *et al.* (1976) reported a stillbirth with a deletion 13(q14.3qter). Although no diagnosis of retinoblastoma is given, the retina consisted of rosettes of undifferentiated epithelium. Although this result may suggest 13q14.3 as the important site, Harnden and Herbert (1982) commented that

the pictures as reproduced do not allow precise breakpoint identification. Involvement of 13q14.3 would conflict with the translocation reported by Motegi *et al.* (1982) in which the breakpoint is distal 13q14.1, unless some position effect is involved.

Two translocations to chromosome X are of interest. The patient of Cross *et al.* (1977) was originally reported to be a translocation of 13q14-qter to the active X. Riccardi *et al.* (1979) suggested that the patient was a mosaic with a line having an incomplete translocation with loss of 13q14 material. Reinvestigation of this patient by Nichols *et al.* (1980) uncovered no evidence of mosaicism and showed that the breakpoint in 13 was at the junction of 13q12 and q13 and that 13q13-13qter was translocated to the late replicating X. Spread of inactivation to the translocated 13 material would produce a patient functionally hemizygous for 13q14. Fibroblasts of the patient of Hida *et al.* (1980) with a (X;13)(p22;q12) karyotype had a late replicating translocated X in which the 13 material replicated later than the nontranslocated 13 (Ejima *et al.*, 1982a), although the normal X was reported as late replicating in lymphocytes (Hida *et al.*, 1980).

Davison *et al.* (1979) report a chromosome 13 translocation in a retinoblastoma patient in which band 13q14 is not involved. The patient had unilateral retinoblastoma, and the translocation could be coincidental.

Mosaics for deleted 13s have been reported (Thompson and Lyons, 1965; Taylor, 1970; Sparkes *et al.*, 1980; Orye *et al.*, 1982). Three patients mosaic for 13q14 subband deletions (q14.1–14.3) have been reported by Motegi (1982). Only a minority of retinoblastoma patients have a constitutional chromosome 13 deletion (1 out of 33, Francois *et al.*, 1978; 1 out of 15, Wilson *et al.*, 1977; 0 out of 31, Gardner *et al.*, 1982b), but the use of high resolution banding techniques may increase the frequency of small deletions detected.

Patients with substantial deletions of chromosome 13 might be expected to show abnormalities other than retinoblastoma. Johnson *et al.* (1982) found 3 out of 12 retinoblastoma patients with 13q deletions; two of these, one of which had a subband deletion, had developmental and/or mental retardation. Jensen and Miller (1971) reported a significant increase in severe mental retardation among retinoblastoma patients (15 out of 1,077). Although other abnormalities were not at a frequency higher than expected, they were the types associated with abnormalities of 13q, eczema, microcephaly, colobomas, malformed ears, high arched palate, cleft palate, and multiple skeletal abnormalities. Bonaiti-Pellie *et al.* (1975) found 7 out of 598 retinoblastoma patients that had congenital abnormalities, including 4 with cleft palate.

By analogy with Rb gene carriers it would not be expected that every carrier of 13q14 deletion would develop retinoblastoma. Aronson *et al.* (1975) and Serena-Lungarotti *et al.* (1979) describe such patients.

Retinoblastoma patients with a chromosome 13 abnormality have a high frequency of bilateral retinoblastoma (25 out of 31) as do hereditary retinoblastoma patients. Therefore it becomes important to ask whether hereditary retinoblastomas carry a deletion (or other chromosomal lesion) too small to be recognized by conventional cytogenetic techniques. If the location of the retinoblastoma "gene" were known, then it could be determined whether there is any evidence of deletion of associated genes.

Attempts to confirm the presence of the Rb gene at 13q14 using cytogenetic methods have so far failed. The segregation of the Rb gene with fluorescent Q-band chromosome 13 polymorphisms at the centromere and at 13p has been studied (Knight *et al.*, 1980; Morten *et al.*, 1982), and a recombination frequency of 38.6% has been obtained, which is not significantly different from 50%. The estimated recombination frequency between the centromere and 13q14 is between 27 and 37% in male meiosis (Palmer and Hulten, 1982), so this result neither refutes nor confirms the possibility that the Rb gene is located at 13q14.

Esterase D maps to 13q14 and shows appropriate dosage effects in retinoblastoma patients with 13q14 deletions and their families (Sparkes *et al.*, 1980; Rivera *et al.*, 1981; Junien *et al.*, 1982). Screening of 100 patients with retinoblastoma for abnormalities of esterase D revealed only 3 patients with deficiencies (Murphee *et al.*, 1981). Subsequently 2 of the 3 were shown to have deletions of chromosome 13. This could suggest that only a small proportion of hereditary retinoblastomas are due to cryptic deletion, but until we have a specific localization of the gene, this is not certain. However, Sparkes *et al.* (1983) studied the segregation of esterase D electrophoretic types in three families with hereditary retinoblastoma and found a suggestion of very close linkage between the two loci.

Also, the bilateral retinoblastoma patient reported by Benedict *et al.* (1983) is of interest because, even though he was cytogenetically normal at the 550 band level, he had only 50% of normal esterase D activity, which is indicative of a small deletion in 13q14. Examination of direct chromosome preparations from tumor material from one of the patient's eyes revealed two stem lines both lacking a chromosome 13 which was not obviously present in marker chromosomes. No esterase D activity was found in the tumor cells. This result indicates that the tumor cells have lost both copies of the 13q14 esterase D gene and probably the closely linked retinoblastoma gene. This would support the suggestion discussed previously that retinoblastoma is a recessive condition at the cellular level.

Matsunaga (1980b) observed that although the age at diagnosis of retinoblastoma patients with the 13q deletion was the same as for Rb gene carriers, there was a higher than expected frequency of unilateral retinoblastoma patients. Matsunaga suggests that the Rb gene has a higher

oncogenic potential than the deletion and that it is probably not located at 13q14.

The effect of the 13q14 deletion may be to produce a set of unopposed hemizygous normal genes on the homologous chromosome 13 which may result in retinoblastoma. There may not be a site at 13q14 at which a point mutation could lead to retinoblastoma. Riccardi *et al.* (1979) term such a site a *haplicon*. However, as discussed in Section III,A,1 retinoblastoma may result from two mutations at different loci, one of which could be 13q14. In some hereditary cases the inherited mutation could be a 13q14, while in others it may be at a different site. Hereditary patients would therefore fall into two classes, with the probability of bilateral retinoblastoma being dependent on which locus is the inherited mutant.

Of the 33 chromosome abnormalities involving 13q14, 26 have 13q14 as one of the breakpoints. Harnden and Herbert (1982) commented that the important effect of the deletion may not be loss of material but inappropriate positioning of the remaining 13q sequences, analogous to the translocation of 8q24, the site of *onc* gene c-*myc*, to sites of human *Ig* genes in B-cell lymphomas. However, in the family reported by Strong *et al.* (1981) only individuals with loss of the 13q14 material were affected; those with translocated 13q14 were not and neither were the mothers of the patients of Riccardi *et al.* (1981) and Rivera *et al.* (1981), who also carried 13q14 translocations. The high proportion with breakpoints in 13q14 is nevertheless curious.

The only suggestion that some cases of Gardner syndrome of FPC might be associated with a chromosomal lesion comes from the reports of Gardner *et al.* (1980, 1982a). They report that all Gardner syndrome and polyposis coli patients studied as well as two Gardner syndrome carriers without polyps but with increased aneuploidy in cultured skin cells showed a heteromorphism in the size of chromosome 2, tentively identified as a deletion within 2(q14.3–q21.3). This report has not yet been confirmed. The nature of the chromosomal lesion in these diseases and the chromosomal location are at present still unknown.

Renal cell carcinoma, generally thought to be derived from kidney tubule cells, is the most common of the kidney cancers, and its occurrence is most often sporadic. However, in some exceptional families there is clearly an unusually high incidence of this malignancy. It also occurs in about 20% of patients with von Hippel Lindau disease (Table I). There is little cytogenetic information on most of these families or on von Hippel Lindau disease. Cohen *et al.* (1979) reported a remarkable family with 10 renal cell carcinoma patients (who did not have von Hippel Lindau disease) in three generations. The ages at diagnosis ranged from 37 to 59 years; six patients were bilaterally affected and eight had multifocal tumors in one or both kidneys. The average age at diagnosis in the general population of the United States is about 60 years, and only 1–2% are bilaterally affected. Five

survivors with renal cell carcinoma had a balanced translocation t(3;8)(p21;q24), and this karyotype was inferred in three additional cases from the pedigree. No patient examined had a normal karyotype, but five known and one inferred carrier of the translocation did not have the tumor. Two of these had died, one aged 67, from bladder carcinoma and undifferentiated abdominal adenocarcinoma; the remaining four were under 35 and one had a renal cyst. Cohen and co-workers calculated an 87% probability of renal cell carcinoma by the age of 59 for the translocation carriers.

Another three-generation family with a history of renal cell carcinoma or renal calculi was reported by Pathak *et al.* (1982). Twenty-two out of thirty direct tumor chromosome preparations had a translocation t(3;11) (p13 or 14;p15). The breakpoint on the 3 is not the same as in the cases of Cohen *et al.*, and the breakpoint on the 11p is close to the site of the known oncogene on 11p(ras^H), although the breakpoint on the 8 in the cases of Cohen *et al.* (1979) is close to the site of the c-*myc* gene.

A high-resolution banding study on lymphocytes of 35 renal cell carcinoma patients with affected close relatives, bilateral tumors, or early onset (<45 years) showed no constitutional chromosome 3 abnormalities (Kantor *et al.*, 1982). A constitutional inversion 2(p13q11) and an inversion leading to duplication 9(p11→q13) was found in two early onset unilateral patients. A bilaterally affected patient was a 46,XX/45,X/47,XXX mosaic and also had abnormalities of the renal caliceal collecting system.

There is little doubt that the constitutional chromosome complement of BCNS patients is normal (Ferrier and Hinrichs, 1967; Howell and Anderson, 1976; Southwick and Schwartz, 1979; Featherstone, 1980). Earlier reports of an abnormality of chromosome 1 in some members of one family (Yunis and Gorlin, 1963) can be explained, with hindsight, as a polymorphism for the C-band region of chromosome 1 in this family. Other reports of constitutional chromosome abnormalities in BCNS (e.g., the abnormal 19 described by Mills and Foulkes, 1967) are probably of no etiological significance in view of the known frequencies of such abnormalities in the general population. Certainly no anomaly is specifically associated with BCNS.

Ten patients in five families with MEN 2 had a possible deletion 20p12.2; another patient in another family did not show this abnormality (van Dyke *et al.*, 1982). So far there is no confirmation of these reports. This may reflect the great technical difficulty in resolving very small deletions and distinguishing them from interhomolog variation in some cells of normal individuals.

2. *The Gene Causes Chromosomal Instability*

Cytogenetic change is a feature of many malignant cells, although its significance in the etiology of cancer is unclear. A gene could predispose to a particular malignancy by increasing the probability of a specific cytogenetic

change. Alternatively, a general chromosome instability could be expected to produce a wider range of cancers. Kinsella and Radman (1980) suggested that cancer could result from homozygosity at a significant locus brought about by chromosomal rearrangement.

There is no firm evidence of spontaneous chromosomal instability in the normal somatic cells of retinoblastoma patients. However, Czeizel *et al.* (1974) looked for such instability in lymphocytes of 12 retinoblastoma patients of whom 3 hereditary and 2 nonhereditary patients had had radiation therapy. The remaining 7 patients were nonhereditary and had had no radiation therapy. The frequency of aneuploidy, gaps, breaks, and stable chromosome aberrations (including two Dq^- cells) was significantly higher in the retinoblastoma patients than in the controls. The frequency of chromatid aberrations was slightly higher in the irradiated than in the nonirradiated patients but both were higher than the control group. However, the bloods were in transit a long time, and very few metaphases from retinoblastoma patients (389) were examined compared to the number of controls (1363). Knight *et al.* (1979) cocultivated lymphocytes of 12 retinoblastoma patients with age-matched controls of the opposite sex. Eight out of nine bilateral patients and one out of three unilateral retinoblastoma patients had had radiation therapy. Although the level of aberrations varied between individuals, no significant difference was found between retinoblastoma patients and controls. This finding was confirmed by Morten (1980) who found no significant difference between the levels of spontaneous chromosome damage in 18 hereditary retinoblastoma patients, 8 normal subjects, and 5 apparently non-gene-carrier relatives of the patients.

It is also of interest to know whether there is evidence of specific chromosomal rearrangements at 13q14 or at any other locus in retinoblastoma tumors. Such studies are hampered by the difficulty in obtaining high-quality preparations. Nevertheless, several banding studies have now been done on tumors from patients with normal chromosome constitutions. Balaban *et al.* (1982) reported four out of four tumors from which short-term cultures showed a deletion involving 13q14. This is consistent with the earlier study of Hashem and Khalifa (1975) who found a Dq^- marker in four out of five tumors studied. Some cells of a tumor examined by Balaban *et al.* (1982) had two normal chromosome 13s, although they had other marker chromosomes in common with cells having the 13q deletion. This may indicate that the 13q rearrangement is a secondary event in progression of these tumors. Gardner *et al.* (1982b) found only one tumor monosomic for 13q14 in a study of ten tumors. Only two of these tumor karyotypes were obtained directly from the tumor; the others, including the $13q^-$ cells, were from cultures or cells grown in nude mice. Another tumor had a translocation, t(12;13) leading to trisomy 13(q22→qter) and monosomy for part of 13p. The most common

abnormalities seen were trisomy 17q in seven tumors and trisomy 1(q25→q32) in 6 tumors, but they were due to different breakpoints.

Other banding studies of direct tumor preparations have not demonstrated 13q14 deletions (Inoue *et al.*, 1974; Hossfeld *et al.*, 1976; Hossfeld, 1978; Morten, 1980). Generally different marker chromosomes are seen in different tumors, although one tumor examined by Morten (1980) showed a marker chromosome 1 apparently the same as the M1 marker of Inoue *et al.* (1974). It is concluded that consistent cytogenetic abnormalities have been demonstrated in retinoblastoma but that the exact frequency of 13q14 deletions is unclear; some nonrandom changes occur as in other tumors.

A number of reports suggest that chromosomal instability could be important in the initiation and progression of neoplasia in Gardner syndrome and FPC. Danes (1976) observed an increased level of tetraploidy in skin cultures derived from skin epithelium of 7 Gardner syndrome patients when compared to similar cultures from 12 control subjects. Cultures from these patients derived from skin, lipomas, sebacous cysts, and colonic polyps which contained only fibroblasts had normal levels of tetraploidy, as had lymphocyte cultures. Cultures from colonic polyps containing epithelial cells showed a comparable high level of tetraploidy.

These observations were extended (Danes, 1978) to include two patients with FPC and two variant Gardner syndrome patients without osteomas, fibromas, or sebacous cysts. A higher frequency of occurrence of tetraploid cells was observed in cultured colonic mucosa (12–20%) and colonic polyp material (18–22%) compared to dermal, epidermal, mesenteric, and colon wall material which had the same frequency of tetraploids as cultures from normal individuals (0–4%). Cells from these sites in eight Gardner syndrome patients gave a similar frequency of tetraploids, except in epidermal cultures (11–29%), although the two Gardner syndrome variants had normal polyploidy levels in epidermal cultures. Six out of eight Gardner syndrome gene carriers without colonic polyps but with extracolonic lesions showed a normal level of polyploidy in epidermal culture (Danes and Gardner, 1978).

In these studies increased tetraploidy was observed only in epithelial cell–containing cultures from tissue in which tumors develop. Danes and Gardner (1978) suggest that polyploidy identifies *in vitro* a population of cells likely to undergo malignant transformation *in vivo*. However, in a study of epidermal cultures from the patients with adenomatosis of the colon and rectum, elevated frequency of polyploid cells was seen in four out of six patients without associated sebacous cysts and in three out of four with sebacous cysts (Danes and Alm, 1979). The authors suggest this is due to genetic heterogeneity in these conditions. These results may reflect an abnormality in the regulation of cell replication, but it is unclear how representative the *in vitro* cells were of the *in vivo* tissue.

Delhanty *et al.* (1980) studied skin biopsies from 13 patients with FPC. Only three lines showed a greater number of tetraploids than the control range, and two of these had no epithelial outgrowth in the primary culture. Of two brothers with symptoms closest to Gardner syndrome, one had a high frequency of tetraploidy (38%), the other a low frequency (1.4–3.0%). Tetraploid populations were probably present *in vivo*, and separate biopsies from patients and controls gave different levels of polyploidy depending on culture conditions, particularly the use of human serum.

An increase in aneuploidy (24.7%) was reported in lymphocytes from six Gardner syndrome patients as compared to age-matched controls (9.8%) (Tice *et al.*, 1975).

In a study using fibroblasts and lymphocytes, Gardner *et al.* (1982a) demonstrated excessive, though random, numerical chromosome aberrations in 16 Gardner syndrome patients with polyps (14 from one family); 2 young Gardner syndrome family members without polyps; and 3 FPC patients. This was in comparison to 18 controls, 2 Gardner syndrome family members without polyps, and 3 patients with familial discrete polyps. Of the cells from Gardner syndrome and FPC patients 97% were in the near diploid range, some of the cells with 46 chromosomes having monosomy and trisomy in the same cell.

Mitelman *et al.* (1974) banded direct chromosome preparations, four from sporadic benign adenomas and five from a FPC patient. Cytogenetic abnormalities were seen in 26 karyotyped cells and involved C and/or D group chromosomes. In 12 banded cells these chromosomes were identified as chromosome 8 (7 cells) and chromosome 14 (8 cells). These abnormalities were seen in both sporadic and FPC polyps but at a higher frequency in the familial form. Chromosomal instability and clonal rearrangements were observed in epithelial-like cells from colon and polyp as well as in early passage skin fibroblasts and lymphocytes from FPC patients (Delhanty and Davis, 1981). There is considerable evidence that chromosome aberrations of many different kinds occur in patients with Gardner syndrome and FPC. It appears that in both conditions one of the effects of the dominant gene may be to destabilize the replication of the chromosome. Quite extensive chromosome change precedes malignant change in the polyps (Mitelman *et al.*, 1974), and this may be associated with the mode of action of these two genes in causing malignant disease.

There have been a number of reports of increased spontaneous chromosome aberrations in lymphocytes and fibroblasts from BCNS patients. Indeed Hecht and McCaw (1977) classify BCNS as a "chromosome breakage syndrome." The evidence for this, however, is far from clear. Happle *et al.* (1971) presented chromosome analyses on PHA-stimulated lymphocyte cultures from six members of a family which had a single case of BCNS. The unaffected family members had normal blood lymphocyte chromosomes, but

the patient had a relatively high number of all types of chromatid and chromosome aberrations. Fibroblast cultures from a normal area of skin from the patient and from his mother showed some abnormalities but are considered to be unremarkable. Another patient who showed chromosome breakage was described by Happle and Kupferschmid (1972). Additional patients with slightly elevated levels of chromosome damage have been reported by Happle and Hoehn (1973), Moynahan (1973), and Horland *et al.* (1975). In the latter case a high level of aberrations was also found in asymptomatic family members; in other cases interpretation is complicated by prior radiotherapy treatment. Featherstone (1980) examined the chromosomes of 10 BCNS patients and 9 controls (Table II). There is a slight excess of cells with rearrangements but this is attributable to one patient who was known to have had prior radiation therapy. It is concluded that increased levels of spontaneous chromosome aberrations in either fibroblasts or lymphocytes is not a regular feature of patients with BCNS.

Sasaki examined skin fibroblasts from patients with a variety of autosomal dominant genes predisposed to cancer (Sasaki *et al.*, 1980; Sasaki, 1982). Cultures from a patient who had MEN 2 showed two clones with chromosome 17 translocations having breaks at 17q23. In a family with a history of childhood leukemia consistent with very low penetrance autosomal dominant inheritance, 5 out of 12 subjects had karyotypically abnormal fibroblasts. Eight different translocations involving 1p22 were seen. Such abnormalities were not seen in either leukemic cells or PHA-stimulated lymphocytes nor were they seen in another family with a recessive or multifactorial pattern of familial childhood leukemia. Cytogenetically abnormal fibroblasts clones are seen in cultures from normal adult donors (Harnden *et al.*, 1976); nevertheless it is possible that site-specific rearrangements seen in cultured cells of carriers of autosomal dominant cancer-associated genes reflect an instability which may be related to the origin of malignancy through cytogenetic change.

C. Susceptibility to Carcinogenic Agents

The autosomal recessive conditions in which there is predisposition to malignancy and an increased sensitivity *in vivo* and *in vitro* to DNA-damaging agents are among the most extensively studied examples of genetic predisposition to cancer in man (see review by Friedberg *et al.*, 1979). Evidence for a similar association in autosomal dominant conditions is less clear.

1. Basal Cell Nevus Syndrome

There are several reports of clinical radiosensitivity in BCNS patients. Two different types of responses are seen. Berendes (1971) and Happle (1973) both reported a disastrous clinical course in a BCNS patient following

TABLE II

CHROMOSOME STUDIES ON PHA-STIMULATED PERIPHERAL BLOOD LYMPHOCYTES HARVESTED AT 48 HOURS AND CULTURED FIBROBLASTS

	Number of patients[a]	Number of cells analyzed	Chromosome number (%)			Chromatid aberrations (%)	Fragments (%)	Chromosome rearrangements (%)
			<46	46	>46			
I Lymphocytes								
BCNS	10	550	5.2	94.0	0.7	9.5	0.9	3.8[b]
Control	10	450	1.3	98.5	0.2	9.3	0.9	1.6
II Fibroblasts								
BCNS	5	500	16.0	82.4	1.6	6.6	0.2	5.0[c,d]
Control	6	370	7.3	92.0	0.7	4.6	0	4.3[e]

[a] Mean age of BCNS patients is 27 years and of controls is 27 years, but note that the range of age of the BCNS patients was much greater.

[b] 9 out of 21 rearrangements are from one patient who also had a dicentric quadriradial.

[c] 12 out of 25 contributed by 1 patient (same patient as in footnote *b*).

[d] Clones of 4,4 and 10 cells counted only once.

[e] One clone of 2 cells and one clone of 5 cells counted only once each.

radiotherapy of a basal cell carcinoma (BCC). In both cases the tumor appeared to be made worse with extensive invasion into the surrounding tissue. Radiotherapy does not always give this response, as illustrated by the review of Southwick and Schwartz (1979) of 36 BCNS patients. Twelve received radiotherapy for BCCs of whom nine were treated successfully and three recurred in the treated site; of these one developed into an advanced infiltrating tumor.

The second type of response is the apparent induction of BCCs, which appears very rapidly following exposure to ionizing radiation. Scharnagle and Pack (1949) described a 5-year-old child, who shortly after birth received X-ray therapy for an enlarged thymus. Subsequently he developed numerous (at least 1000) tiny basal cell epitheliomas (histologically indistinguishable from BCC) within the irradiated area. The boy had several physical features suggestive of BCNS. Strong (1977a) has drawn attention to tumor induction in BCNS children irradiated during treatment for medulloblastoma. Thirteen such patients, all previously reported, developed multiple BCC within 6 months to 3 years in the area that received radiation. Strong (1977a) compared survivors of other childhood tumors (including medulloblastoma) with these patients and noted that they did not develop BCC with such a high frequency or within such a short time. The latent period for a radiogenic BCC is usually greater than 10 years (Good *et al.*, 1980), and Martin *et al.* (1970) recorded that, while radiogenic tumors are often multiple, the mean number is about five. These figures are in contrast to a mean latent period of less than 1 year and the hundreds of tumors that occur in BCNS patients. Strong (1977a) also draws attention to two female BCNS patients who developed ovarian tumors at an unusually early age following radiotherapy and suggests that this also is a radiation effect on predisposed tissue.

A more recent observation that suggests a possible radiosensitivity in these patients was made by Golitz *et al.* (1980). A 5-year-old girl who suffered from BCNS was treated for eczema of the hands by X-ray treatment. At the age of 28 years this patient had 26 BCCs on the palms and dorsa of her hands. BCCs are only rarely seen at these sites in normal individuals and are unusual even among BCNS patients.

Experimental studies have failed to show any unusual sensitivity of BCNS fibroblasts to cell killing by X rays, γ rays, α particles, carbon X rays, and mitomycin C (MMC) (Taylor *et al.*, 1975; Featherstone, 1980). The ability of BCNS fibroblasts to carry out potential lethal damage repair after X irradiation was also normal (Featherstone *et al.*, 1983). There was, however, a small significant increase in the number of chromosome aberrations induced by X rays in BCNS patients as compared with controls (Featherstone *et al.*, 1983) (Table III). In the same study it was found that spontaneous MMC-induced

TABLE III

CHROMOSOME ABERRATIONS IN PHA-STIMULATED PERIPHERAL BLOOD LYMPHOCYTES FROM CONTROL AND BCNS PATIENTS IRRADIATED AT G_0

	200 r				400 r			
	BCNS		Control[a]		BCNS		Control	
Case	Dic[b]	Fgt[c]	Dic	Fgt	Dic	Fgt	Dic	Fgt
1	23	29	8	12	—	—	—	—
2	22	40	20	23	56	120	60	104
3	39	49	25	30	113	198	107	182
4	37	73	3	12	92	174	58	95
5	21	43			83	130		
6	28	39	21	31	59	96	87	98
7	24	39			88	150		
8	39	73	29	42	127	252	95	186
9	41	67	16	36	126	213	70	148
Total	274	452	122	186	744	1333	477	813
Aberrations per cell	0.61	1.0	0.35	0.53	1.86	3.3	1.59	2.71

[a] In each case the BCNS and control bloods were irradiated and processed simultaneously. For cases 5, 6, and 7, who were all members of the same family, there was only one simultaneous control.

[b] Dic, dicentric.

[c] Fgt, fragment.

and X-ray-induced levels of sister chromatid exchanges were no different in BCNS patients as compared with controls.

Three BCNS fibroblast cell lines showed a slightly increased sensitivity to the killing effect of ultraviolet light compared with normal cell lines (Lehmann, 1977). However, the influence of caffeine as a continuous postirradiation treatment was normal. The same three lines were found to be indistinguishable from normal fibroblasts in their ability to carry out excision repair after UV irradiation, as measured by unscheduled DNA synthesis and loss of UV light-specific endonuclease sensitivity sites. However Ringborg *et al.* (1981) found that there was a 25% reduction in UV-induced DNA repair in peripheral leukocytes from 7 BCNS patients (all with BCC) compared with cells from 39 controls.

2. *Retinoblastoma*

Hereditary retinoblastoma patients develop second primary neoplasms within the field of therapeutic radiation. However, since unilateral reti-

noblastoma patients are usually treated by enucleation rather than by radiation, there exists no absolute control group matched for age, radiation dose, and field to test the hypothesis that hereditary retinoblastoma patients have a predisposition to radiation-induced malignancy. Strong (1977b) made a comparison study between the follow-up data of Sagerman *et al.* (1969) on 243 radiation-treated retinoblastoma patients, of whom 21 developed malignancy within the radiation field, and the data of Li *et al.* (1975) for 288 survivors of other childhood neoplasms, of whom 14 developed radiation-induced malignancy. All patients had received at least 1000 rads, but the retinoblastoma patients had received a higher dose over a smaller field. The excess of radiation-induced tumors seen in the bilateral retinoblastoma patients was composed almost entirely of osteogenic sarcomas (9 out of 21 for retinoblastoma patients in contrast to 0 out of 14 for other childhood cancers). This could be due to the amount of bone with high radiation absorption in the field of irradiation for retinoblastoma patients, or it could reflect the genetic predisposition to osteogenic sarcoma enhanced by radiation, rather than suggesting a general sensitivity to ionizing radiation.

Studies on the sensitivity of fibroblasts from hereditary retinoblastoma patients to the cell-killing effects of ionizing radiation are equivocal.

Weichselbaum *et al.* (1977, 1978, 1980) have reported a lower level of colony formation following X irradiation for a fibroblast cell line AG 1142 from a retinoblastoma patient with a deletion 13(q14q22) compared to six control lines. The D_0 for AG 1142 was 94 rads compared to a control range of 140–152 rads. Fibroblasts from six hereditary retinoblastoma patients showed a level of cell survival intermediate between AG 1142 and the controls (92–131), whereas fibroblasts from seven nonhereditary patients were on average less sensitive than the controls (D_0 135–168 rads). However, fibroblasts from a retinoblastoma patient with a deletion 13(q12q14) were normal in their sensitivity to X rays (D_0 139 rads). Fibroblasts with a deletion 12(q14-qter) from a nonretinoblastoma patient and fibroblasts trisomic for proximal 12q14 to 13pter were not X-ray sensitive, but two fibroblast strains, one trisomic for all 13q and the other trisomic for distal 13q14→13qter, were sensitive (Nove *et al.*, 1979). The authors postulate that there are two loci in band 13q14, the proximal one involved in retinoblastoma, the distal one involved in DNA repair. X-Ray sensitivity would result from deletion or duplication of the DNA repair gene. The extent of the lesion in hereditary retinoblastoma patients would determine whether or not their cells were sensitive to killing by X rays.

An increased sensitivity to cell killing following γ irradiation was also reported for AG 1142 (D_0 89 rads) and for two fibroblast strains from hereditary retinoblastoma patients (D_0 72 and 84 rads) when compared to two control lines (D_0 124 and 160 rads) by Arlett and Harcourt (1980). The

parents of one of the retinoblastoma patients had D_0 values of 92 and 98 rads. Caution must be applied, however, in the interpretation of these reports of radiosensitivity because of difficulties in defining the normal range and possible variation between biopsies from the same patient. In an earlier study that examined subjects showing abnormal responses to clinical ionizing radiation, Weichselbaum *et al.* (1976) described a control subject strain with a D_0 of 112 rads, although Weichselbaum *et al.* (1980) suggested that this result was due to variable culture conditions subsequently standardized. Arlett and Harcourt (1980) compare all lines studied to two reference lines from normal subjects; however, they also report a normal strain with a D_0 of 101 rads. Weichselbaum *et al.* (1980) suggest that their line and the lower reference line of Arlett and Harcourt (1980) may be heterozygous for one of the recessive conditions with radiosensitivity, but no one has yet (convincingly) shown such heterozygotes to be sensitive. The results of Cox and Masson (1980) show a broad distribution in X-ray sensitivity (D_0 from 98 to 160 rads) in 10 normal foetal and 34 postfoetal normal skin samples and suggest that these strains with low D_0 values reflect the normal range and that hereditary retinoblastoma patients lie within that range.

For four subjects studied by Arlett and Harcourt (1980) independently derived fibroblast strains were available, and no significant difference was seen between biopsies. Differences between biopsies were referred to by Cox and Masson (1980). A greater difference is seen in two independently derived strains of the same hereditary retinoblastoma patient reported by Weichselbaum *et al.* (1978, 1980): AG 1408 has a D_0 of 119 rads whereas AG 1980 has a normal D_0 of 140 rads.

In this laboratory survival curves following γ radiation of AG 1142 give a D_0 of 113 rads (Morten, 1980; Harnden *et al.*, 1980) which is not significantly different from seven normal strains (D_0 122–136 rads) and is within the normal range for this laboratory. D_0 values for nine hereditary retinoblastoma patients (116–140 rads) did not differ significantly from the normal range (Morten, 1980). Zampetti-Bosseler and Scott (1981) found the D_0 for AG 1142 (104 rads) within their normal range (78–101 rads) following X irradiation. No difference was found in sensitivity to the cell-killing effects of γ irradiation between fibroblasts of ten bilateral retinoblastoma patients and those of eight control subjects, nor was any difference found between affected and unaffected sibs in three families (Kossakowska *et al.*, 1982).

Survival curves for X-irradiated fibroblasts from five retinoblastoma patients with a 13q14 abnormality [three with a deletion, of which one involved loss of all 13q14, one deletion mosaic, and one t(X;13) translocation], three subjects with 13q abnormalities (but not retinoblastoma and not involving 13q14), and one trisomy 13 patient fell within the range of seven normals (Ejima *et al.*, 1982b). Plots for seven hereditary and two nonhereditary

patients had survival curves within the normal range at lower doses but appeared to be radio-resistant at doses above 600 rads. The plots were based on a single experiment per individual, and some strains showed a considerable shoulder below 400 rads. A similar shoulder is apparent on the plots of Weichselbaum *et al.* (1978) at less than 100 rads, although all points plotted are above this dose.

At the doses of radiation used in these survival experiments (<1000 rads), a major component of the lethal effects of ionizing radiation is visible chromosome damage (Carrano, 1973; Grote *et al.*, 1981). The relationship between cell killing and chromosome abnormalities is not, however, a simple one (Scott and Zampetti-Bosseler, 1982). Morten *et al.* (1981) studied the level of induced chromosome damage in lymphocytes from 11 hereditary retinoblastoma patients X irradiated in Go. After 400 rads, 10 patients showed a small increase in the number of induced rings and dicentrics compared to simultaneously irradiated normal lymphocytes. This difference was not detected at 200 rads. Although statistically significant the elevation of damage was so small as to be unlikely to be a major effect of Rb gene. Furthermore, the level of induced damage in hereditary retinoblastoma patients' lymphocytes was less than in simultaneously irradiated Down syndrome lymphocytes. Since fibroblasts from Down syndrome patients are not significantly different from normal fibroblasts in terms of X-ray cell-killing effects (Weichselbaum *et al.*, 1980), it is unlikely that the small difference between hereditary retinoblastoma patients and normal subjects would be detected on a survival curve.

Zampetti-Bosseler and Scott (1981) found no difference between AG 1142 and normal subjects in the number of chromosome bridges and fragments at anaphase or telophase, mitotic delay, or spindle defects following X irradiation.

The one fibroblast strain from a hereditary retinoblastoma patient tested for sensitivity to 313-nm UV, showed normal levels of cell survival (Smith and Paterson, 1981).

The spontaneous and MMC-induced levels of sister chromatid exchanges (SCE) in lymphocytes from two related retinoblastoma patients were the same as for the two control subjects. Unscheduled DNA synthesis following MMC was normal. After treatment with the alkylating agent *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG), a reduced level of UDS was seen in the retinoblastoma patient lymphocytes compared with treated normal subjects (Fabricant *et al.*, 1982).

Two studies on fibroblasts from hereditary retinoblastoma patients have demonstrated essentially normal DNA repair following γ rays (Woods *et al.*, 1982) and X rays (Cleaver *et al.*, 1982). Woods *et al.* found normal induction and rejoining of single and double strand breaks using alkaline elution and

nondenaturing elution, respectively. Cleaver *et al.* found normal levels of rejoining of single strand breaks, of repair replication, and of poly(ADP-ribose) synthesis. DNA replication following X irradiation of the hereditary retinoblastoma fibroblasts was not inhibited to the same extent as in normal fibroblasts, although the difference was small and is seen as increased chain elongation. This increased ability to replicate and join DNA strands opposite damaged parental strands, which would not occur in normal cells until the damage was repaired, could explain the small increased frequency of rings and dicentrics observed by Morten *et al.* (1981) and may increase the probability of mutation.

A four- to fivefold increase in the frequency of transformation by Moloney murine sarcoma virus was reported for fibroblasts from bilateral retinoblastoma patients compared with normal subjects' fibroblasts (Miyaki *et al.*, 1983). Fibroblasts from hereditary unilateral patients showed an approximately twofold increase, whereas those from nonhereditary unilateral retinoblastoma patients and retinoblastoma patients with chromosome 13 abnormalities, including those with bilateral retinoblastoma, had normal levels of transformation. These observations are concordant with the suggestions of Matsunaga (1978, 1980b) that the genetic background of the Rb gene effects its expression and that abnormality of chromosome 13 may be a different oncogenic mechanism to the Rb gene. Furthermore, they imply that the host-resistance mechanism is, to some extent, dependent on innate properties of the cell rather than on the cellular environment.

3. *Gardner Syndrome and FPC*

Although there is no clinical evidence suggesting an abnormal sensitivity to carcinogenic agents in patients with FPC or Gardner syndrome, the apparent preneoplastic condition of cells from these syndromes has led to a number of experimental studies on the effects of oncogenic agents on such cells.

Little *et al.* (1980) found a reduction in the colony-forming ability of fibroblasts from two related patients with Gardner syndrome compared with normal subjects following X irradiation, 254-nm UV, and MMC. The D_0 values following X irradiation were 102 and 111 rads and, as discussed previously, may be in the lower part of the normal range. Sensitivity to UV (D_0 values of 1.2 and 1.4 J/m^2 compared with 2.7 and 2.9 J/m^2 for controls) was not as great as xeroderma pigmentosum group A fibroblasts (D_0 0.8 J/m^2) and was not affected by postirradiation incubation with caffeine. Repair of potentially lethal damage was almost completely defective following UV irradiation and was slower following X rays in the Gardner syndrome patients' fibroblasts than in controls. Unscheduled DNA synthesis was normal after UV. This result is interesting since it is unusual for cells of syndromes

associated with a DNA repair defect to be sensitive to more than one type of DNA damage, although fibroblasts from Rothmund–Thompson syndrome, an autosomal recessive condition exhibiting sun sensitivity and predisposition to malignancy, were found to be abnormal in their response to both X irradiation and near UV (Smith and Paterson, 1979).

Barfknecht and Little (1982) extended their study by examining the cell-killing effects of methylmethane sulfonate (MMS), ethylmethane sulfonate (EMS), MNNG, and 4-nitroquinoline 1-oxide (4NQO) on fibroblasts from a patient with Turcot syndrome and an FPC patient as well as from the two previously studied Gardner syndrome patients. They suggest that the Gardner syndrome cells were abnormally sensitive to MMS and EMS; the FPC cells to MMS, EMS, and 4NQO; and the Turcot syndrome cells to MMS. However, in some cases the sensitivity was seen in the shape of the curve (D_{10}) but not in its exponential slope (D_0).

Sensitivity to MMC was also reported by Sasaki (1978) for Gardner syndrome, FPC, and Peutz–Jegher syndrome cells. Miyaki *et al.* (1980a) found FPC fibroblasts sensitive to MMC and 4NQO but not to UV or MNNG, while Peutz–Jegher fibroblasts had normal sensitivity to all of these agents. Kopelovich and Sirlin (1980) refer to normal repair of UV and X-ray damage in FPC fibroblasts.

MNNG treatment during late S and G_2 produced approximately twice as many breaks, mainly chromatid gaps and deletions, in fibroblasts from four FPC patients compared with four normal subjects and one Peutz–Jegher syndrome patient (Hori *et al.*, 1980). The spontaneous level of damage was normal. The two FPC strains tested also showed greater induced mitotic delay compared with two normal strains.

Treatment of a fibroblast strain (PF) from an asymptomatic FPC carrier with MNNG resulted in morphologically transformed cells having increased saturation density and MNNG resistance which formed colonies in soft agar but which were not tumorigenic in nude mice (Rhim *et al.*, 1980). Similar results were obtained with fibroblasts from two FPC patients treated with 4NQO and MNNG (Miyaki *et al.*, 1980b, 1982). Morphologically transformed cells acquired chromosomal abnormalities while at the same time being able to form colonies in soft agar.

Increased sensitivity of FPC fibroblasts to transformation by Kirsten (Ki) and Moloney (Mo) murine sarcoma virus (MuSV) (Pfeffer and Kopelovich, 1977; Rasheed and Gardner, 1981; Miyaki *et al.*, 1980c) and by SV40 virus (Kopelovich and Sirlin, 1980) has been reported.

Pfeffer and Kopelovich (1977) report a 100 to 1000-fold increase in sensitivity to transformation by KiMuSV in 10 FPC patients and in 6 out of 9 asymptomatic progeny. KiMuSV infected cells from FPC patients and asymptomatic patients with a high susceptibility to transformation had a

lower serum requirement, produced colonies in methocel at a higher frequency than infected normal fibroblasts, and produced tumors intradermally in *nude* mice. No difference was found in viral adsorption or in viral replication between FPC and normal fibroblasts.

Four out of five lines of fibroblasts from Gardner syndrome patients demonstrated a 100 to 1000-fold increased sensitivity to transformation by KiMuSV compared with normal subjects, and two out of three strains from FPC and the remaining Gardner syndrome strain showed a 10 to 100-fold increased sensitivity (Rasheed and Gardner, 1981). Variation in the susceptibility of asymptomatic relatives was detected, but a high frequency of transformation was detected in fibroblasts from two asymptomatic relatives in unaffected branches of the Gardner syndrome and FPC families. In another study Miyaki *et al.* (1980c) reported that as much as a fivefold increase in the number of transformed foci was seen in fibroblasts of 20 FPC patients, 2 Gardner syndrome patients, and 5 Peutz–Jegher syndrome patients compared with 7 normal strains, following MoMuSV infection. Again nonmanifesting relatives' fibroblasts showed high and low sensitivities.

Increased SV40 T-antigen positive cells and focus production was reported following SV40 infection of fibroblasts from Gardner syndrome and FPC patients (Kopelovich and Sirlin, 1980). Although this generally correlated with the presence of a "preneoplastic" phenotype, a high level of T-antigen production was seen in cells from one clinically asymptomatic Gardner syndrome relative who was normal by the authors' other criteria. The transformed foci were not tumorigenic in athymic mice and they had a finite life span. SV40-adeno 12 hybrids gave similar results to KiMuSV (Rasheed and Gardner, 1981).

Fibroblasts from ACR patients treated with the tumor promotor 12-*O*-tetradecanoylphorbol-13-acetate (TPA) and inoculated into the anterior chamber of the eye of *nude* mice have been reported to produce moderately differentiated fibrosarcomas (Kopelovich *et al.*, 1979); however, the human origin of the cells growing in the eye has yet to be established (Kopelovich, 1982). The histology of the nodules is not convincingly malignant, and it is clear that the TPA treatment does not produce stable anchorage independence nor infinite lifespan on these cells. Antecol and Mukherjee (1982) were unable to reproduce the results of Kopelovich *et al.*

It is hard to reconcile all these various reports of sensitivity of Gardner cells or FPC cells to environmental agents either to cell killing or cellular transformation (Table IV). A similar situation arose in the study of the recessive disease ataxia-telangiectasia; gradually it became apparent that there was general agreement on sensitivity to a relatively small number of agents. It is probably better to reserve judgment on FPC and Gardner syndrome until such a concensus also emerges for these.

TABLE IV
SENSITIVITY TO ENVIRONMENTAL AGENTS OF CELLS FROM PATIENTS WITH POLYPOSIS[a]

Agent	Familial polyposis coli	Gardner syndrome	Peutz–Jegher syndrome	Turcot syndrome	Reference
		1. Sensitivity to cell killing			
X rays		+			Little *et al.* (1980)
UV		+			Little *et al.* (1980)
Mitomycin-C		+			Little *et al.* (1980)
	+	+	+		Sasaki (1978)
	+		−		Miyaki *et al.* (1980a)
EMS	+	+		−	Barfknecht and Little (1982)
MMS	+	+		+	Barfknecht and Little (1982)
MNNG	−	−		−	Barfknecht and Little (1982)
	+		−		Miyaki *et al.* (1980b)
	+		−		Hori *et al.* (1980)
4NQO	+	−		−	Barfknecht and Little (1982)
	+		−		Miyaki *et al.* (1982)
		2. Sensitivity to transformation			
Kirsten MuSV	100–1000×				Pfeffer and Kopelovich (1977)
	10–100×	100–1000×			Rasheed and Gardner (1981)
Moloney MuSV	5×	5×		5×	Miyaki *et al.* (1980c)
SV40	+	+			Kopelovich and Sirlin (1980)
TPA	+				Kopelovich *et al.* (1979)
	−				Antecol and Mukherjee (1982)
MNNG	+				Rhim *et al.* (1980)
	+				Miyaki *et al.* (1980b)
4NQO	+				Miyaki *et al.* (1980b)

[a] +, sensitive; −; tested but not sensitive; 5×, fivefold more sensitive than controls. Blank spaces indicate not tested.

4. *Other Dominant Syndromes*

There is some clinical evidence for sensitivity to environmental agents in patients with dysplastic nevus syndrome. Reimer *et al.* (1978) reported sun sensitivity in six patients, facial melanoma following UV treatment for acne

in two patients, and melanoma developing at the site of a hair-setting solution burn in another patient.

Smith *et al.* (1982) used survival curves to investigate UV sensitivity in fibroblasts from six patients with hereditary cutaneous malignant melanoma and dysplastic nevus syndrome. Responses ranged from the lower end of the normal range to a twofold increase in sensitivity. Sensitivity to γ-ray-induced cell killing was normal as were repair synthesis, inhibition, and recovery of DNA synthesis after γ and UV irradiation. The role of UV radiation in the development of melanoma in these patients is unclear, and the wavelength used in this study (254 nm) is not environmentally relevant because of atmospheric absorption.

Two families with melanoma showed a dominantly inherited sensitivity to UV light as measured by the colony-forming ability of EBV lymphoblastoid cell lines (Ramsey *et al.*, 1982). These patients did not have dysplastic nevus syndrome and not all UV-sensitive subjects in the family had melanoma. Lines from sporadic melanoma patients were not UV sensitive. Sensitive cells showed normal DNA repair synthesis, unscheduled DNA synthesis, inhibition of DNA replication, and sister chromatid exchange.

Four fibroblast lines from three patients with tuberous sclerosis were studied for sensitivity to γ radiation (Paterson *et al.*, 1982). Sensitivity compared with normal strains varied according to whether irradiation was under oxic or anoxic conditions and whether D_0 or D_{10} was used as a measure of radiation sensitivity. Using D_{10} values three strains from two patients were significantly more sensitive than normal strains.

Two fibroblast strains from a neurofibromatosis patient showed normal γ-ray sensitivity (Paterson *et al.*, 1979).

A family with an apparently autosomal dominant condition of sensitivity to sunlight and facial senile keratosis and basal and squamous cell carcinomas was reported by Cleaver *et al.* (1981). Fibroblasts from the proband had normal incision steps of excision repair and normal postreplication repair.

D. Effect of Dominant Cancer Susceptibility Genes on Development and Cellular Differentiation

1. Introduction

Thus far we have mainly considered the idea that a gene predisposed to cancer acts either by increasing the susceptibility of gene-carrying cells to oncogenic agents or by rendering the cells liable to further change if certain specific genomic events occur. The production of abnormal populations of cells either during development or differentiation could be another mechanism by which these genes express their effect.

It is characteristic of this group of dominant conditions that the tissue in which the cancer arises shows some form of hyperplasia or other abnormal behavior prior to the recognition of frank malignancy. Retinoblastoma is exceptional since there is no evidence of preneoplastic tissue hypertrophy. Abnormalities of this kind are always focal so that most cells, even in the affected differentiated tissue which carry the gene, behave quite normally. It is this fact which makes the idea, that some other event must occur to produce the abnormal behavior, so attractive. Since the abnormal proliferative behavior is tissue specific this strongly suggests that the genotypic environment is vital in the expression of the abnormal phenotype. The change in the abnormal tissue may itself constitute a preneoplastic phenotype with the cells showing one or more of the properties of malignant cells. Alternatively, the gene may not produce cells which have a defect directly on the pathway to malignancy but may cause an abnormally differentiated tissue in which there is an increased probability of a malignant cell arising or progressing. These possibilities are not mutally exclusive and there is some evidence for both mechanisms.

There is an association between some kinds of developmental abnormalities and cancer, e.g., aniridia with Wilms tumor and hypoplasia of the thumbs and radii in Fanconi anemia with acute myeloblastic leukemia. This field has been reviewed by Bolande (1977). In the case of the dominant cancer susceptibility genes, however, the majority of gene carriers in all of the disorders appear normal at birth.

2. *The Occurrence of Abnormalities in the Differentiated Tissue in Which Neoplasia Arises*

a. Retinoblastoma. There is some evidence for the involvement of the retinoblastoma gene in retinal differentiation. Although it may or may not be the location of the Rb gene, chromosome 13 is clearly involved in the development of retinoblastoma (Section III,B,1). One of the features of trisomy 13 is retinal dysplasia which may resemble or give rise to retinoblastoma (Allen *et al.*, 1977). Retinal dysplasia was also seen in a five-day-old child with 13q-mosaicism; the dysplastic retina contained rosettes around large undifferentiated cells but no retinoblastoma (Weichselbaum *et al.*, 1979). Hittner *et al.* (1980) reported pronounced atrophy of rod outer segments with retention of cone outer segments, vacuolation of the photoreceptors, and absence of synaptic connections between photoreceptors, bipolar cells, and horizontal cells in the outer plexiform layer in a retinoblastoma patient with a congenital 13q deletion (Riccardi *et al.*, 1979). This suggests that there are genes on chromosome 13 which are involved in retinal development.

There are a few studies which have shown abnormalities of the nonmalignant retina of retinoblastoma patients which suggest that the retina in which

a retinoblastoma develops is abnormal and possibly incompletely differentiated (Uga *et al.*, 1978; Nakao, 1978; Nakao *et al.*, 1974). However, it is difficult to assess whether these abnormalities are a direct effect of the Rb genes or whether they are due to the tumor or to the degenerative effects of retinal detachment.

Mitotic figures were seen in nontumorous retina (Teng and Katzin, 1955, Nakao, 1978) These could be seeded tumor cells or new lesions, though ultrastructurally they resemble Muller cells (Nakao, 1978).

Examination by light microscopy of the nontumorous retina in 25 cases of retinoblastoma showed no structural abnormalities (R. W. Cox, personal communication), although it is not known how many cases were hereditary. The nonmalignant retina of two hereditary retinoblastoma patients and one sporadic unilateral patient (1-month-old at diagnosis and probably hereditary) was also normal. However, another sporadic unilateral patient (also diagnosed at 1 month) showed the rods to be entirely absent; the unaffected eye of this patient had normal vision (D. R. Barry, personal communication).

Nonprogressive retinal lesions have been reported in retinoblastoma patients and in unaffected Rb gene carriers but may also occur sporadically. They have recently been termed *retinomas* (Gallie *et al.*, 1982). Smith (1974) and Gallie *et al.* (1972) concluded that these structures did not result from ischemia, and Smith (1974) suggested that they represented tumors which had regressed due to maturation. Gallie *et al.*, (1982) suggest the attractive hypothesis that they result from the "second event" occurring in a retinal cell nearer to terminal differentiation than that which would give rise to retinoblastoma.

Retinal disruption can also produce retinoblastoma-like structures. Ohnishi (1977) produced both Homer–Wright and Flexner–Wintersteiner rosettes by rotation of cultures of dissociated chick embryo neural retina. Goldstein and Wexler (1931) reported rosettes in the eyes of five human fetuses irradiated at 2 months, when the presumptive retinal cells could have separated into the inner and outer neuroblastic layer, and terminated at 3–7 months. Morten (1980) produced nonmalignant tumor-like masses following inoculation of immature rat neural retinal cells into the vitreous of older syngeneic rats. However, additional events other than structural or temporal disruption must be needed in these cases to produce a malignant tumor.

The association between retinoblastomas and pineal tumors is of interest since in lower vertebrates the pineal has a photoreceptor function. Differentiated structures observed in pinealoblastoma resembled rosettes and fleurettes seen in retinoblastoma (Stefanko and Manshot, 1979; Herrick and Rubinstein, 1979). As with the genes for neural crest-related tumors, it is not

surprising that a gene for retinal tumors should also predispose to tumors in an embryologically related organ, especially if it acts through an abnormality of differentiation. The association between retinoblastoma and osteogenic sarcoma is difficult to explain, although it is of interest that the embryologically related pigment epithelium, derived from the outer cell layer of the optic cup, is capable of bone formation.

b. FPC and Gardner Syndrome. In normal colon, proliferation of the epithelial cells is largely confined to the basal and middle regions of the crypts. DNA synthesis and mitosis are suppressed as the cells migrate toward the mouth of the crypts. In contrast, cells in the surface mucosa of patients with FPC fail to repress DNA synthesis as determined by incorporation of tritiated thymidine in short-term *in vitro* culture. Since this has been observed in apparently normal mucosal epithelial cells of FPC patients as well as in the polyps and also in symptom-free members of FPC families, this abnormality cannot be due to the abnormal cellular arrangement in the polyps. An abnormal labeling pattern is sometime seen in the mucosa of control subjects with no gastrointestinal problems and has also been observed at a higher frequency in patients with a single adenomatous polyp or villous papilloma (Deschner and Lipkin, 1975) or primary colon cancer (Maskens and Deschner, 1977). Cells in which DNA synthesis has not been repressed accumulate to produce adenomatous or villous polyps from which carcinomas arise. This field has been reviewed by Lipkin (1978).

The polyps are composed of proliferating, nonterminally differentiated cells in an abnormal cellular environment. The number of target cells for malignant transformation increases with time, thus when the age-specific incidence of tumors (P) in polyposis and normal subjects is plotted against time, as was done for retinoblastoma (Section III,A,1), the effect of the single mutation in FPC on the function $P = kt^r$ is to alter the range of values of r from 6–7 to 4–5 (Knudson, 1977).

Not all types of gastrointestinal polyps give rise to malignant cells. The polyps in juvenile polyposis are generally hamartomatous and are not linked to carcinoma (Erbe, 1976), although Goodman *et al.* (1979) reported a rectal adenocarcinoma arising among polyps in a patient who also had gastric polyps. This patient had adenomatous polyps and large adenomas but lacked the minute adenomas seen in patients with polyposis coli. Adenomatous juvenile polyps associated with malignant transformation were also reported by Rozen and Baratz (1982). That the differentiation pattern of the polyps is important is suggested by the observations of Spjut and Estrada (1977) who considered that adenocarcinoma or carcinoma *in situ* only arose in lesions which had villous as well as adenomatous components. Trau *et al.* (1982) reviewed 12 cases of Peutz–Jegher syndrome in which malignancy probably

arose from a hamartomatous polyp. However, gastrointestinal tumors in these patients tend to be in the stomach, duodenum, and colon, whereas polyps tend to develop in the jejunum and ileum.

Delhanty and Davis (1981) reported a pattern of isozymes in the polyps and carcinomas of polyposis coli resembling that of the fetus or liver rather than that of the adult colon.

c. BCNS. The nature of many of the abnormalities associated with BCNS inevitably means they are present at birth, for example, the musculoskeletal abnormalities, the facial characteristics, the calcification of the dura, etc. In a few patients congenital forms of hydrocephalus and blindness have been seen. The gene must therefore influence events during morphogenesis. Only in rare cases are multiple basal cell tumors present at birth (Kahn and Gordon, 1967). It is more usual for the BCCs to be recognized in early adulthood, although they do occasionally appear in children. It is unclear whether or not the basal cell nevi are present at birth since BCNS is rarely diagnosed at birth. The distinction between the nevi and the basal cell carcinomas is unclear; from the earliest stage the pathology resembles classic BCC (Mason *et al.*, 1965). Cells of the nevus may grow into the dermis so that the lesion may resemble premalignant fibroepithelioma. A fibrous stroma may be induced in the dermis but the reticular dermis is not usually invaded at early stages. The lesions may be pigmented, and calcification is not uncommon. Since any or all of the lesions of BCC may occur in the nevoid tumors, it is not possible to say whether the malignant tumor is preceded by a local benign lesion.

Interestingly, the great majority, if not all, of the abnormalities associated with this syndrome are observed (albeit at a much lower frequency) in the normal population. The BCNS gene, therefore, increases the risk of carriers developing a whole range of abnormalities which also occur sporadically suggesting that the function of the normal allele of the BCNS gene may be critical for normal development in many different tissues.

d. Dysplastic Nevus Syndrome. Again the preneoplastic lesion is the main feature of the syndrome. While normal acquired melanocytic nevi have a regular outline and orderly melanocytes and the dermal component may show neurotization, dysplastic nevi have a uniformally cellular dermis without neurotization and an irregular outline. The intraepidermal melanocytic component is similar to normal nevi. However, regions, particularly of the larger dysplastic nevi, show distinctive abnormal histological changes including intraepidermal abnormal melanocytic hyperplasia and dermal fibroplasia with lymphocyte and macrophage infiltration, neovascularization, and large melanocytes. The abnormal melanocytes are large, spindle-shaped or sometimes epithelioid cells with fine melanin granules filling the cytoplasm. They are mitotically active and show some structural features of malignant

melanocytes but tend to be located at the junction of the dermis and epidermis (Clark *et al.*, 1978; Greene and Fraumeni, 1979).

There is direct evidence of malignant melanoma arising from large dysplastic nevi (Reimer *et al.*, 1978; Clark *et al.*, 1978; Lynch *et al.*, 1980), although they are not essential for the development of melanoma as evidenced by Clark *et al.* (1978) who refer to two members of dysplastic nevus syndrome families who developed malignant melanoma but not dysplastic nevi. The dysplastic nevi can therefore be regarded as preneoplastic lesions in which there is an increased probability of malignancy arising.

Since the atypia of the dysplastic nevi appear focally among the larger nevi, Clark *et al.* (1978) suggest that these arise during the progression of the nevus rather than being a direct result of the autosomal dominant gene. Both hereditary and sporadic cutaneous melanomas will arise from preexisting nevi (Greene and Fraumeni, 1979). A gene could predispose to malignancy by increasing the probability of malignant transformation within these lesions. However, the differences in number, body distribution, and pattern of onset with age between the dysplastic nevi and the normal acquired melanocytic nevi indicates that the gene affects the occurrence of the nevi as well as affecting the probability of malignant progression. The nature of the defect leading to dysplastic nevus formation is not known.

e. Neurofibromatosis. The observation that fibrosarcomas and neurofibrosarcomas arise at the site of preexisting neurofibromas in both neurofibromatosis patients and patients with solitary neurofibromas (Knight *et al.*, 1973) indicates the premalignant nature of these lesions. Von Recklinghausen disease has been classed as one of the phacomatoses—a group of disorders, originally an association of skin malformation with eye defects, in a class somewhere between congenital malformations and tumors (Van der Hoeve, 1932). However, it has also been classed as one of the hamartoses, i.e., lesions which begin as systemic tissue malformations but which tend to develop into tumors (Warkany, 1977), or as having a neural crest origin (Schimke, 1977). This latter view is favored by a majority of workers in the field. A neural crest origin is supported by the association of neurofibromatosis with pheochromocytoma, with agangliosis of the distal colon, and possibly with neuroblastoma and ganglioneuroma, which may indicate a defect in neural crest cell migration or differentiation. However, Hope and Mulvihill (1981) point out that some tumors occurring in excess in these patients, such as Wilms tumor and juvenile chronic myeloid leukemia, are clearly not of neural crest origin while some cancers of known neural crest origin, such as melanoma, do not appear to occur in excess.

Localized neurofibromatosis has been reported and reviewed by Miller and Sparkes (1977). This can be explained by somatic mutation in a neural crest cell which subsequently populates a particular segment. One case of

Nicholls (1969) had a mediastinal neurofibroma and five neurofibromata in the segmentally corresponding skin. The cellular origin is of considerable interest since study of 14 neurofibromas from 2 glucose 6-phospate dehydrogenase (G-6PD) heterozygotes with neurofibromatosis showed both A and B enzymes present in the same pattern as the normal skin of the patient (Fialkow *et al.*, 1971). Histology showed that the material assayed was composed of 90–95% tumor cells. The authors suggest that the starting number of cells in a neurofibroma is at least 150 but may be as high as several thousand. Friedman *et al.* (1982) have shown that, while benign tumors of a neurofibromatosis patient heterozygous for G-6PD were of mixed phenotype, one primary neurofibrosarcoma and four metastases were all of the A phenotype; this strongly suggests that a single clone had given rise to the malignant tumor.

Further evidence of widespread involvement of neural crest cells comes from a study of the café-au-lait spots and "freckles" of neurofibromatosis patients. Such lesions contain a higher density of DOPA positive melanocytes than does the surrounding skin, and the melanocytes contain giant pigment granules. In contrast, café-au-lait spots on subjects without neurofibromatosis have fewer melanocytes than does the surrounding skin and do not have giant pigment granules. More melanocytes than expected were found in the normal pigmented skin of the neurofibromatosis patients (Johnson and Charneco, 1970).

Riccardi (1977) has suggested that the expression of the transformed phenotype may be masked by the proximity of normal cells and that this leads to a mosaic expression of the phenotype. Only when an adequate number of transformed cells are in close proximity will a neoplastic lesion develop.

It has been suggested by Schenkein *et al.* (1974) that there is an excess of nerve growth factor (NGF) in patients with Von Recklinghausen disease. It now seems clear that while elevated levels of NGF cross-reacting protein are found in the sera of a high proportion of patients who have CNS involvement (with or without peripheral involvement) and in the sera of a lower proportion of patients with peripheral disease only, there is no excess of NGF physiological activity in the positive serum, suggesting that an abnormal molecule may be produced in these cases (Rubenstein *et al.*, 1981).

f. Multiple Endocrine Neoplasia Type 2. Medullary thyroid carcinoma in MEN 2 is derived from the parafollicular C cell, which has a neural crest origin as have the cells of the adrenal medulla that give rise to pheochromocytoma. More specifically these cells belong to the "APUD" series characterized by their ability to take up amine precursors, utilize L-aromatic amino acid decarboxylase, and synthesize and secrete amine neurotransmitters and specific peptide hormones (Pearse and Polak, 1974).

From a study of the adrenal glands of 19 MEN 2 patients, Carney *et al.* (1976) suggested that diffuse or nodular medullary hyperplasia precedes development of pheochromocytoma. Multifocal C cell hyperplasia was detected in three subjects at risk of medullary thyroid carcinoma (Wolfe *et al.*, 1973). There was no evidence of tumor and no abnormal nuclei or vascular invasion but the thyroid showed increased numbers of C cells and clusters of C cells not seen in control thyroids.

A clonal origin was demonstrated in both medullary thyroid carcinoma cells and multiple samples of pheochromocytoma from a MEN 2 patient heterozygous for G6PD (Baylin *et al.*, 1976). Study of three more G6PD heterozygotes with inherited medullary carcinomas also indicated a monoclonal origin. One tumor appeared to consist of several separate nodules of different G6PD phenotypes (Baylin *et al.*, 1978). The primary effect of the MEN 2 gene could be the occurrence of multifocal hyperplasia from which a single cell will progress to an overt tumor by somatic mutation or epigenetic events.

The hyperplastic and tumor cells are functional. The patients of Wolfe *et al.* (1973) showed an abnormally elevated serum calcitonin level in response to infused calcium, and in the early stages medullary thyroid carcinomas may be otherwise asymptomatic. Likewise pheochromocytoma may lead to excess catecholamine production but some are asymptomatic (Schimke, 1977).

The third component of MEN 2, parathyroid hyperplasia, is discussed by Schimke (1977) and Baylin (1978). It has not been demonstrated that these cells have a neural crest origin, unlike other tumors associated with MEN 2 (gliomas, glioblastomas, and meningiomas), but it is possible. Parathyroid hyperplasia is not a usual feature of medullary thyroid carcinoma (Block *et al.*, 1975), although it is possible that in MEN 2 it is induced by early elevation in calcitonin levels resulting from C cell hyperplasia. However, Melvin *et al.* (1972) reported increased levels of parathyroid hormone in members of MEN 2 families who had no excess calcitonin, and in MEN 3 parathyroid hyperplasia does not occur despite the presence of medullary thyroid carcinoma and elevated calcitonin. Baylin (1978) suggested that if parathyroid hyperplasia is a primary effect of the MEN 1 and MEN 2 genes, then the thyroid lesions (MEN 1 and MEN 2) and the pancreatic islet lesions (MEN 1) could result from hypercalcemia.

Finally, it is of interest to note the large number of autosomal dominant genes which predispose to pheochromocytoma often with other tumors of neural crest origin. There is an autosomal dominant gene for simple pheochromocytoma. In addition, pheochromocytoma is associated with neurofibromatosis; with MEN 2; with medullary thyroid carcinoma, mucosal neuromas, neurofibromas, and café-au-lait spots (MEN 3); with pancreatic

cysts, papillary cystadenomas, and islet cell tumors, renal cell carcinoma, liver cysts, and hemangioblastomas of the retina and cerebellum (von Hippel–Lindau syndrome), and with carotid body tumors.

3. *In Vitro Properties of Cells*

Pfeffer *et al.* (1976) reported that 13 skin fibroblast strains from FPC patients grew in multilayers like embryonic fibroblasts; they had a 20–40% higher saturation density than normal adult fibroblasts and proliferated in medium with 1% fetal calf serum. Fibroblasts from some asymptomatic family members behaved in the same way. While Rasheed and Gardner (1981) found no abnormality in the morphology of fibroblasts from five Gardner syndrome and three FPC patients, they did confirm that the saturation density of fibroblasts from FPC patients was approximately twice that of normal fibroblasts and that the plating efficiency in medium with 1% fetal calf serum was higher for FPC cells. Miyaki *et al.* (1980c, 1982) reported no difference in the growth rate of FPC and normal fibroblasts in medium containing 1% fetal calf serum but a 10–20% increase in saturation density and a longer lifespan for FPC fibroblasts.

Additional differences between FPC and normal fibroblasts are reviewed by Kopelovich (1982). The plating efficiency of FPC cells in agar was not increased by hydrocortisone, unlike that of normal fibroblasts. A decrease in the percentage of cells with actin cables was reported in five out of seven lines of FPC fibroblasts (Kopelovich *et al.*, 1980). Elevated levels of plasminogen-dependent protease were observed in FPC cells, and the potential across the mitochondrial membrane of FPC cells was different from normal. Heim (1983), however, found no difference between fibroblasts from FPC and Gardner patients as compared with normal controls with respect to plating efficiency, cloning efficiency, cultural morphology, anchorage, dependency, and contact inhibition at saturation density.

It is unclear how (if at all) changes in properties in fibroblasts could relate to the abnormal cell proliferation pattern which in the case of FPC is restricted to the intestinal mucosa. If the primary effect of the FPC involves control of cell proliferation, it must be that the activity of this particular gene is less important in other tissues or else it is not expressed. The benign tumors of the skin in Gardner syndrome could be a less extreme manifestation of the defective control of proliferation, but in this case, the effect of the gene is not confined to one tissue. It is also possible that failure to control proliferation could make the occurrence of cytogenetic changes more probable and/or increase sensitivity to transforming agents.

Growth characteristics of fibroblasts from normal and neurofibromatosis subjects were compared by Krone *et al.* (1981). Although lines of fibroblasts derived from neurofibromatosis patients showed a capacity to grow in 1%

fetal calf serum-supplemented medium more readily than did control strains (as measured by the number of population doublings, saturation density, or ratio of initial to final amounts of protein), there was high intrastrain variability and some differences between strains could reflect the variablity of the biopsy site in the neurofibromatosis patients.

Kossakowska *et al.* (1982) demonstrated a small increase in the ability of fibroblasts from bilateral retinoblastoma patients to grow in 0.5% FCS-supplemented medium compared with the same aged controls.

E. The Involvement of Viruses in Dominant Cancer Susceptibility

A vertically transmitted virus could mimic the effects of an autosomal dominant gene. Alternatively, a gene could predispose to cancer by making its carrier more susceptible to the effect of an oncogenic virus. We have already considered the relationship between animal tumor viruses and dominant inheritance.

The only one of this group of diseases in which the role of virus in the etiology of the disease has been seriously considered is retinoblastoma. Even in retinoblastoma, however, there is no direct evidence for a viral origin. Albert *et al.* (1974) found no evidence of viral particles in electron micrographs of 50 tumors. Suckling and Fitzgerald (1972) found no evidence for time/space clustering in retinobastoma cases in New Zealand over a 20-year period, although this does not rule out susceptibility to a ubiquitous virus.

Although RNA-dependent DNA polymerase-like activity has been detected in extracts of 10 retinoblastomas and in the Y79 retinoblastoma cell line, such activity has also been demonstrated in fetal, though not in adult, retina. It is not certain whether this activity was viral or fetal in origin, and it may vary with the degree of tumor differentiation (Albert and Reid, 1973; Reid *et al.*, 1974; Reid and Russell, 1974).

Retinoblastoma is not known to occur naturally in species other than man (Albert *et al.*, 1974); however, Albert *et al.* (1977) induced a retinoblastoma-like tumor in an eye of a newborn kitten inoculated with feline leukemia virus. This could suggest an effect on a critical stage of eye development rather than a specific virus effect. A more extensively studied *in vivo* system is the production of retinal tumors following inoculation of human adenovirus 12 into the eyes of newborn to 7-day-old rats, newborn inbred mice, and newborn baboons (Mukai and Murao, 1975; Mukai *et al.*, 1977, 1980; Mukai and Nishida, 1978). These tumors histologically and electron microscopically resembled relatively undifferentiated retinoblastomas except those induced in baboons, which had Flexner–Wintersteiner rosettes (Mukai *et al.*, 1977, 1980).

Human adenovirus 12 has a gross cytopathic effect on cultured human embryonic retinal cells (Morten, 1980). However, using cloned DNA of the left-hand end of adenovirus 12 (map units 0–15.5), Byrd *et al.* (1982) transformed these cells to give a line tumorigenic in *nude* mice with differentiation structures like those seen in retinoblastoma. No evidence was obtained for the presence of adenovirus 12 DNA or RNA in 6 lines from retinoblastomas (Mak *et al.*, 1982) or for T-antigen in cells from four tumors (Morten, 1980).

F. Immunological Mechanisms

Unlike the recessive disorders that have immune deficiency as a feature (sometimes the main feature) of cancer susceptibility, there is little evidence of immunological deficiency in this group of dominant disorders.

A decreased response in one-way mixed lymphocyte reaction and low T and B lymphocyte levels was seen in four families prone to cutaneous malignant melanoma (Dean *et al.*, 1979). However, this applied to unaffected blood relatives and spouses as well as to the melanoma patients and carriers of precursor nevi and therefore cannot be due to the gene associated with melanoma, although it may affect malignant progression. This is not a feature of melanoma-susceptible patients.

Bertrams *et al.* (1973) suggested that the histocompatibility antigen HLA BW35 or absence of B12 could increase susceptibility to retinoblastoma, although there was no clear correlation between HLA haplotype and retinoblastoma. However, Gallie *et al.* (1977) could not confirm this association nor was any correlation found between HLA types and spontaneous regression. Jones (1974) found no association between HLA haplotype or mixed lymphocyte reactivity and retinoblastoma in a large family.

Lynch *et al.* (1975) found an association between $HLAA_2$/A12 in a cancer family predisposed to carcinoma of the endometrium and colon. In one cancer prone branch 20 out of 21 patients were $HLAA_2$/A12.

IV. Conclusions

This survey of dominantly inherited cancer susceptibility and the consideration of the general nature of dominant inheritance allows a number of tentative conclusions to be reached.

1. There is no reason to suppose that, in terms of the genetic lesion at the DNA or chromosome level, dominant genes for cancer susceptibility will prove to be any different from other dominant or recessive genes, and they

may in fact be base changes, frame shifts, deletions, inversions, duplications, etc.

2. Implicit in the nature of dominance and so unlike recessive genes, the expression of the gene will be critically dependent on the response of the normal allele to the presence of a gene with abnormal function, and also to somatic damage to, or malfunction of, the normal allele. Expression will depend either on (1) interference with normal structure or function by a product of the abnormal gene or on (2) absence of dosage compensation because of failure of the normal allele to respond to the abnormal intracellular environment or because of damage to the normal allele.

3. There is little evidence that members of this group of genes are expressed in an overt way during embryogenesis. The exception is basal cell nevus syndrome for which there is clear evidence of morphogenetic abnormalities. The possibility that a less obvious effect, such as the production of a relatively small number of imperfectly differentiated cells within a specific tissue, cannot be ruled out in the other syndromes.

4. Again, with the exception of BCNS, these genes, in their nonneoplastic effects, do not directly affect multiple systems of the body. Where multiple system involvement appears to occur, it is usually (1) indirect, e.g., the neurological signs in neurofibromatosis, or (2) the result of cells of common origin distributed throughout many systems of the body, e.g., the neural crest cells in MEN 2. It is reasonable to conclude that very precise tissue specificity is a feature of these diseases. Nevertheless some unexpected combinations of tumors not readily explained by common embryological origin do occur, e.g., retinoblastoma and osteosarcoma. A detailed knowledge of the developmental biology of these systems may eventually provide an insight into the mechanisms of such associations.

5. It is characteristic, however, that the majority of cells in the susceptible tissue do not normally show evidence of the effect of the gene, e.g., the majority of the retinal cells in a patient with inherited retinoblastoma are normal. Thus, another characteristic is that the diseases are focal in origin. Though the number of foci may be quite high in some cases, it is still small relative to the number of cells at risk.

6. These focal abnormalities usually precede the occurrence of overt malignancy. Retinoblastoma is again exceptional since only occasionally are there clear retinal abnormalities prior to malignancy. It is unclear when the malignant change actually occurs in BCNS, but the age of patients with recognized malignancies suggests a prolonged premalignant phase.

7. In several of these syndromes there is some evidence that cells, not necessarily from the target tissue, may respond to the usual conditions of cell culture in a different way to normal cells and/or may show abnormal re-

sponses to external, potentially carcinogenic, stimuli. These abnormalities seem difficult to reproduce and are certainly not as clear cut and dramatic as the abnormal responses reported for the recessive diseases, such as A-T and XP.

8. Several hypotheses have been put forward to explain the action of these genes in producing focal, tissue-specific cancer susceptibility. These ideas overlap to some extent and may not be mutually exclusive. All have certain features in common (1) all assume that the primary effect of the gene on the differentiated tissue is *not* to cause cellular transformation; (2) all require further events which occur in only a (perhaps very small) proportion of the cells of the differentiated tissue (i.e., it is not cancer but susceptibility that is inherited); and (3) all, therefore, assume that the process of carcinogenesis is multistep.

9. The main differences among the hypotheses concern whether or not the inherited mutation is itself considered as part of this pathway leading to cellular transformation and neoplasia.

If one regards neoplasia as a two-step process and one step is inherited, undoubtedly this would lead to increased susceptibility to neoplasia, but this crude form of the two-mutation hypothesis does not fit with the facts or with theoretical considerations. If, however, the inherited mutation along with a randomly occurring second event is responsible for the focal tissue disturbance and the conditions which exist within that disturbed focus are such that neoplasia is more likely to occur, this would help considerably in meeting the criticism of this hypothesis. Retinoblastoma would then have to be considered as a special case where the onset of malignancy has such a high probability that the intermediate stage of focal abnormality is not normally recognized.

The nature of the second or subsequent event(s) is still a matter for discussion. There may be a mutation in the normal allele at the same locus as the susceptibility gene or in a totally different gene at another locus. Cells may emerge which have been inappropriately differentiated as a result of the action of the mutant gene during embryogenesis. It should eventually be possible to distinguish between these ideas using recombinant DNA techniques.

The second main type of hypothesis is that the inherited abnormality is not itself part of the process of carcinogenesis but, rather like the defect of excision repair in XP, that it makes it more probable that events of significance in carcinogenesis may occur. The tissue-specific patterns of malignancy in these syndromes are not, however, consistent with the notion that the gene acts through a general mechanism of increasing the probability of malignancy in all cells. Furthermore, the absence of solid evidence of susceptibility to environmental agents or even of increased spontaneous chro-

mosomal events for most of these diseases makes it difficult at present to suggest that this is the major route of gene action. However, the clear evidence for the importance of the genetic background in the expression of at least some of these genes does support the general idea that the inherited defect confers susceptibility on the cells rather than itself constituting a step in the pathway to malignancy. It could be that the slightly elevated level of sensitivity to external agents that has been reported in some cases indicates a general propensity toward mutation and chromosomal rearrangement important in cancer development and progression.

Each of these two hypothetical mechanisms can be applied to the dominant syndromes considered in this article, and it is possible that both could contribute to the pathogenesis of any one disease. It is important that future work should continue to explore the fundamental mechanisms underlying these diseases.

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MULTIPLE MYELOMA, WALDENSTRÖM'S MACROGLOBULINEMIA, AND BENIGN MONOCLONAL GAMMOPATHY: CHARACTERISTICS OF THE B CELL CLONE, IMMUNOREGULATORY CELL POPULATIONS AND CLINICAL IMPLICATIONS

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I. Introduction

With the development of electrophoretic techniques, γ-globulins with restricted electrophoretic mobility were detected in sera from patients with various malignant and nonmalignant diseases and from apparently healthy elderly persons. Such M components consist of identical immunoglobulin molecules suggesting that they derive from a single expanded immunoglobulin-producing clone. The early contention that M proteins were abnormal immunoglobulins is not generally true. In most cases of multiple myeloma (MM), Waldenström's macroglobulinemia (WM), and benign monoclonal gammopathy (BMG), the M proteins seem to be functionally normal and complete immunoglobulin molecules (Slater *et al.*, 1955; Kunkel, 1960).

Multiple myeloma is the most well recognized of these diseases and has attracted great interest only for the clinicians but also for immunologists and chemists. Thus, myeloma cells in mouse and in man represent clonal malig-

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nant counterparts to normal immunoglobulin secreting cells, each clone producing a single molecular species of normal immunoglobulins. Knowledge of their general nature has enabled the formation of various hypotheses of clonal selection of antibody response, the structural and functional characterization of immunoglobulins, and the development of the hybridoma techniques for production of monoclonal antibodies.

Human myeloma is a disease with poor prognosis. In untreated patients the survival duration is in the majority of cases less than 12 months. Even with the use of intensive multiple drug chemotherapy, a cure has not been achieved (see Durie and Salmon, 1982). Consequently, there is a need for more efficient therapeutic means. In this context the pathobiology of multiple myeloma and related diseases has attracted particular interest.

The hallmark of clonal B cell tumors is the expression of the clonal immunoglobulin product carrying unique antigenic (idiotypic or Id) determinants in the variable region. According to the network hypothesis by Jerne (1974), idiotypic structures may serve as targets for the immune regulation of the normal immune response. Evidence that this is also the case in B cell neoplasia is beginning to emerge. Thus, antigen and idiotype-specific T cell immunity may develop in murine myeloma (Lynch *et al.*, 1979; Abbas *et al.*, 1982; Milburn *et al.*, 1982). Moreover, malignant B cells expressing idiotypic surface structures may be eradicated by the exposure to exogenous antiidiotypic antibodies (Hamblin *et al.*, 1980; Miller *et al.*, 1982; Ritz and Schlossman, 1982). It is inherent in this hypothesis that the targets for an antiidiotypic immune response are B cells in stages of differentiation when surface membrane immunoglobulins (smIg) are exposed while plasma cells lacking smIg are not attacked. Hence, antiidiotypic immune regulation in multiple myeloma and related plasma cells tumors should occur at the level of plasma cell precursors. Therefore, search for clonal B cell precursors in these diseases is an important goal for immunological studies.

This review is devoted to a summary of the present knowledge of the immunology of the B cell clone and immunoregulatory T cell subsets in multiple myeloma, Waldenström's macroglobulinemia, and benign monoclonal gammopathy. The presentation focuses on recent progress while older data are summarized in greater detail in previous reviews (Holm *et al.*, 1977; Mellstedt *et al.*, 1982a).

II. Characterization of the B Cell Clone

A. Multiple Myeloma

1. Clonal B Lymphocytes

Abnormalities within the lymphocyte compartment in MM have been known for many years. In 1920 Wallgren observed bone marrow lympho-

cytosis in untreated cases. MM patients may show a relative increase in blood lymphocytes, although the total counts remain within the normal range (Mellstedt *et al.*, 1982a). Moreover, blood lymphocytes with atypical features may appear in cases with active disease (Biberfeld *et al.*, 1977).

The composition of lymphocytes in bone marrow and blood of MM patients could be studied after the introduction in the early 1970s of techniques for the identification of lymphocyte subpopulations. Thus, an increase of lymphocytes with a receptor for human complement (C3b), as a B lymphocyte marker, with a concomitant T lymphocytopenia in untreated cases was found (Mellstedt *et al.*, 1973). These observations suggested that an increase of non-T cells mainly contributes to the lymphocytosis. Although some B lymphocytes may carry C3b receptors, the true nature of the expanded C3b receptor-carrying population was not known. It was later shown that there is also a rise in cells carrying receptors for IgG-Fc in some cases, although the mean value of the group did not deviate from normal (Pettersson *et al.*, 1980a). However, a relative and often absolute increase of smIg-bearing blood lymphocytes was observed (Mellstedt *et al.*, 1974). The identity of the enlarged lymphocyte population in bone marrow remained unresolved.

The following two hypotheses were favored to explain the observations: (1) the expanded population of complement receptor-bearing lymphocytes may represent B cells belonging to the malignant clone as progenitors to the myeloma plasma cells; or (2) the alteration of blood lymphocyte subpopulations may reflect a host reaction against the tumor.

The first hypothesis has been approached by techniques aimed to identify the clonal B cells by the use of fluoresceinated antisera against idiotypic structures on the myeloma protein (Lindström *et al.*, 1973; Mellstedt, 1974; Mellstedt *et al.*, 1974). By this approach it was expected to reveal B cells expressing surface and/or cytoplasmic immunoglobulins with idiotypic determinants, characteristic of the myeloma clone. The rationale for this was based on the knowledge about the normal B cell differentiation. Antigen triggers antigen-reactive B cells carrying surface IgM with or without IgD to differentiate into plasma cells producing antibodies against the same antigenic determinants. This may suggest that the same or similar idiotypic structures are exposed on the smIg and the final product of the clone (Ada, 1970; Raff *et al.*, 1973; Wigzell, 1974). Because these studies require at least one antiserum for each patient, only a limited number of cases have been evaluated so far.

More suitable for larger population studies is to identify B cells carrying the light chain isotype of the myeloma protein. The presence of clonal B cells is suggested by an abnormal ratio between lymphocytes carrying κ chains and lymphocytes expressing λ chains on the surface (Pettersson *et al.*, 1980a–c; Mellstedt *et al.*, 1982a). This approach has the theoretical disadvantage that small numbers of monoclonal B cells may "hide" within a

normal ratio. Moreover, an abnormal ratio does not prove monoclonality because it would also be seen if there was a preferential raise of one light chain B cell phenotype not belonging to the clone. However, a close correlation between abnormal κ:λ ratios and the presence of monoclonal idiotype-bearing lymphocytes has been described (Mellstedt *et al.*, 1982a).

The original findings of blood lymphocytes carrying surface immunoglobulins with idiotypic characteristics of the myeloma clone (Lindström *et al.*, 1973; Mellstedt *et al.*, 1974) have been confirmed in a number of reports (Abdou and Abdou, 1975; Preud'homme *et al.*, 1976, 1977b; Holm *et al.*, 1977; Husz *et al.*, 1977; Mathieu, 1977; Burns *et al.*, 1978; Sato *et al.*, 1978; Van Acker *et al.*, 1979; Van Camp *et al.*, 1979, 1981; Kubagawa *et al.*, 1979, 1980; Schedel *et al.*, 1979, 1980; Pettersson *et al.*, 1980a,b; Boccadoro *et al.*, 1981; Bast *et al.*, 1982; Bruyn, 1982; Mellstedt *et al.*, 1982a). Such lymphocytes have also been found in bone marrow (Kubagawa *et al.*, 1979, 1980; Van Camp *et al.*, 1981; Mellstedt *et al.*, 1982a). In a study of 41 unselected and untreated cases, abnormal blood lymphocyte κ:λ ratios were noted in 22 of the cases (Pettersson *et al.*, 1981). Similar figures were reported by Sato *et al.* (1978) and Nagai *et al.* (1981). However, monoclonal lymphocytes were seen in the bone marrow of all cases (Mellstedt *et al.*, 1982a).

Evidence for the presence of malignant blood lymphocytes in MM has also been obtained by cytological techniques. Barlogie *et al.* (1982) used DNA flow cytometry for studies of 30 patients with overt myeloma all lacking circulating plasma cells. Among 22 individuals with marrow aneuploidy, 4 showed the presence of a aneuploid blood lymphocyte subpopulation displaying marrow concordant DNA index values with a mean frequency of 22%, suggesting the presence of malignant cells. Moreover, karyotypic abnormalities in myeloma are not only confined to bone marrow cells. Thus, abnormal metaphases have been found in 54% of bone marrow examinations from patients with myeloma and in blood lymphocyte samples from about 60% of the patients studied (see Anday *et al.*, 1974).

2. *Differentiation Markers on Clonal B Lymphocytes*

During the normal maturation of a B cell clone some cells switch from the expression of IgM to the production of other immunoglobulin classes (Gathings *et al.*, 1977; Parkhouse and Cooper, 1977; Preud'homme *et al.*, 1977a). Embryonic V_H genes are first rearranged in pre-B cells and transcribed together with the μ gene leading to the synthesis of μ chains in the absence of light chains in the cytoplasm (Leder, 1982). This is followed by the rearrangement of light chain genes and the expression of complete IgM on the cell membrane while cytoplasmic μ chains have disappeared. The gene for the δ chain is close and usually transcribed together with the μ

chain (Liu *et al.*, 1980). Mature antigen-reactive lymphocytes simultaneously express surface IgM and IgD. During further development some cells switch from IgM to IgG or from IgM to IgA, which is probably accomplished by DNA recombination with deletion of the genes for μ and δ. Some cells committed to the secretion of IgG may express surface IgG along with IgM and IgD during a transitional stage (Kuritani and Cooper, 1982). Similarly, IgA is expressed on IgA-committed cells. Later smIgD and smIgM are lost. The most mature B lymphocytes committed to IgG or IgA secretion express only smIgG or smIgA, respectively, with or without cytoplasmic Ig (cIg) detectable by immunofluorescence (IFL). The mature plasma cell contains cIg but has no smIg. Hence, the heavy chain isotype pattern of malignant monoclonal B cells may be useful in the determination of the actual stage of maturation.

Receptors for IgG-Fc and C3b are present on some B cells and absent on mature plasma cells (Möller, 1974; McCornell and Hurd, 1976). Ia-like antigens may also be useful as markers for B cell differentiation because they are not expressed on mature plasma cells (Schlossmann *et al.*, 1976).

Monoclonal hybridoma antibodies against B lymphocyte antigens have also been exploited to identify and further strengthen the B lymphocyte nature of the clonal cells. The B1 antigen is present on all B cells except for mature plasma cells (Stashenko *et al.*, 1980; Nadler *et al.*, 1981b). The expression of the B1 antigen on the cell surface varies quantitatively during the B cell maturation. Using chronic lymphocytic leukemia B lymphocytes as a model, it was found that the intensity was strong on secretory B lymphocytes but weak on pre–B cells (Gordon *et al.*, 1983). The B2 antigen (Nadler *et al.*, 1981a) is essentially restricted to the itermediate, mature, and secretory B lymphocytes. It is usually, but not invariably, accompanied by smIgM and smIgD (Gordon *et al.*, 1983). Interestingly, EBV-induced conversion of the EBV-negative Ramos Burkitt lymphoma cell line which induces IgD expression (Spira *et al.*, 1981) may also induce the appearance of B2 (Klein *et al.*, 1983). The 2H7 monoclonal antibodies identify a pan-B antigen, presumably a separate epitope on the B1 molecule, and HB2 antibodies detect resting B cells (E. Clark, personal communication).

$B1^+$ and Ia^+ lymphocytes have been studied in six myeloma patients and in one patient with Waldenström's macroglobulinemia. All had circulating monoclonal lymphocytes, which were identified by an abnormal κ:λ ratio in six cases and by the presence of lymphocytes bearing surface idiotypic Ig structures in one case (Table I). There was a good correlation in healthy subjects and in the patients between the percentage of $B1^+$ lymphocytes and the number of cells carrying smIg stained by a polyvalent antiserum ($smpv^+$) or anti-light chain sera. $B1^+$ cells were increased and $smpv^+$ cells were at the upper range limit in the secretory myeloma cases. Very high

TABLE I

B1+ AND Ia+ BLOOD LYMPHOCYTES IN SIX MYELOMA PATIENTS AND ONE MACROGLOBULINEMIA PATIENT[a]

M Component	smpv+ (%)	smκ+ (%)	smλ+ (%)	Ia+ (%)	B1+ (%)	smId+ (%)	cIg+ (%)
Nonsecretory	53	<0.25	52	47	45	nd[b]	0.5
IgG-κ	8.5	7.5	1.0	5.5	10.5	nd	0.5
IgG-λ	6.5	2.0	6.0	7.0	8.0	nd	1.0
IgG-κ	7.0	7.0	1.5	17.5	12.0	nd	1.0
IgA-κ	9.0	nd	nd	1.5	9.5	8.0*	0.5
IgG-κ	8.0	7.5	1.5	6.0	8.0	nd	0.5
IgM-κ	24.5	18.5	<0.25	28.5	27	nd	nd
Controls							
Mean ± SE	4.1 ± 0.26	2.8 ± 0.2	1.4 ± 0.1	3.1 ± 0.4	3.3 ± 0.5	—	—
n	68	68	68	24	12	6	15
Range	1.0–8.5	0.25–6.5	0.25–3.5	0.5–7.0	1.0–7.0	<0.2	≤1.0

[a] Immunoglobulins have been identified by direct IFL with $F(ab')_2$ fragments of the antisera except for one experiment (*) in which indirect IFL were used (Pettersson *et al.*, 1978). Ia+ and B1+ cells were identified in indirect IFL using goat antimouse IgG in the second step (Mellstedt *et al.*, 1982b).

[b] nd, Not done.

numbers of smpv$^+$ and B1$^+$ cells were found in one patient with a nonsecretory myeloma and in one patient with Waldenström's disease. In four cases a good correlation was seen between the number of Ia$^+$ cells and the percentage of smpv$^+$ and B1$^+$ lymphocytes, while in the other three patients a discordance was found. The reason for this is unknown but may be explained by a varying expression of the Ia antigens during the B cell maturation.

Lymphocytes carrying monoclonal immunoglobulin were identified by double staining in IFL using a clonal marker (anti-light chain or antiidiotypic sera) in combination with one monoclonal antibody against lymphocyte surface antigens. In all cases most cells which stained with anti-B cell monoclonal antibodies to B1, B2, 2H7, and Ia also carried the marker of the myeloma clone (Table II). The expression of B2 antigen is more restricted and seems to coincide with the presence of smIgD. However, it may also appear on some mature B cells suggesting compartmentalization of B cells (Gordon *et al.*, 1983). This could explain the inability of anti-B2 to stain a fraction of cells which carried pan-B cell antigens in some cases (i.e., the BJ-κ and the IgA-λ myeloma in Table II). Clonality of HB2$^+$ cells was less consistently revealed. In contrast, no or only few OKT3$^+$ (PAN-T) cells had clonal characteristics.

In addition, a fraction of cells from three cases identified by the clonal markers did not react with anti-B monoclonals. The nature of these cells is not known. Some of them may be B cells not identified by the monoclonal antibodies. It might be assumed that these B cell antigens are lost before smIg is lost during the maturation toward plasma cells. Thus, two patients (one IgG-κ and one IgG-λ myeloma) with a high number of both smIg$^+$ and cIg$^+$ lymphocytes and a comparatively low number of lymphocytes reacting with the monoclonal B cell antibodies. It should be noted that less than 1% of the blood lymphocytes in these cases had plasma cell morphology. As there was no staining for clonal Ig on T cells the experiments gave strong support to the notion that monoclonal B cells are present in the blood and bone marrow of myeloma patients as part of the malignant clone. Van Camp and co-workers using gold labeled antiidiotypic antibodies in combination with cytochemical staining have reached the same conclusion (de Waele *et al.*, 1981, 1983; B. van Camp, personal communication).

Further support for the concept that myeloma is a differentiating tumor has been obtained by the simultaneous use of rosetting technique to identify surface IgG-Fc receptors or complement receptors and IFL to reveal surface or cytoplasmic immunoglobulin structures (Pettersson *et al.*, 1980a,b: Mellstedt *et al.*, 1982a). The following phenotypically different monoclonal B cells also showing some morphological disparity could be identified: (1) cells with monoclonal cIg only, lacking IgG-Fc receptors and complement receptors, were large major plasmacytoid cells; (2) cells with monoclonal

TABLE II

SURFACE DOUBLE LABELING[a] OF LYMPHOCYTES IN MULTIPLE MYELOMA USING MONOCLONAL ANTIBODIES AGAINST T AND B CELL ANTIGENS AND ANTISERA TO MONOTYPIC IG STRUCTURES

Myeloma type Lymphocyte source	Monoclonal lymphocytes (surface staining) (%)	cIg+ (%)	Lymphocytes stained by monoclonal antibodies (%)											
			B1		B2		HB2		2H7		Ia		OKT3	
			Total	Clonal[b]	Total	Clonal	Total	Clonal	Total	Clonal	Total	Clonal	Total	Clonal
BJ-κ	9.0 (κ+)	1.0	4.5	4.5	1.0	1.0	14.0	5.0	6.0	6.0	nd	nd	nd[c]	nd
Blood	0.5 (λ+)													
IgG-κ	21.0 (κ+)	11.0	8.5	7.5	5.75	5.25	7.0	3.5	8.5	5.5	14.0	nd	75.0	<0.25
Blood	0.5 (λ+)													
IgA-λ	2.0 (κ+)	0.5												
Blood	12.0 (λ+)		12.0	12.0	5.0	5.0	nd		15.0	12.75	12.0	12.0	77.0	<0.25
IgG-λ	0.25 (κ+)													
Blood	14.5 (λ+)	15.0	4.0	4.0	5.0	5.0	nd		2.5	2.5	1.5	1.5	71.0	1.0
	8.5 (Id+)[d]		3.5	3.5	6.0	6.0	2.0	2.0	3.0	3.0	1.0	1.0	72.0	<0.25
IgG-λ														
Blood non-T[e]	0.5 (κ+)													
	40.0 (λ+)	nd	36.0	32.5	38.0	38.0	34.0	25.5	21.0	19.0	nd		2.0	<0.25
Bone marrow	1.0 (κ+)													
	26.0 (λ+)	nd	22.0	22.0	17.0	17.0	28.0	21.0	31.0	25.0	nd		47.0	<0.25
Blood	2.0 (κ+)													
	14.0 (λ+)	1.5	8.0	8.0	3.0	3.0	8.0	4.75	11.5	8.0	9.0	9.0	62.0	0.5
	10.0 (Id+)													

[a] In double labeling, preincubated cells were stained at 4°C with $F(ab')_2$ fragments of anti-light chain/idiotypic antisera, monoclonal antibodies and conjugate in that order using different fluorochromes on the monotypic Ig antisera and conjugate, respectively. The goat anti-mouse conjugate alone stained <0.25% cells. Double labeling with anti-light chain/idiotypic antisera and conjugate alone revealed no double-stained cells.

[b] Lymphocytes double stained with the monoclonal antibody and the anti-light chain/idiotypic antiserum.

[c] nd, Not done.

[d] Indirect IFL with $F(ab')_2$ fragments in both steps.

[e] Non-T cells obtained after Ficoll-Isopaque centrifugation of lymphocytes binding neuraminidase treated sheep red blood cells.

smIg and cIg, some of which had IgG-Fc receptors but lacking complement receptors, were large lymphocytes or lymphoplasmacytoid cells; (3) cells with monoclonal smIg only but no cIg, the majority of the cells carrying IgG-Fc receptors and to a varying degree complement receptors, were small lymphocytes.

3. *The Extent of the Clonal Involvement*

The clonal B cell differentiation in IgA and IgD myeloma was studied by Kubagawa *et al.* (1979, 1980). They used two kinds of antiidiotypic antisera. One group was heavily absorbed to monospecificity for idiotypic determinants of the individual myeloma protein, while the other less absorbed antisera reacted with a family of myeloma-related idiotypic structures. B lymphocytes seemed to express the individual myeloma-specific idiotype along with heavy chain isotypes (IgM, IgD, IgA, and/or IgG) in all three cases. However, the majority of cells had the same heavy chain isotype as that secreted by the myeloma plasma cells. The results were interpreted to indicate that the oncogenic event had hit cells before the heavy chain switch. The number of cells with pre-B phenotype which expressed the myeloma-specific idiotype in the cytoplasm was increased further suggesting that the tumor involvement includes clonal precursor cells. A family of myeloma-related idiotype-bearing B cells mainly expressing smIgM and smIgD were also detected by the use of cross-reacting antiidiotypic antisera. T cells expressing idiotypic determinants were not found. Kubagawa and co-workers concluded that the initial oncogenic event in multiple myeloma may occur in pre-B cells or in B stem cells.

Van Camp and associates also studied the clonal event in multiple myeloma by using heterologous antiidiotypic antisera (Van Camp, 1980; Van Camp, *et al.*, 1981; Bast *et al.*, 1982). The authors were also able to identify blood and bone marrow lymphocytes bearing idiotypic structures. The idiotypic immunoglobulin on the lymphocytes was always restricted to the heavy chain isotype secreted by the plasma cells. They concluded that the clonal differentiation in multiple myeloma may start in the bone marrow memory B lymphocytes. Memory B lymphocytes, whose main homing site is the bone marrow compartment, express only the final isotype (Benner *et al.*, 1977; Coffman and Cohn, 1977).

In our studies of human IgG and IgA myeloma, the majority of idiotype-bearing lymphocytes which were detected by mono-specific rabbit antisera expressed the same heavy chain isotype as secreted by the plasma cells. In a few IgG myeloma patients a small fraction of monoclonal lymphocytes with smμ/smδ/smγ might also exist (Pettersson *et al.*, 1980b; Mellstedt *et al.*, 1982a). Such transitional cells may be committed to the production of IgG (Kuritani and Cooper, 1982).

Studies of MOPC-315 and other murine myelomas have also provided evidence for a progressive differentiation of myeloma cells during *in vivo* growth in spleen colonies and in diffusion chambers placed in the peritoneal cavity. Maturation was manifested as a progressive change from small, stem cell-rich, nonsecretory lymphoid cells to larger, myeloma protein-secreting plasma cells (Rohrer and Lynch, 1977; Lynch *et al.*, 1979).

4. Monoclonal Ig Secretion

Provided that the monoclonal B lymphocytes in multiple myeloma serve as progenitors for the myeloma plasma cells, they might differentiate to monoclonal Ig-secreting cells after *in vitro* stimulation. The protein A plaque assay has been used to enumerate cells spontaneously secreting Ig of a given idiotype or isotype (Smith *et al.*, 1981; Nagai *et al.*, 1981; Mellstedt *et al.*, 1982a). By this approach cells secreting the heavy chain and/or light chain isotype of the myeloma protein were identified in blood of patients with active disease. The cell in the center of a plaque had the morphology of a small lymphocyte. In the majority of cases no cells with plasma cell morphology could be detected in the blood. However, attempts to generate plaque-forming monoclonal cells by stimulation with B cell mitogens have so far been unsuccessful.

The immunoglobulin production of cultured blood lymphocytes from myeloma patients was also studied with an ELISA technique. Idiotypic antisera were used to quantitate monoclonal Ig production in microcultures (Peest *et al.*, 1982). Blood mononuclear cells from four myeloma patients secreted the myeloma protein. The kinetics of the release of monoclonal Ig was different from that of polyclonal Ig. Patients' lymphocytes synthesized monoclonal Ig spontaneously. The monoclonal Ig production was not enhanced by pokeweed mitogen (PWM). Supernatant monoclonal Ig increased rapidly during the first 2 days of incubation while the rate of release decreased from Day 3. There was a rapid drop of cellular monoclonal Ig during the first 1–2 days of culture. The release of Ig during the first phase was not inhibited by the addition of puromycin, a protein synthesis inhibitor, while the later phase was puromycin sensitive. It was concluded that the first phase represented release of already synthesized myeloma protein while *de novo* synthesis was required for the second phase. No cells with plasma cell morphology were found in blood of any of the four patients, and the number of lymphoid cells containing cytoplasmic Ig were identical to that of normal individuals ($\leq 1\%$). It was concluded that cells secreting monoclonal immunoglobulin were present in the blood of myeloma patients. Because plasma cells as well as postswitch B cells in general have the ability to secrete Ig, B cells in various stages of terminal differentiation may contribute to the secretion of Ig in cultures (Gordon *et al.*, 1983). However, it remains to be shown

whether cells recruited by differentiation from clonal nonproducer precursor cells participate in the late puromycin-sensitive Ig production.

Further support for the role of lymphocytes in the propagation of the myeloma clone was achieved by autoradiographic studies of [^3H]thymidine incorporation into bone marrow cells from three multiple myeloma patients (Mellstedt *et al.*, 1977). Antiidiotype antisera were used to identify lymphocytes and plasma cells belonging to the malignant cell clone. Overall, a higher labeling index was observed in monoclonal cells than in normal bone marrow B cells. Few labeled plasma cells were revealed (2.5–5%), while lymphocytes constituted the major fraction (11.5–14%), having a larger degree of labeling. The results support the notion that not only malignant plasma cells but also monoclonal B lymphocytes proliferate in multiple myeloma.

The conclusion from these studies is that B cells in various stages of differentiation and carrying immunoglobulins with immunological characteristics of the myeloma clone are present in bone marrow of all cases and in blood of many patients with multiple myeloma. A crucial question is How early in the B cell differentiation can clonal B cells be traced? The work by Cooper's group has suggested the presence of clonal pre-B cells and early B cells with smIgM and smIgD in IgA and IgD myeloma, respectively. However, these results still remain unconfirmed. The present writers have not detected cells with characteristics of pre-B cells or early B cells in multiple myeloma nor in Waldenström's macroglobulinemia (see later) using a number of rabbit antiidiotypic sera. Other groups have also failed to disclose clonal pre-B cells in myeloma (see previous discussion). On the other hand, cells with postswitch clonal B cell characteristics have been consistently demonstrated by various groups. The reactivity with monoclonal hybridoma anti-B cell antisera has labeled the majority and, in many cases, all clonal cells as B lymphocytes (Table II). These findings also imply that monoclonal Ig was not passively attached to IgG-Fc- (or IgA-Fc-) receptor-carrying T cells or non-T/non-B cells, which gives further support to the notion that true monoclonal B cells are present in myeloma.

5. *Morphology*

The morphology of the majority of myeloma blood cells bearing surface membrane monoclonal or idiotypic immunoglobulin structures was that of small lymphocytes. In most cases the number of cIg^+ cells was as low as observed in healthy persons ($\leq$1%). However, in about 5% of untreated myeloma patients cIg^+ cell counts were as high as 70% (Pettersson *et al.*, 1980b; Mellstedt *et al.*, 1982a). The lymphoid morphology was characterized by a cell pleomorphism with lymphocytes, lymphoblasts, lymphoplasmacytoid cells, and plasmacytoid cells (Biberfeld *et al.*, 1977, 1979; Holm *et al.*,

1977). Immunoelectronmicroscopy studies revealed the presence of small- to medium-sized lymphocytes sometimes with irregular nuclei and nuclear invaginations, relatively large Golgi zones, and conspicuous villous surfaces. Some patients had a high number of blood cells carrying cytoplasmic and surface idiotypic Ig and also displayed pronounced lymphocyte heterogeneity at the ultrastructural level. Signs of plasmacytoid differentiation were obvious with circular arrays of endoplasmic reticulum and a conspicuous Golgi zone. Many of these cells also had irregular, lobated, and segmented nuclei.

B. Waldenström's Macroglobulinemia

The bone marrow cell morphology in Waldenström's macroglobulinemia is more heterogeneous than that in multiple myeloma and is characterized by a mixture of small lymphocytes, lymphoblasts, lymphoplasmacytoid cells, and plasma cells. The tumor morphology of this disease is suggestive of a differentiating B cell tumor. The morphology of blood lymphoid cells in the majority of patients is that of small- to medium-sized lymphocytes. Some cells may have a lymphoplasmacytoid appearance but only occasionally are plasmacytoid cells detected (Pettersson *et al.*, 1980c).

Monoclonal lymphocytes are present in bone marrow and blood of the patients (Preud'homme and Seligmann, 1972a; Seligmann *et al.*, 1973; Pettersson *et al.*, 1980c; Schedel *et al.*, 1980; Axelsson *et al.*, 1981; Van Camp *et al.*, 1981). As in multiple myeloma the monoclonal B lymphocytes may also express B1 antigens and Ia-like antigens, as is suggested by the results shown in Tables I and III. Most bone marrow lymphocytes carrying the light chain of the M component also stain by antiidiotypic antisera (Pettersson *et al.*, 1980c). This observation strengthens the validity of light chain monospecificity as a useful marker for monoclonality. The monoclonal lymphocytes express μ chains with or without δ chains. It is not known whether the smIgD and the macroglobulin share idiotypic structures by analogy to that observed in chronic lymphocytic leukemia (Fu *et al.*, 1974). Some monoclonal lymphocytes in Waldenström's disease contain cIgM.

Additional evidence for heterogeneity within the monoclonal B lymphocyte population in Waldenström's macroglobulinemia has been obtained by the simultaneous recording of smIg, cIg, and either IgG-Fc receptors or C3b receptors (Pettersson *et al.*, 1980c; Schedel *et al.*, 1980). The majority of small lymphocytes and lymphoplasmacytoid cells were $smIg^+$. cIg was present in lymphoplasmacytoid cells and in mature plasma cells. IgG-Fc receptors were found on $smIg^+$ cells with or without cIg while complement receptors were present on cells with smIg only.

Monoclonal blood lymphocytes from patients with macroglobulinemia se-

TABLE III

MONOCLONAL BLOOD LYMPHOCYTES IN RELATION TO THE CLINICAL COURSE OF THE DISEASE IN WALDENSTRÖM'S MACROGLOBULINEMIA

Patient	M component type	Time of test and clinical status	OKT3 (%)	smpv+ (%)	smκ+ (%)	smλ+ (%)	Ia+ (%)	cIg+ (%)	S-IgM[a] (g/l)
M. M.	IgM-κ	4 years after diagnosis	62	nd	1.0	1.0	3.0	<0.25	26.0
		After 5 years	88	1.0	1.0	0.5	0.5	<0.25	24.0
J. N.	IgM-κ	At diagnosis	70	3.5	3.75	<0.25	3.5	0.5	16.5
		After 6 months	61	13.0	12.0	0.5	13.0	1.0	88.0
A. B.	IgM-κ	At diagnosis	78	5.5	5.0	2.0	5.5	1.0	13.0
		After 6 months	71	21.5	21.0	1.5	5.5	1.0	34.0

[a] Normal values 0.4–2.0 g/liter. For control values of lymphocyte subpopulations see Tables I and V.

crete the monoclonal immunoglobulin as determined by a plaque assay using antiidiotypic antibodies (Hammarström *et al.*, 1980, 1981). After stimulation of blood and bone marrow cells with polyclonal B cell activators (PBA), cells within the malignant clone developed into immunoglobulin-secreting cells. The spontaneous secretion of monoclonal immunoglobulin could be abolished by the addition of the appropriate antiidiotypic antiserum.

It is concluded that the monoclonal malignant cell fraction in patients with Waldenström's macroglobulinemia contains B cells in different stages of maturation. These cells can differentiate into immunoglobulin plaque-forming cells *in vitro* after activation by PBA.

C. Benign Monoclonal Gammopathy

BMG is the benign counterpart of multiple myeloma and Waldenström's macroglobulinemia. Usually less than 10% plasma cells are found in bone marrow of these patients. No morphological abnormalities within the lymphoid cell compartment have been described. Using antiidiotypic and antilight chain antisera monoclonal B cells have been identified in the bone marrow. The cells express the same heavy and light chain isotypes as the secreted monoclonal immunoglobulin (Van Camp, 1980; Van Camp *et al.*, 1981). Idiotype-bearing small lymphocytes may also be found in blood (Lindström *et al.*, 1973; Van Camp, 1981; Olsen *et al.*, 1981; Bast *et al.*, 1982; Carmagnola *et al.*, 1983). Accordingly abnormal blood κ:λ ratios indicating the presence of monoclonal B lymphocytes were noted in 5 out of 37 BMG patients (14%) (Pettersson *et al.*, 1981, unpublished observations). The few monoclonal blood B cells detected in BMG always had the morphology of small lymphocytes, which is based on the cytoplasm:nuclear ratios and the centrally located nuclei (Pettersson *et al.*, 1981; Bast *et al.*, 1982).

Mononuclear cell suspensions containing less than 1% cIg^+ cells from a BMG patient produced monoclonal immunoglobulin in 7-day-old *in vitro* cultures. The kinetics of polyclonal and monoclonal Ig production were similar to that seen in myeloma patients (see Section II,A,4). Thus, the monoclonal Ig production was not enhanced by PWM. The polyclonal Ig synthesis in unstimulated cultures was low but could be increased by PWM stimulation. The Ig production was inhibited after suppression of protein synthesis by puromycin. It was concluded that BMG blood B cells may actively synthesize monoclonal Ig (Peest *et al.*, 1982).

III. T Lymphocytes in Multiple Myeloma

T lymphocytes play an important role in B lymphocyte regulation. The normal activation, proliferation, and differentiation of B cells may be regulated by a network of antigen, T cells, and antibodies (Jerne, 1974). Two

categories of regulating T cells are known. One is antigen-binding T cells sharing idiotypic structures with B cells binding the antigen and modulating B cell function by activation or suppression. The second is antiidiotypic T cells reactive against idiotype-bearing cells.

Also, malignant B cells seem to submit to T cell regulation. This has been clearly shown in murine plasmacytomas (see Milburn *et al.*, 1982). Conclusive evidence for immune regulation of monoclonal B cells in human myelomas and also in Waldenström's macroglobulinemia and BMG is lacking. However, various immunocytological and functional abnormalities of regulatory T cells have been disclosed.

A. Immunocytology of T Cells

The relative and total number of blood T lymphocytes are low in untreated multiple myeloma and Waldenström's macroglobulinemia patients. The decrease is most evident in advanced clinical stages (Mellstedt *et al.*, 1974, 1982a,b; Jones and MacFarlane, 1975; Pettersson *et al.*, 1980c). The percentage of blood T lymphocytes normalizes in remission. Blood T lymphocytes are within the normal range in the majority of BMG cases (Pettersson *et al.*, 1981) (Table IV).

Blood T lymphocyte subpopulations have been studied in untreated patients with MM and WM and in patients with BMG. T lymphocytes and subpopulations of T lymphocytes were identified by monoclonal mouse antibodies of the OKT series: OKT3 (PAN-T), OKT4(inducer/helper T lymphocytes), and OKT8 (cytotoxic/suppressor T lymphocytes) purchased from Ortho Pharmaceutical Corp., Raritan, New Jersey. The cells were stained by indirect IFL using fluoresceinated goat anti-mouse IgG in the second step (Meloy Lab, Springfield, Virginia) (Mellstedt *et al.*, 1982b). A slight reduction of the $OKT4^+/OKT8^+$ lymphocyte ratio was seen in BMG (Fig. 1). However, the percentage of $OKT4^+$ T lymphocytes was significantly decreased (Mellstedt *et al.*, 1982b). The ratio was also decreased in MM, particularly in advanced stages. The low ratio was mainly caused by a profound reduction of the $OKT4^+$ cells. The imbalance between the two T lymphocyte subsets was even more pronounced in WM. When absolute cell counts were considered, $OKT4^+$ cell counts were significantly decreased while $OKT8^+$ cell counts were raised (Mellstedt *et al.*, 1982b). The imbalance was aggravated with progression of the disease. It may be concluded that the depression of the lymphocyte $OKT4^+/OKT8^+$ ratio is more pronounced in malignant than in benign gammopathies and that it is also more pronounced in patients with a large tumor load. The imbalance is mainly attributed to an absolute reduction of $OKT4^+$ lymphocytes. A similar observation was made in patients with non-Hodgkin lymphomas mainly of the B cell type. Simultaneous enumeration of $OKT4^+$ and $OKT8^+$ cells in blood

TABLE IV

CLINICAL FINDINGS IN FIVE PATIENTS WITH BENIGN MONOCLONAL GAMMOPATHY AND MONOCLONAL BLOOD LYMPHOCYTES

Patient	M component type	smpv$^+$ (%)	κ:λ ratio	E$^+$ (%)	ESR mm/hr	Serum immunoglobulins (g/liter)[a] IgG	IgA	IgM	Bone marrow cytology
L. O.	IgG-κ	5.0	4.70	56	10	19.7	0.4	0.2	8% plasma cells
G. A.	IgM-λ	7.0	0.7	67	72	4.8	0.9	18.5	8% plasma cells and an increased number of lymphocytes and a few lympho-plasmacytoid cells; no mast cells
H. E.	IgM-κ	46	17.2	30	41	12.4	0.7	9.6	7% plasma cells; no increase in lymphocytes but a few lymphoplasmacytoid cells; no mast cells
O. S.	IgG-λ	5.0	0.6	73	50	37.7	0.4	0.2	4% plasma cells
O. E.	IgG-λ	2.0	0.5	39	72	29.4	0.1	0.3	5% plasma cells

[a] Normal values: IgG 7.0–15.0 g/liter; IgA 0.8–3.0 g/liter; IgM 0.4–2.0 g/liter.

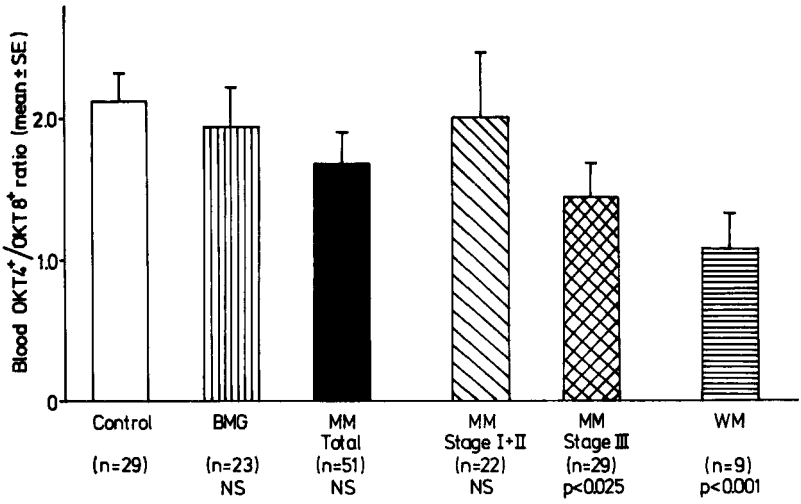

FIG. 1. Ratio between OKT4+T lymphocytes and OKT8+T lymphocytes in benign monoclonal gammopathy (BMG), multiple myeloma (MM), Waldenström's macroglobulinemia (WM), and control donors. Comparisons are made between patients and controls (Student's *t* test). NS, not significant.

and lymph nodes may suggest a reduced pool of OKT4+ lymphocytes in most cases (Lindemalm *et al.*, 1983).

The imbalance of T lymphocyte subsets in patients with multiple myeloma has also been envisaged by studying T lymphocyte bearing Fc-receptors for IgM (Tμ) and for IgG (Tγ) (Moretta *et al.*, 1977). Tγ cell counts were consistently high while Tμ cell counts were low (Oken and Kay, 1981; Pezzutto *et al.*, 1981). Moreover, T cells with low affinity binding of sheep red blood cells predominated in peripheral blood and bone marrow of MM patients (Giustolisi *et al.*, 1981). Such T cells supposedly represent suppressor cells while helper T cells predominate among high affinity binding cells (West *et al.*, 1977).

B. T Lymphocytes with Idiotype- or Isotype-Specific Receptors

Idiotype-bearing structures have been identified on the surface of T helper and T suppressor cells (see Jensenius and Williams, 1982). The idiotypes of the receptors on T lymphocytes may be shared with idiotypic receptors on antigen-reactive B cells. The biochemical nature of the T cell receptors is still unknown. Earlier suggestions that they are represented by immunoglobulin heavy chain V regions have not been supported by molecular

genetic evidence (Kurosawa *et al.*, 1981). Surface molecules reactive with heterologous antiidiotypic sera raised against the monoclonal immunoglobulin have been described on about 10% of T lymphocytes in some patients with myeloma and BMG (Preud'homme *et al.*, 1977b; Lea *et al.*, 1979; Olsen *et al.*, 1980; Boccadoro *et al.*, 1981). The idiotypic determinants were identified on T cells which did not stain with light and heavy chain-specific antiimmunoglobulin sera. These results may suggest the presence of idiotypic T lymphocytes lacking conventional immunoglobulin structures in MM and BMG. However, other workers have failed to detect idiotype-bearing T cells identified by E-rosetting or anti-T cell sera (Kubagawa *et al.*, 1979, 1980; Pettersson *et al.*, 1980b; Mellstedt *et al.*, 1982a) (Table II). Thus far, idiotype-bearing T cells have neither been disclosed in Waldenström's macroglobulinemia (Pettersson *et al.*, 1980c).

Lynch and associates have shown that BALB/c mice with secretory MOPC-315 tumors develop a large number of blood and spleen T lymphocytes with surface membrane M-315 protein (Gebel *et al.*, 1979; Hoover *et al.*, 1981a). The cells were postthymic lymphocytes of host origin and were not circulating myeloma cells (Hoover *et al.*, 1982). M-315 were attached to class-specific IgA receptors (Hoover and Lynch, 1980). The presence of Tα cells in various IgA-secreting plasmacytomas correlated with the serum IgA concentration. Thus, no Tα cells could be detected in mice with nonsecretory or low secretory IgA plasmacytomas. Moreover, Fc-α receptors could be induced on T cells in a dose-dependent manner by incubation with purified polymeric IgA (Hoover *et al.*, 1981a). It was concluded that the Tα cell expansion in mice with IgA myelomas was related to the high IgA level and not to the plasmacytoma burden per se. A similar expansion of Tα and Tγ cells has been described in human IgA and IgG myelomas, respectively (Hoover *et al.*, 1981b,c). Similarly, the induction of class-specific IgE receptors by IgE has been previously reported (Yodoi and Ishisaka, 1979).

Lynch and co-workers have also shown that class-specific Fc receptors on T cells are capable of absorbing the corresponding Ig molecules *in vitro* and *in vivo* (Hoover *et al.*, 1981b). A 24-hr precultivation of the cells in Ig-free medium was undertaken to uncover the receptors. It is conceivable that unless lymphocytes are preincubated at 37°C, monoclonal Ig, which is bound *in vivo* to Fc receptors on T and non-T lymphocytes, might be stained by antiidiotypic antisera (Pettersson *et al.*, 1978). Because myeloma patients may have up to 60% IgG-Fc receptor-bearing blood lymphocytes (Pettersson *et al.*, 1980a), absorption of monoclonal IgG may result in a high number of idiotypic lymphocytes in some cases. Under such conditions it is possible that monoclonal Ig may be attached to some Fc receptor-bearing T cells. However, attempts to identify monoclonal Ig by IFL on E-rosetting T cells were unsuccessful (Pettersson *et al.*, 1980b). After removal of absorbed IgG

by 37°C preincubation, the fraction of cells carrying monoclonal IgG was reduced. However, in many cases a large fraction of idiotype-bearing cells remains (Pettersson *et al.*, 1980b). Moreover, under the present experimental conditions, no monoclonal Ig was found on T cells (Table II).

In conclusion, increased numbers of lymphocytes within the T cell population carrying Fc receptors for the M component isotype may be seen in myeloma. However, it should be noted that the idiotypic structures found on myeloma and BMG T lymphocytes were presumably not immunoglobulins because they did not react with antisera against isotypic heavy and light chains (Preud'homme *et al.*, 1977b; Lea *et al.*, 1979; Olsen *et al.*, 1980; Boccadoro *et al.*, 1981). Such T cells may represent cells with idiotypic receptors produced by the cells.

C. Function of T Lymphocytes

Some basic principles for the immune regulation of myeloma cells have been uncovered by the studies of the MOPC-315 murine plasmacytoma (see Lynch *et al.*, 1979; Milburn *et al.*, 1982). This monoclonal tumor produces immunoglobulin with antibody affinity for the TNP-hapten. The growth and differentiation of the tumor cells can be regulated by antigen-specific and idiotype-specific signals. MHC-restricted, antigen-specific T helper and T suppressor cells may balance the proliferation of myeloma cells, the expression of surface myeloma protein, and the secretion of such protein (Rohrer and Lynch, 1978, 1979).

Regulatory signals specific for the idiotype on the surface membrane-bound M-315 protein include idiotypic-specific T cells and antiidiotypic antibodies produced by immunization with M-315 protein. Idiotype-specific T cells inhibiting the myeloma growth are responsible for the protection of immune animals against challenge with lethal numbers of tumor cells (Lynch *et al.*, 1972, 1979; Flood *et al.*, 1980).

A second, probably distinct, antiidiotypic T cell subset rapidly and specifically inhibit M-315 secretion *in vivo* via a soluble product. The cells do not affect tumor cell growth or the expression of surface M-315 (Milburn and Lynch, 1982). T Helper cells specific for V_H determinants on the M-315 protein have also been described (Jörgensen and Hannestad, 1982). Moreover, antiidiotypic antibodies inhibit the production of M-315 protein and MOPC-315 growth (Mahony *et al.*, 1981).

Fc receptors for different immunoglobulin classes are present on T and B cells (see Spiegelberg, 1981). The number of class-specific, Fc-bearing cells is raised in animals with increased levels of the corresponding immunoglobulin class. This may suggest that these cells are involved in class specific regulation of immunoglobulin production. Hoover and Lynch (1983)

showed that Tα-rich spleen cells derived from mice with MOPC-315 suppressed the number of anti-TNP IgA plaque-forming cells after injection into orally immunized mice. There was no effect on IgG and IgM anti-TNP plaque-forming cells. Like idiotype specific T suppressor cells the isotypic-specific suppressor cells seem to regulate late B cells.

Immune regulation of the tumor cell clone in human myeloma has not been disclosed. Most studies have been devoted to the normal B cell function. Originally, Broder *et al.* (1975) described suppression of normal immunoglobulin production of blood cells in three out of six myeloma cases. The suppression was primarily mediated by phagocytic, adherent monocytic cells. However, studies of 29 patients disclosed no qualitative alterations of blood monocyte regulation of PWM-induced polyclonal immunoglobulin production (Twomey *et al.*, 1982).

Monocyte-depleted blood lymphocytes from myeloma patients exerted increased suppressor cell activity against antigen-induced blast transformation, PWM-induced autologous or allogeneic polyclonal Ig production and the allogeneic-mixed lymphocyte reaction (Paglieroni and McKenzie, 1977, 1980). The majority of suppressing cells had IgG-Fc receptors but did not form rosettes with sheep red blood cells. They lacked receptors for EBV and complement and did not react with anti-B cell sera. However, the exact identity of the suppressor cell subset remains unresolved.

Ozer *et al.* (1981) have described abnormal regulation of PWM-induced polyclonal immunoglobulin secretion in myeloma by nonadherent blood cells. In spite of a low polyclonal immunoglobulin secretion, T helper function for polyclonal immunoglobulin secretion was intact and the suppressive effect of the Tγ subsets was also normal. However, a marked deficiency in radiosensitive and concanavalin A-induced suppressor activity was noted and localized to T non-γ suppressor subset. The relation of these abnormalities to the myeloma tumor progression remains unexplained.

It may be concluded that evidence for regulation of the tumor clone in human myeloma is lacking. Experimental studies have shown a disturbed regulation of normal immunoglobulin production in human myeloma and also of other regulatory functions, but the results remain inconclusive.

IV. Clonal B Lymphocytes in Relation to the Clinical Course

A. Multiple Myeloma

Treatment of myeloma induces a characteristic mode of tumor reaction (Durie *et al.*, 1980). After an initial rapid phase of tumor regression, a plateau state is reached with minimal change of the tumor cell mass. This plateau can be maintained by continuous treatment for many years with a

tumor load 1–2 logs smaller than the pretreatment tumor burden. During plateau phase the incorporation of tritiated thymidine into bone marrow monoclonal cells, dominated by idiotype-bearing lymphoid cells, is low (Pileri *et al.*, 1981). At this stage tumor cell death is also low, supporting a low tumor cell growth and turnover. Aneuploid tumor cells, which are detected in about 80% of untreated patients, disappear in remission (Barlogie *et al.*, 1982). This and other tumor kinetic observations are in accordance with the shift from predominant S phase plasma cells to dormant functional G_0 cells with surface immunoglobulins.

Studies of cell kinetics are in general agreement with the distribution of monoclonal cells in bone marrow and blood of untreated and treated patients. Thus, monoclonal lymphoid cells were detected in the bone marrow of myeloma patients in all phases of the disease (Kubagawa *et al.*, 1979, 1980; Van Camp *et al.*, 1981; Mellstedt *et al.*, 1982a). The myeloma plasma cells in the bone marrow were large in numbers compared to the percentage of monoclonal B lymphocytes in patients with active disease. Patients in clinical remission had few bone marrow plasma cells but had a proportionately large monoclonal lymphocyte compartment in comparison to patients with active disease (Mellstedt *et al.*, 1982a).

The relative and total number of blood monoclonal lymphocytes in untreated patients varies with the clinical stage. Thus, about 25% of stage I patients had a small population of circulating monoclonal B lymphocytes. The corresponding percentage for stage II disease was 40% and for stage III disease 65%. Monoclonal blood B lymphocytes were also present in two IgD myelomas and in one nonsecretory myeloma (Pettersson *et al.*, 1980a, 1981; Mellstedt *et al.*, 1982a).

Following cytoreductive therapy with melphalan monoclonal blood lymphocytes decreased and were not detected in clinical remission (Mellstedt *et al.*, 1974, 1976, 1980, 1982a; Abdou and Abdou, 1975; Holm *et al.*, 1977; Van Camp *et al.*, 1979; Kubagawa *et al.*, 1980; Bast *et al.*, 1982). The variation in the monoclonal blood lymphocyte subpopulation with disease activity is exemplified in Table V. A good correlation was noted between the level of monoclonal blood lymphocytes and the disease activity in all types of myelomas.

A main conclusion from these studies is that the tumor mass as reflected by the clinical stages is an important factor in determining the export of tumor cells to the circulation. This is also consistent with the appearance of tumor cells during rapidly expanding tumors in relapse patients (Mellstedt, 1974; Björkholm *et al.*, 1976; Mellstedt *et al.*, 1976, 1980). However, other previously unrecognized factors may also be responsible for the transport of tumor B cells into the circulation.

Human leucocyte interferon (IFN-α) has some antitumor effect in selected myeloma patients. Patients with IgA and Bence–Jones myelomas seem to

TABLE V
MONOCLONAL BLOOD LYMPHOCYTES IN RELATION TO THE CLINICAL COURSE OF THE DISEASE IN MULTIPLE MYELOMA

Myeloma type	Time of test and clinical status	κ:λ[a] ratio	E+[a] (%)	M component	
IgG-λ + BJ-λ	At diagnosis stage I A	2.25	59	S-IgG[b]	36.5 g/liter
				U-BJ[c]	3.6 g/24 hr
	After 4 years first relapse	0.75	40	S-IgG	38 g/liter
				U-BJ	12 g/24 hr
	After 4½ years second remission	2.75	62	S-IgG	11.4 g/liter
				U-BJ	1.0 g/24 hr
	After 6½ years second relapse	0.04	33	S-IgG	12.4 g/liter
				U-BJ	8.5 g/24 hr
IgG-κ	At diagnosis stage III A	7.0	73	S-IgG	51 g/liter
	After 4½ years first relapse	16.0	40	S-IgG	87.7 g/liter
	After 5 years and intensified cytostatic treatment	2.0	67	S-IgG	38.6 g/liter
IgG-κ + BJ-κ	At diagnosis stage III B	161.0	20	S-IgG	35.6 g/liter
				U-BJ	9.9 g/24 hr
	After 2 months and 2 courses of high dose cyclophosphamide/prednisone	3.0	67	S-IgG	18.2 g/liter
				U-BJ	0.5 g/24 hr
BJ-κ	At diagnosis stage III B	124.0	28	U-BJ	7.5 g/24 hr

have the same response rate as those treated with intermittent high doses of melphalan/prednisolon, while there is no apparent effect in IgG myeloma patients (Mellstedt *et al.*, 1979; Lönnqvist *et al.*, 1982; Åhre *et al.*, 1983). The mechanisms for the therapeutic effect of IFN-α are unknown but two main possibilities have been suggested: (1) a direct antiproliferative effect on the tumor cells, and (2) augmenting possible antitumor activities of the immune system (e.g., natural killer cells). As exemplified by the results in Table V the ratio between κ-bearing and λ-bearing lymphocytes normalized after treatment with IFN-α suggesting that the monoclonal blood cell fraction had disappeared. In parallel the serum M component concentration decreased.

Similarly, cells secreting the idiotypic immunoglobulin were present in mononuclear cell suspensions from patients with active disease while no

TABLE V (*Continued*)

Myeloma type	Time of test and clinical status	κ:λ[a] ratio	E+[a] (%)	M component	
	After 1 month and 1 course of high dose cyclophosphamide/prednisone	14.0	nd[d]	U-BJ	3.0 g/24 hr
	After 6 months	4.0	71	U-BJ	<0.5 g/24 hr
IgD-λ + BJ-λ	1 month from diagnosis and 1 course of high dose cyclophosphamide/prednisone	1.5	60	U-BJ	2.0 g/24 hr[e]
	After 7 months in progression	0.06	55	U-BJ	12.0 g/24 hr[e]
IgA-κ	At diagnosis Stage II A	6.0	62	S-IgA	18.0 g/liter
	After 1 month daily IFN-α treatment (3×10^6 IE/day)	1.0	63	S-IgA	2.9 g/liter

[a] Controls: $n = 68$, mean ± SE; range: κ:λ ratio 2.1 ± 0.08; 1.0–3.3; E+ 66.0 ± 1.0; 41.0–81.0.

[b] S, Serum.

[c] U-BJ, Urine Bence–Jones protein excretion.

[d] nd, Not done.

[e] sIgD, not quantitated.

myeloma protein was produced in 7 day culture from patients in remission (Peest *et al.*, 1982). Using a plaque assay, Nagai *et al.* (1981) found that about two-thirds of myeloma patients had blood lymphocytes secreting immunoglobulin that carried the light chain isotype of the M protein. In the majority of cases no blood cells with cytoplasmic Ig were found. The number of monoclonal immunoglobulin-secreting cells fluctuated with the variation of the disease activity. Similar results have been presented by our group (Mellstedt *et al.*, 1976, 1980, 1982a; Pettersson *et al.*, 1980b) (Table V). Normalization of the ratio between κ-secreting and λ-secreting cells was a more rapid sign of response to therapy than, for instance, the decrease of serum M protein concentration and the increase of hemoglobin concentration. The increase in cells secreting monotypic Ig preceded other laboratory findings that indicated relapse. These observations are in good agreement

with earlier reports showing that idiotype-bearing lymphocytes could be detected in blood of myeloma patients up to 6 months before clinical relapse (Mellstedt *et al.*, 1976, 1980).

Hence, analysis of monoclonal blood lymphocytes may be a useful tool to monitor patients during the course of the disease. Detection of monoclonal blood cells may serve in guiding therapy, particularly because such cells may be responsible for the dissemination of the disease to the entire bone marrow compartment (Warner and Kreuger, 1978). Such tumorogenic lymphoid cells have been found in the circulation of mice bearing plasmacytomas (Warner *et al.*, 1977).

B. Waldenström's Macroglobulinemia

Monoclonal B lymphocytes bearing smIgM with idiotypic antigenic determinants of the serum M component can always be detected in bone marrow of patients with Waldenström's macroglobulinemia (Seligmann *et al.*, 1973; Pettersson *et al.*, 1980c). Cells secreting IgM with idiotypic determinants are also present (Hammarström *et al.*, 1980). Monoclonal lymphoid cells are also found in the lymph nodes and spleen.

Among 27 untreated patients with Waldenström's macroglobulinemia having normal lymphocyte counts, 55% had circulating monoclonal B lymphocytes. The percentage of B lymphocytes in patients with abnormal κ:λ ratios varied from normal values up to 80%. The number of monoclonal blood B lymphocytes was correlated with the disease activity, as exemplified by results from 3 patients shown in Table III. In patient M. M., assumingly with a small and stationary tumor mass, no circulating monoclonal B lymphocytes could be found. Two other patients (J. N. and A. B.) had progressive disease as judged by increased serum IgM concentrations and decreased hemoglobin concentrations as well as platelet counts. Patient J. N. also developed constitutional symptoms. During this period a large monoclonal B lymphocyte fraction appeared in both cases. A parallel increase of Ia^+ lymphocytes was noted in J. N. but not in patient A. B. Because the cell suspensions contained >98% lymphocytes, the Ia-like antigens were likely to be present on B lymphocytes. The discrepancy with regard to $smpv^+$ and Ia^+ lymphocytes may suggest that the monoclonal B lymphocytes were in different stages of maturation in these two cases; Ia antigens are sequentially lost during the B cell maturation (Winchester and Kunkel, 1979; Gordon *et al.*, 1983).

C. Benign Monoclonal Gammopathy

The serum M component in patients with BMG is small. Less than 10% plasma cells are usually found in the bone marrow suggesting a small tumor

volume (Thuresson, 1975). Not unexpectedly, the fraction of monoclonal bone marrow lymphocytes present in all BMG patients is small (Van Camp, 1980; Van Camp *et al.*, 1981).

BMG, which is a relatively common disease in elderly patients, has to be distinguished from its malignant counterparts. In the present study the diagnosis was based on the following criteria (Pettersson *et al.*, 1981): (1) immunoglobulin M component in serum or urine with no rise in the concentration during a >2-year observation period; (2) <10% plasma cells in the bone marrow; in patients with IgM M components no cytological picture of WM including no mast cells; (3) hemoglobin concentration >120 g/liter; (4) platelet counts $>150\times10^9$/liter; (5) no roentgenographic osteolytic lesions in the skull, vertebrae, or pelvic bones; and (6) no clinical symptoms.

Determination of the lymphocyte κ:λ ratio showed that a small subpopulation of blood monoclonal lymphocytes could be found in 3 out of 21 BMG patients (Pettersson et al., 1981). In the extended series of 37 patients, 5 had an abnormal κ:λ ratio (Table IV). Patient O. S. had had a stationary disease during 5 years before testing. Two years after testing she developed a clinically overt myeloma and died 6 months later from a cerebral hemorrhage. Autopsy revealed an advanced amyloidosis. Patient O. E. was observed 2½ years before testing. During a check-up 2 years later, the M component concentration (IgG) had raised from 29.0 to 41.0 g/liter. The bone marrow showed a myeloma-like picture. Patient H. E. had an advanced peripheral neuropathy which is sometimes seen in BMG but more frequently in malignant proliferation. The bone marrow pictures in patients L. O. and G. A. were possibly indicative of malignant plasma cell disease, but a definite diagnosis could not be made. As yet there has been no reason to question the diagnosis of BMG in the remaining 32 cases. Some of them have been retested, and the κ:λ lymphocyte ratios remained within the normal range.

Blood lymphoid cells bearing idiotypic immunoglobulin structures (1–4%) have also been detected in 3 out of 11 patients with BMG (Van Camp, 1980; Bast *et al.*, 1982). These 3 patients were classified as high risk according to the multivariate rating system by Morell *et al.* (1978), which is based on M component concentration, paraprotein synthetic rate, and bone marrow morphology. Such high-risk BMG patients have an increased risk to developing multiple myeloma. Thus, the finding of monoclonal blood B lymphocytes in BMG might indicate an imminent malignification. BMG patients having circulating monoclonal lymphocytes revealed by IFL should be checked carefully with regard to development of MM or WM, while other BMG patients can be checked less frequently. It should also be noted that a large monoclonal blood lymphocyte fraction may be revealed in BMG cases by use of more sensitive methods such as fluorescence-activated cell sorter (Carmagnola *et al.*, 1983).

The presence of monoclonal blood B lymphocytes in BMG has also been documented by studying monoclonal Ig production (Peest *et al.*, 1982). Blood mononuclear cells from an IgG-κ BMG patient produced idiotypic immunoglobulin in PWM-stimulated 7-day cultures. Polyclonal Ig production was decreased, which is in line with a low polyclonal serum Ig concentration in about half of the BMG patients (Lindström and Dahlström, 1978).

V. Concluding Discussion

Studies of human myeloma by the use of clonal immunoglobulin markers have clearly demonstrated the presence of B lymphocytes belonging to the malignant clone in bone marrow of all cases and in blood of some patients with active disease. Clonality has been defined from the pre-B cell level by the use of antiidiotypic antisera in some studies. These observations may suggest recruitment of malignant B cells from malignant precursor B lymphocytes. Experimental support for proliferation within the monoclonal B cell compartment gives further evidence for this notion. A similar B cell clonality has been described in murine plasmacytoma. The designation of human multiple myeloma as a differentiating B cell tumor (Fig. 2) raises three main questions. First, what is the ultimate malignant precursor cell in myeloma? Second, is the myeloma clone part of a nonmalignant blood stem cell clone? Third, are the malignant B cells susceptible to immune regulation, and if so, which regulatory signals participate?

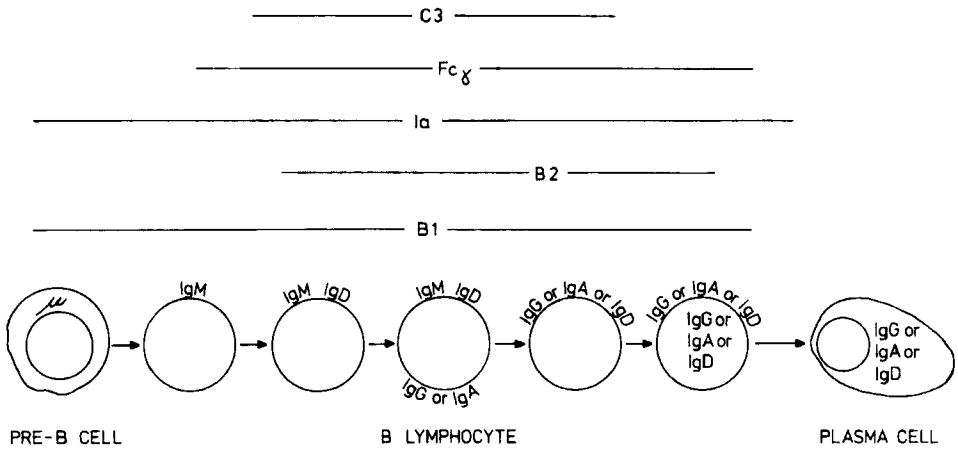

FIG. 2. Various maturation stages of the B cell lineage in multiple myeloma identified by antiidiotypic antisera and different B cell markers shown to be present on cells within the clone.

Pre-B cells were the earliest cells suggested to belong to the myeloma clone. However, pre-B cells were also the earliest B cells which could be identified by the use of antiidiotypic antisera. Since no studies with other markers of clonality (i.e., isoenzymes) have been performed, the possibility of a hematopoietic stem cell origin of human myeloma was not formerly excluded.

Such stem cell clones have been described in polycytemia vera and in chronic myelogenous leukemia by using A and B allelic forms of glucose-6-phosphatase dehydrogenase, with the X chromosome marker and the Philadelphia chromosome as clonal markers. Cells in various stages of differentiation emerging from a common stem cell have been identified. Apparently normal erythrocytes, granulocytes, megakaryocytes, and B lymphocytes also belong to the same clone of cells derived from a single multipotent hematopoetic stem cell (Adamson *et al.*, 1976; Fialkow *et al.*, 1977, 1978). Cairns (1975) and Mintz *et al.* (1978; Mintz and Illmensee, 1975) made a general suggestion that stem cells are invariably the target cell for malignant transformation; the affected stem cells in mouse embryonic teratocarcinomas may continue to differentiate in a normal manner following the oncogenic event. However, such a stem cell origin of multiple myeloma remains a speculative alternative open for testing.

The results of Cooper and co-workers suggest the expansion not only of monoclonal pre-B and $smIgM^+$ B cells but also of myeloma-related $smIgM^+$ and $smIgD^+$ B cell clones. The latter could also be identified in a small number of healthy subjects. Pre-B cells carrying cross-reacting but no individual specific idiotypes were not found. The myeloma clone and myeloma-related clones may emerge from a common stem cell which could generate a number of subclones, only one of which could fully differentiate into mature plasma cells. However, it should also be noted that myeloma-related clones are nonmalignant representatives of a clonal B stem cell expansion. Only one of the subclones undergoes malignant transformation and gives rise to the myeloma tumor. As a third alternative myeloma-related clones might expand as a result of a normal network regulation of the myeloma clone (Jerne, 1974).

Provided that the clonal event has taken place at the B stem cell level it is anticipated that multiple malignant B cell clones could appear in some patients. Such double clones have been described repeatedly, and the serum M components have been shown to share idiotypic specificities in some cases (Natvig and Kunkel, 1973; Wolfstein-Todel *et al.*, 1974; Scolari *et al.*, 1978; Kyle *et al.*, 1981). Moreover, the coexistence of chronic lymphocytic leukemia or lymphosarcoma and multiple myeloma seems to be more frequent than expected by chance. This observation has been used as an argument for a common precursor cell in such neoplasms (Vander and Johnson, 1960;

Preud'homme and Seligmann, 1972b; Narasimhan *et al.*, 1975; Holm *et al.*, 1977).

Sequential appearance of two B cell malignancies has also been described. Patients with a diagnosis of multiple myeloma have subsequently developed lymphocytic leukemia and vice versa (Fitzgerald *et al.*, 1973; Cryer and Kissane, 1975; Polliack *et al.*, 1977). An interesting case has been reported of a patient who had chronic lymphocytic leukemia of B lymphocyte type with appearance of δ-heavy chains and λ-light chains on the lymphocyte surface (Buchi *et al.*, 1982). Later this patient developed a typical plasma cell myeloma with an IgD-λ M component and IgD-λ chains in the plasma cell cytoplasm. The authors suggest that the disease in this patient may represent the evolution of the malignant lymphoproliferative disease with low aggressiveness into another highly malignant lymphoproliferative disorder. This might be a consequence of a switch-on in a previously blocked lymphocyte transformation, but it may also represent two related clones. However, as idiotypic cell markers have not been used in these studies, evidence for identity or relationship between the clones is lacking.

It is well recognized that human myeloma cells in contrast to murine myeloma cells are notoriously difficult to culture *in vitro*. Thus, to our knowledge, only two human myeloma cell lines have been established so far (Nilsson *et al.*, 1970; Karpas *et al.*, 1982). Moreover, although myeloma cells kept in short-term cultures spontaneously produce myeloma protein, differentiation into Ig-producing cells can not be enhanced by various T cell dependent or independent B cell mitogens such as EBV (Peest *et al.*, 1982, unpublished observation). This is in sharp contrast to the ease with which mitogens activate chronic lymphocytic leukemia cells to mature into immunoglobulin-producing cells (Robért, 1979). It may be that malignant myeloma precursor B cells are already fully turned-on to continuous and autonomous maturation into myeloma protein secreting cells. Autonomous propagation of the clone mainly within late immunoglobulin-producing cells that are unable to respond to mitogens may also contribute. Moreover, the malignant cells may lack mitogen receptors or otherwise be incapable of responding to mitogenic signals. However, in spite of such unresolved problems, the evidence for the presence of idiotype-bearing T cells and the expansion of T cells with class-specific Fc receptors in human myelomas may suggest that the tumor clone has elicited an immune response in the host. Idiotypic determinants on tumor B cells may serve as targets for immunological effector mechanisms, not only playing a role in the host regulation of the tumor but also providing attemptive and sometimes successful treatment, as seen in both experimental and human B cell tumors (for references, see Ritz and Schlossmann, 1982). Whether this is also the case in human

multiple myeloma and Waldenström's macroglobulinemia remains a goal for further research.

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IDIOTYPE NETWORK INTERACTIONS IN TUMOR IMMUNITY

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I. Introduction

It is well known that immune responses to tumors are very complex. Even within a given tumor model, different classes of T and B cells, natural killer cells, macrophages, cytotoxic or blocking antibodies, and complement may all play a part in the response to the tumor. It is not the purpose of this article to review comprehensively the various forms of tumor immunity, nor will I discuss all of the possible mechanisms that may regulate the delicate balance of tumor-specific responses (for review, see Naor 1979; Hellström *et al.*, 1977; Nossal, 1974; Prehn, 1976; Kamao and Friedman, 1977; Wheelock *et al.*, 1981). Most of the mechanisms that are proposed to regulate tumor immunity are not exclusive or antithetical. In this article, I will concentrate on discussing the regulation of tumor immunity by idiotype network interactions, reactions which so far have been given rather little consideration by tumor immunologists, although they hold particular promise for the selec-

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tive manipulation of immune responses in cancer patients. Thus, the purpose of this review is to discuss idiotype interactions which may be relevant and useful for understanding immune responses to tumors so that, in the long term, new or novel approaches to cancer immunotherapy may be developed.

II. Principle of Idiotype Network Interactions

One of the many mechanisms capable of regulating an immune response involves the interaction between idiotypes and antiidiotypes (for review, see Rowley *et al.*, 1973; Jerne, 1974; Urbain, 1979; Eichmann, 1978). The mechanism results because the receptor molecules on lymphocytes have unique conformational structures which not only specifically bind a given antigen but also act themselves as unique antigens (Kunkel *et al.*, 1963; Oudin and Michel, 1963). This antigenicity of the receptors is a composite of multiple antigenic determinants called *idiotopes*, the combination of which is termed *idiotype* (Oudin and Michel, 1963). Thus, T or B cell receptors can act as unique antigens which are characteristically different for each lymphocyte clone. These idiotypes can be recognized by other T or B lymphocytes, and the receptor of an antigen-specific lymphocyte can therefore not only bind to the antigen but can also interact with an antiidiotypic lymphocyte. The basic principle of such idiotype–antiidiotype interactions is shown in Fig. 1. Experimental studies have clearly demonstrated that such complementary lymphocytes are simultaneously present in the same individual and can interact with each other in a regulatory fashion (for review, see Eichmann, 1978). B and T lymphocytes specific for the same antigens may share idiotopes (Ramseier and Lindenmann, 1972; Binz and Wigzell, 1975a–c; Eichmann, 1978; Cosenza *et al.*, 1977; Weinberger *et al.*, 1979; Nadler *et al.*, 1982), even though there may be little similarity in the primary structure of the receptors (Jensenius and Williams, 1982; Nakanishi *et al.*, 1982). Thus, both types of lymphocytes may be affected simultaneously by an antiidiotypic antibody or cell. (For convenience, I will refer to these antiidiotypic T cells or antibodies as a probe.) Antigenic similarity of T and B cell receptors can occur either because short sequences or domains are shared or because pairs of proteins with little or no sequence homology can show very similar three-dimensional structures, hapten binding, and antigenic determinants (Richardson *et al.*, 1976; Young and Williams, 1978; Oliveira *et al.*, 1979; Volanakis and Kearney, 1981).

Since antiidiotypic immunity is directed against antigenic determinants on an individual's own immunoglobulin or T cell receptor, it follows that antiidiotypic lymphocytes also have their own idiotypic determinants on their antigen receptor. Therefore, these lymphocytes can in turn be recognized

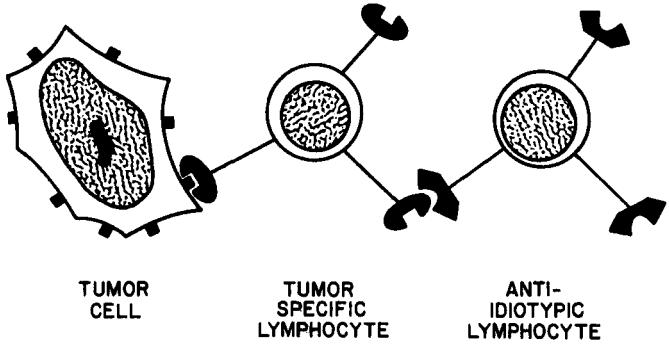

FIG. 1. Principle of idiotypic network interactions in tumor immunity. Tumor-specific and antiidiotypic lymphocytes have receptors which are complementary to one another. Clones of both types of lymphocytes coexist in the same individual and can potentially suppress or stimulate each other depending upon their functional subclass. Suppressive antiidiotypes may abrogate immunologic tumor-resistance, while stimulatory antiidiotypic immunity may help to enhance the antitumor response. (Reprinted from Schreiber *et al.*, 1982, with permission of Elsevier, Inc.)

by other complementary lymphocytes (Urbain *et al.*, 1977; Cazenave, 1977; Pollok *et al.*, 1982) so that one can conceive of a continuous chain of interacting lymphocytes. Jerne (1974) originally proposed the existence of such a complex network of idiotypically interacting lymphocytes, and he also suggested that it provided a basis for physiological regulation of immune responses. Although it is now clear that the immune system does include lymphocytes which have complementary idiotypes (for review, see Urbain, 1979), the relative importance of these network interactions in the physiological regulation of immune responses is still questionable. However, experiments have demonstrated that active or passive antiidiotypic immunity can very specifically and effectively either suppress or stimulate immune responses. It would thus appear that antiidiotypic probes may be a useful means of specifically manipulating immune responses to the advantage of the patient with cancer.

III. Idiotype-Specific Suppression of Immune Responses

A. Suppression by Administration of Antiidiotypic Reagents

A large number of studies have shown unequivocally that experimental animals can mount immune responses against their own idiotypes of either immunoglobulins or T cell receptors (Rodkey, 1974; Binz and Wigzell, 1976;

Andersson *et al.*, 1976; McKearn, 1974; Sakato *et al.*, 1977; Flood *et al.*, 1980a, 1981) by producing antiidiotypic antibodies or T cells. Antiidiotypic antisera recognizing idiotypic determinants on antigen receptors of animal or human lymphocytes can also be generated effectively by allogeneic or xenogeneic immunization followed by appropriate absorptions of the antisera. In this way, antiidiotypic antibodies to human immunoglobulin idiotypes can be raised (Kunkel *et al.*, 1963). Furthermore, a human syngeneic antiidiotypic suppressor T cell line has been isolated *in vitro* which suppresses a helper T cell line (Lamb and Feldmann, 1982). Passive administration of idiotype-specific antisera has been used to suppress humoral or cellular responses to a given antigen. For example, small amounts of antiidiotypic antibodies can effectively suppress immune responses to pneumococcal *C* polysaccharide, streptococcal *A* carbohydrate, *p*-azophenylarsonate, and histocompatibility antigens (Cosenza and Köhler, 1972; Hart *et al.*, 1972; Eichmann, 1974, 1975; McKearn, 1974).

Effective suppression of the immune response to an antigen can be achieved if the antiidiotypic antisera consist of multiple specificities for the idiotypes of all the different clonotypes involved in the immune response, or when monoclonal antiidiotypic antibody recognizes an idiotope on a dominant clone or a common idiotope which is shared among different clonotypes involved in the immune response to the same antigen (Claflin and Davie, 1974). The mechanism of suppression by antiidiotypic antisera may differ depending on the experimental condition. For example, antiidiotypic antisera given to adults may only induce short-term suppression (e.g., weeks to months) with subsequent reappearance of the same clonotypes, while the same antisera given to neonates may induce chronic, often life-long suppression, possibly caused by clonal elimination (Köhler, 1975). When idiotypically suppressed mice eventually regain responsiveness, they do so with new antibodies that are frequently products of minor (Urbain 1976) or previously silent clones (Augustin and Cosenza, 1976; Cosenza *et al.*, 1977; Flood *et al.*, 1980a). Such secondary antigen-reactive clones have escaped the suppression because they express idiotypes which are different from the original clones. Thus, the overall results are consistent with the notion that antiidiotypic immunity can eliminate responses to an antigen or profoundly alter the clonotypes of responding antigen-specific lymphocyte clones.

B. Suppression by Exposure to Idiotype on Antibodies or T Cells

Active immunization of a host with its own purified antigen-specific idiotype (on either T cells, antisera, or monoclonal antibody) evokes an autogenous antiidiotypic response. Such antiidiotypic immunity specifically suppresses the subsequent response to the relevant antigens (Rowley *et al.*,

1976; Binz and Wigzell, 1976; Andersson *et al.*, 1976; Bellgrau and Wilson, 1978; Flood *et al.* 1980a, 1981). For example, active immunization with a monoclonal (produced by a plasmocytoma) antibody specific for the hapten phosphorylcholine suppresses the subsequent active immunization with phosphorylcholine-containing antigens. This suppression included phosphorylcholine-specific antibodies that had a different idiotype than the antibody used for immunization (Rowley *et al.*, 1976). Since different phosphorylcholine-binding idiotypes are known to express a shared or common idiotope (Claflin and Davie, 1974), it is likely that the induced autogenous antiidiotypic immunity included reactivity against common idiotopes. Shared or common idiotopes have been reported in immune responses to several antigens (Sher and Cohn, 1972; Kuettner *et al.*, 1972; Eichmann, 1972; Flood *et al.*, 1981; Kohno *et al.*, 1982). In most immune responses, however, we do not know the degree of idiotypic heterogeneity so that immunization with the idiotype of a single clonotype may not necessarily alter the total response to the antigen but only the clonal profile of the response. In order to achieve effective suppression in these cases, immunization might have to include all the different idiotypes which can participate in the particular immune response. Such an approach may be possible by using as immunogen antigen-reactive lymphoblasts responding in culture to the particular antigen (Andersson *et al.*, 1976; Flood *et al.*, 1980a, 1981). This technique eliminates the need for extensive purification of the relevant idiotypic receptor molecules before using them as immunogen and selects for all the relevant idiotypes by specifically activating them in culture. Using this approach, specific suppression of responses to histocompatibility antigens have been reported (Andersson *et al.*, 1977; Aguet *et al.*, 1978; Binz and Wigzell, 1978).

The immunization of individuals against tumor-specific lymphocytes can have profound effects on tumor resistance and tumor growth (Flood *et al.*, 1980a, 1981). The general principle of such an experiment is shown in Fig. 2. The tumors used were two syngeneic fibrosarcomas, named 1591 and 1316, of C3H mouse origin which were induced with UV light. The tumors expressed non-cross-reacting tumor-specific transplantation antigens (Fisher and Kripke, 1977), and, similar to other UV-induced tumors, these tumors are so immunogenic that they are regularly rejected in normal syngeneic mice (Kripke, 1974, 1977). Tumor-specific lymphocytes were generated in culture from spleen cells of 1591-immunized animals (Flood *et al.*, 1980a, 1981). The 1591 tumor-specific lymphoblasts were then separated from the nonresponding lymphocytes and injected into normal animals. About two-thirds of the mice that had been immunized with 1591-specific lymphoblasts lost the ability to reject a primary tumor challenge of 1591 cells, despite the high immunogenicity of this tumor; however, the mice still rejected the

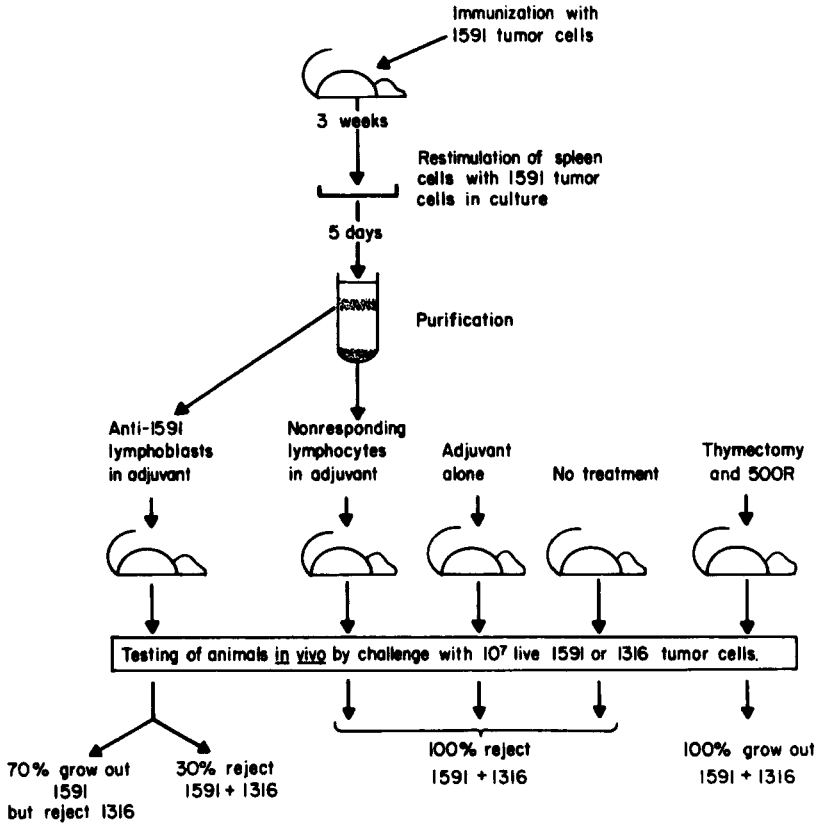

FIG. 2. Experimental protocol for the generation and purification of tumor-specific lymphoblasts, the induction of suppressive antiidiotypic immunity, and subsequent testing of animals by *in vivo* challenge with 1591 and 1316 tumor cells (for details, see Flood *et al.*, 1980a). (Reprinted from Schreiber *et al.*, 1982, with permission of Elsevier, Inc.)

similarly immunogenic but antigenically non-cross-reactive 1316 tumor. Furthermore, it was shown that these mice also failed to produce 1591-specific T lymphocytes, although they did generate specific lymphocytes reactive to the control tumor. Finally, spleens of these mice contained anti-idiotypic T lymphocytes since they contained T cells which selectively lysed 1591 tumor-specific lymphoblasts. It is interesting that about one-third of mice immunized with lymphoblasts did reject tumors, but these mice developed secondary clonotypes which were idiotypically different from the original clonotypes (Fig. 3).

Suppression of tumor rejection in these mice by immunization with antigen-reactive lymphocytes persisted until they died of progressive tumor

growth. This is different from the suppression of allograft rejection, which was usually only transitory, although it followed an analogous experimental protocol (Binz *et al.*, 1981). It is possible that the specific suppression of tumor resistance mediated by antiidiotypic immunity was also transitory but that the progressively growing tumor could maintain its continued growth by other suppressor mechanisms until the animal died of the tumor. Such suppressor mechanisms have been found to develop in animals with increas-

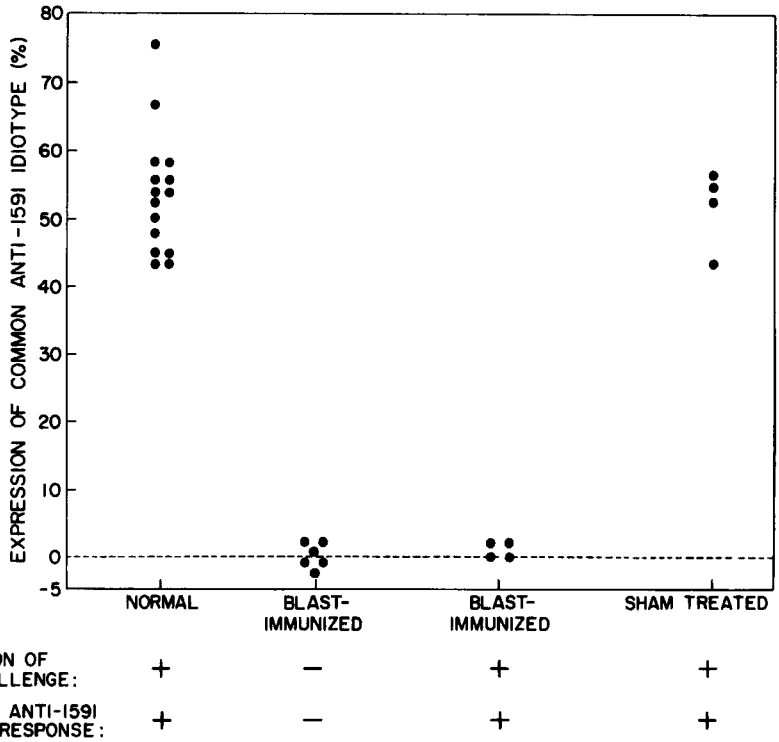

FIG. 3. Expression of a common idiotype on 1591 tumor-specific lymphocytes which is absent on lymphocytes of blast-immunized animals. Lymphocytes from normal, blast-immunized, or nonresponding lymphocyte-immunized (sham-treated) mice were stimulated in primary mixed-lymphocyte tumor cell cultures with 1591 cells. The culture-generated lymphoblasts were purified, ^{51}Cr-labeled, and used as targets in a ^{51}Cr-release assay with antiidiotypic effector cells. Each symbol represents the analysis of an individual animal. Blast-immunized animals consistently lacked the expression of the common anti-1591 idiotype which was regularly expressed by normal or control mice. Some blast-immunized mice could reject a 1591 tumor challenge. However, the 1591-specific lymphocytes from these mice were not lysed by the cytolytic antiidiotypic T cells (for details, see Flood *et al.*, 1980a). (Reprinted from Schreiber *et al.*, 1982, with permission of Elsevier, Inc.)

ing tumor burden (for review, see Naor, 1979). It is therefore possible that the mice that developed progressive tumor growth failed to regain immunoresistance with secondary clonotypes in time before the tumor burden was large enough to establish its own suppression.

Results consistent with the above findings have been obtained more recently using a different tumor model but similar general procedures (Leclerc *et al.*, 1981). Immunization with murine sarcoma virus (MSV)-specific T lymphoblasts drastically enhanced the outgrowth of MSV tumor cells and selectively inhibited the *in vitro* generation of MSV-reactive T cells. Analogous results were also obtained in a study using chemically induced rat tumors (Binz *et al.*, 1982). In addition, this latter study showed a rather striking correlation between the presence of circulating antiidiotypic antibodies and loss of immunologic tumor resistance. However, individuals that develop active antiidiotypic immunity also appear to develop antiidiotypic T cells (Binz and Wigzell, 1978; Flood, 1980a, 1981), so it is not clear to what extent the antiidiotypic antibodies were involved in the suppression of tumor resistance. These studies directly demonstrate the critical importance of tumor-specific lymphocytes in tumor rejection, as implicated in the early studies of Klein *et al.* (1960), and they clearly demonstrate that the induction of antiidiotypic immunity can be a powerful mechanism which suppresses effective tumor immunity.

In all of the studies just discussed, the mode of experimental induction of antiidiotypic immunity was by immunization with idiotype in adjuvant, and this protocol may therefore be irrelevant to any clinical situation. However, it is conceivable that immunotherapeutic injections of Bacille Calmette Guerin (BCG) or other adjuvants into lesions containing tumor-specific lymphocytes may induce the generation of antiidiotypic immunity and therefore stimulate rather than suppress the growth of the tumors. Adverse effects of such adjuvant treatments have been described (Piessens *et al.*, 1970; Kirchner *et al.*, 1975; Glaser, 1978). A number of studies have also shown that the presence of adjuvant is not essential for the induction of antiidiotypic immunity by exposure to idiotype. For example, immunization with antigen–antibody complexes has been shown to elicit the induction of antiidiotypic antibodies to self idiotypes even when given in minute amounts and in the absence of adjuvant (Klaus, 1978). Also, antiidiotypic immunity can develop when antigen-specific T cells (Bellgrau and Wilson, 1978) or T cell lines are transferred to mice without adjuvant (Infante *et al.*, 1982) or after injection of hybridoma antibodies into patients (Colvin *et al.*, 1982). Thus, antiidiotypic immunity may develop as a result of immunotherapy when tumor-specific antibodies or T cells are passively transferred. Clearly, development of such immunity could interfere with the wanted therapeutic effects.

C. Suppression by Exposure to Antigen

Antiidiotypic immune responses may develop not only as a consequence of experimental or therapeutic procedures involving the direct exposure to idiotypic-bearing cells or molecules, but they may also develop spontaneously after simple exposure to antigen. Thus, experiments have suggested that antiidiotypic immunity may provide physiologically significant regulation of immune responses. For example, immunizations to phosphorylcholine antigens (Kluskens and Köhler, 1974), levan (Bona *et al.*, 1978), TNP–Ficoll (Goidl *et al.*, 1979), histocompatibility alloantigens (McKearn *et al.*, 1974; Binz *et al.*, 1979), and tobacco mosaic virus (Urbain 1976) first elicited antibodies to the antigens. Then as these antibodies declined, autoantiidiotypic immune responses were detected which were specific for the idiotypes of the primary antibodies. Although such autoantiidiotypic immune responses have been reported to occur after a single immunization with antigen (Kelsoe *et al.*, 1980), usually repeated chronic antigenic stimulation was needed before a significant decline of the immune response and induction of antiidiotypic immunity was observed.

It is reasonable to assume that development of antiidiotypic immunity may affect the host response to cancer. Carcinomas may persist in a preinvasive form for many years, and signs of chronic irritation and antigenic stimulation may often be observed at the site of development. Possibly, antiidiotypic immunity develops during the continued and increasing antigenic challenge. For example, antiidiotypic antibodies have been found to develop in melanoma patients, and the appearance of these antibodies correlated with the decline of tumor-specific response and spread of the malignant disease (Lewis *et al.*, 1971, 1976; Hartmann and Lewis, 1974; Hartmann *et al.*, 1974; Jerry *et al.*, 1976). However, the functional significance of the observed antiidiotypic immunity was not determined, and further investigation must determine whether development of such antiidiotypic immunity is an important factor in the clinical course of cancer and whether such adverse immunity can result inadvertently from immunotherapeutic procedures. Similarly unanswered is the question of whether antiidiotypic antibodies or antiidiotypic T cell receptor molecules are involved in the composition of some serum-blocking factors that are found in cancer-bearing individuals (Hellström *et al.*, 1969). Since antiidiotypic molecules react with the receptors of tumor-specific lymphocytes, they may block the activation of these lymphocytes, as do tumor antigen-specific blocking factors.

D. Relationship to Age-Related Immune Defects

An exploration of the relationship between antiidiotypic immunity and age-related loss of immune responsiveness is especially important since most

cancers develop in older individuals, beginning in middle age. Furthermore, several studies have implicated antiidiotypic immune suppression as an important factor in the age-related loss of immune functions (Szewczuk and Cambell, 1980; Goidl *et al.*, 1980; Klinman, 1981). The considerable magnitude of the age-related loss of immune responses to tumors can be seen from the example of the highly immunogenic syngeneic UV-induced tumor 1591. This tumor regularly regresses when transplanted into young adult syngeneic mice. [On the rare occasion that the tumor grows progressively, it has lost a major unique tumor-specific transplantation antigen (Urban *et al.*, 1982a).] This tumor will, however, grow progressively in old mice (Flood *et al.*, 1981), with a decrease in primary resistance beginning at 9 to 12 months, which is during the middle age of these mice. At this age, animals also begin to lose the capacity to mount tumor-specific immune responses to this tumor *in vitro*. During this decline, qualitative changes in the response, such as changes in specificity and clonotype, were demonstrated. Thus, the response of normal young mice to the tumor cells *in vitro* was regularly associated with the expression of a dominant common 1591 tumor-specific "idiotype" as defined by an antiidiotypic probe, but at about 8 months of age, this dominant common idiotype began to decline and other tumor-specific lymphocyte clones that were idiotypically different began to predominate. With further increase in age, the response *in vitro* was no longer tumor-specific and showed marked cross-reactivity and autoreactivity with other syngeneic tumor targets and nontransformed cell lines. In the light of these findings of age-related changes in T cell idiotopes, it is interesting that it has also been suggested that the expression of B cell idiotypes also undergoes changes during aging (Szewczuk and Cambell, 1980; Goidl *et al.*, 1980).

Several other studies have indicated that older animals have an increased tendency to generate immune reactivity to autologous and third-party target cells and to generate low affinity antibody-producing cells (Kruisbeek and Steinmeier, 1980; Ben-Nun and Cohen, 1980; Goidl *et al.*, 1976). Although the age-dependent increase in generation of such cross-reactive cells has been demonstrated *in vitro*, there is considerable evidence that autoimmunity also develops *in vivo* with increase in age (for review, see Makinodan and Kay, 1980). While the reasons for the development of autoreactivity with age are poorly understood, such reactivity may well include autoantiidiotypic antibody and T cells. For example, it has been reported that after immunization, old animals have an increased tendency to generate low-affinity antibodies and possibly also autoantiidiotypic antibodies (Szewczuk and Cambell, 1980; Goidl *et al.*, 1980). Furthermore, it was shown that old mice have Igh-restricted T suppressor cells (possibly antiidiotypic) that can suppress antigen-specific B cell responses of young mice (Klinman, 1981).

Although antiidiotypic reactivity was not demonstrated, it was postulated that as a result of a life-long series of antigenic experiences, antiidiotypic suppressor mechanisms might accumulate, causing a down-regulation of the immune responses to much of the antigenic universe (Klinman, 1981).

In all of the previously discussed studies, it is still unclear whether old animals selectively produce antiidiotypic reactivity after immunization or whether such reactivity develops as part of general autoreactivity (Van Snick and Masson, 1980). In other words, Is suppression specific for a particular response or is it the result of generalized immunosuppression? Flood *et al.* (1981) found that aged animals have a decreased capacity to generate cytolytic immune responses to both the tumor antigen and the tumor-specific idiotype. Thus, it appears that old animals may have a generally decreased capability to mount immune responses which are highly specific. However, other antiidiotypic components such as helper T cells were not assayed in these studies. It is therefore still possible that age-dependent changes in the antiidiotypic compartment, such as a loss of responsiveness of an antiidiotypic helper T cell, might contribute to the loss of tumor resistance in old mice. At present, however, it is unknown whether antiidiotypic T helper cells can affect the immune response of T cells, although they can play a role in the response of B cells (see Section IV).

IV. Idiotype-Specific Stimulation of Immune Responses

A. Stimulation by Idiotype-Specific T Cells

In the experiments mentioned in the previous section, antiidiotypic antibodies or T cells, given passively or produced endogenously, had a suppressive effect on the clones carrying the respective idiotype. These results fit the hypothesis that the interactions between complementary clones is predominantly suppressive, an essential feature of a tightly regulated functioning network. As Jerne (1974) pointed out, regulatory networks are not functional unless the negative or suppressive components exceed the positive stimuli. Thus, exposure to antigen may only cause a temporary disturbance in this immunological network of balanced suppression. However, it has also been reported that, at least in some instances, naturally occurring antiidiotypic helper T cells seem to be required for the optimal response of certain B cell clonotypes which express the complementary idiotope (Woodland and Cantor, 1978; Hetzelberger and Eichmann, 1978; Eichmann *et al.*, 1978; Adorini *et al.*, 1979; Bottomly and Mosier, 1979, 1981; Bottomly *et al.*, 1980). These helper T cells apparently act in cooperation with the classical carrier-specific helper T cell that were first defined by Mitchison (1971).

Many other studies have also suggested that two different types of helper T cells can cooperate in the induction of antibody synthesis by B cells (Marrak and Kappler, 1974; Janeway *et al.*, 1977, 1980; Tada *et al.*, 1978). Although it has been suggested that the induction of antiidiotypic T helper cells might depend upon presence of the idiotype in the host environment (Bottomly and Mosier, 1979), this may not necessarily be so (Quintans *et al.*, 1982). Unfortunately, it is not known to what extent antiidiotypic T helper cells influence the immune response of T cells, but it is conceivable that such antiidiotypic T cells may not only alter the clonal profile of responding tumor-specific T cells but also the magnitude of their response to the tumor antigen.

B. Stimulation by Idiotype-Specific Antibodies

Antiidiotypic antibodies can stimulate B and T cells bearing the complementary idiotype. Thus, B cells (Trenkner and Riblet, 1975) and various T cell subsets such as helper cells (Eichmann, 1975; Julius *et al.*, 1978), suppressor cells (Yamamoto *et al.*, 1979), and T cells involved in DTH reactions (Sy *et al.*, 1980; Thomas *et al.*, 1981; Arnold *et al.*, 1982) have been activated by heterologous or isologous antiidiotype antibodies. Specific immune reactions against histocompatibility alloantigens have been induced using autoantiidiotypic antibodies under defined conditions (Frischknecht *et al.*, 1978; Binz *et al.*, 1979; Bluestone *et al.*, 1981). These antibodies have been used as highly potent substitutes for the allogeneic stimulator cells, and they clearly induce high titers of antibody to transplantation antigens. The use of antibody as antigen eliminated the need for antigen during the immunization process (Bluestone *et al.*, 1981; Frischknecht *et al.*, 1978). In most of these experiments, low doses of the passively administered antiidiotypic antibody were required for optimal stimulation of the idiotype-positive B or T lymphocytes while high doses were commonly suppressive (Eichmann, 1978; Kelsoe *et al.*, 1981). An exception to this rule is the Ig class dependence of the effects of heterologous antiidiotypic antisera in the streptococcal A carbohydrate system. In this system one class (IgG_{2a}) of antiidiotypic antibodies suppressed the response while another class (IgG_1), at similar doses, specifically stimulated idiotype-carrying clones (Eichmann, 1974), even in the absence of antigen (Hetzelberger and Eichmann, 1978; Eichmann *et al.*, 1978).

In vitro, antiidiotypic antibodies could also directly induce the development of alloantigen-specific cytolytic T cell responses (Binz *et al.*, 1979; Strong *et al.*, 1979). In these studies, cytolytic T cells were generated in primary cultures using normal Lyt-$1^-2^+3^+$ T cells and antiidiotypic antibodies. In fact, the antiidiotypic antibodies served as a more potent triggering signal than the allogeneic cells themselves, and use of these antibodies

eliminated the need for Lyt-$1^+2^-3^-$ alloantigen-specific helper T cells (Binz *et al.*, 1979). Similarly, antiidiotypic antisera specific for the idiotypes of tumor-specific lymphocytes have been used to restimulate tumor-specific cytolytic T cells in culture in the absence of tumor antigen (Binz *et al.*, 1982; Schreiber *et al.*, unpublished results). Antiidiotypic antibodies can also directly stimulate Lyt-1^+ alloantigen-specific helper T cells to proliferate *in vitro* in the absence of the relevant antigen (Infante *et al.*, 1982). Thus, these experiments show that B cells and various subsets of T cells, such as cytolytic T cells, helper T cells, and delayed-type hypersensitivity (DTH)-mediating T cells, can be specifically activated by antiidiotypic antibody. This is important since DTH-mediating T cells have been implied to play a major role in transplantation resistance to tumors (Leclerc and Cantor, 1980; Bhan *et al.*, 1981) and allografts (Loveland *et al.*, 1981). It has been shown that a monoclonal antibody without detectable tumor reactivity could prime for DTH to a tumor in an Igh restricted fashion (Forstrom *et al.*, 1983). However, antiidiotypic reactivity of this antibody could not be shown leaving the mechanism for the observed stimulatory effect undetermined.

The specific stimulation of immune responses with antiidiotypic antibodies or T cells is only possible because antigen receptors of different specificities apparently express uniquely different idiotypes. However, it is also clear from a number of studies that even lymphocytes with different specificities may express the same idiotypic determinants (Cazenave *et al.*, 1974; Dzierzak and Janeway, 1981; Bluestone *et al.*, 1981). Therefore, non-antigen-binding idiotype-positive clonotypes may be induced as a result of immunization with antiidiotypic molecules. Nevertheless, even in these situations, subsequent immunization with antigen preferentially induces the antigen-specific clonotypes expressing the particular idiotypes (Urbain *et al.*, 1977; Cazenave, 1977).

V. Rescue of Immune Responses by Idiotype-Specific Manipulations

A. Rescue by Elimination of Idiotype-Specific Suppression

It was outlined in Section IV,B that active immunization with antiidiotypic antibodies can induce antigen-reactive lymphocyte clones expressing complementary idiotypes. Because of our present ignorance, it is not known whether this phenomenon is due to the direct induction of idiotype-positive, antigen-specific B cells and helper T cells, or whether it is due to induction of anti-(antiidiotypic) immunity. Such second-degree idiotypic–antiidiotypic interactions may counteract or eliminate antiidiotypic suppressor cells and thereby allow the expression of antibodies possessing the idiotype. It has

been shown that such antiidiotypic T suppressor cells are sometimes "preexisting" and thus may normally suppress the idiotype positive cells (Bona and Paul, 1979; Bona *et al.*, 1979), while in other situations such cells may be generated as a result of single or prolonged antigenic stimulation. As mentioned earlier in Section III,C, the stimulation of suppressive antiidiotypic immunity may occur in cancer patients as a result of chronic antigenic challenge during tumor development and growth or as a result of direct exposure to tumor-specific antibodies or T cells or complexes of these reagents with tumor cells during passive immunotherapy.

When considering the strategies for the elimination of antiidiotypic suppressor T cells, it should be remembered that they react with two different sets of complementary lymphocytes: the tumor-specific, idiotype-positive lymphocytes and the anti-(antiidiotypic) lymphocytes. Either clone may potentially regulate or suppress the antiidiotypic T cells. Alternatively, the regulatory interactions of these complementary clones of lymphocytes may be unidirectional, e.g., the anti-(antiidiotypic) T cells but not the idiotype-positive, tumor-specific T cells, can eliminate the antiidiotypic suppressor T cells. This question is important but unresolved. Obviously, elimination of antiidiotypic suppressor cells would be easier with antigen-specific idiotypic positive reagents than with anti-(antiidiotypic) reagents, since the former are much more readily produced in the laboratory. In the context of the possible elimination of antiidiotypic suppression by idiotype, it is interesting to note that injection of idiotypic antibody into the host can protect or induce the B clone producing it (Rowley *et al.*, 1981; Forni *et al.*, 1980; Kelsoe *et al.*, 1981). However, whether this is due to the elimination of or release from antiidiotypic suppression by idiotype is presently unknown. It has also been suggested that idiotype may be required to protect the particular clonotype when it is being rescued from "stem cells" following elimination of the suppressor system (Rowley *et al.*, 1981).

B. Rescue by Elimination of Internal Image Clones

A variety of mechanisms have been proposed to explain the insufficient or sometimes undetectable immunogenicity of tumor-specific antigens (Herberman, 1974); most of these explanations are neither antithetical nor mutually exclusive. The lack of immunogenicity of some tumors may occur because their tumor-associated antigens are not strictly tumor-specific, i.e, they are similar or identical to self antigens to which the host is tolerant. Molecules which carry such determinants could provide an internal image of the foreign or tumor-specific determinants. In some cases these determinants may be on immune receptors of lymphocytes and are then referred to as "internal image" clones (Jerne, 1974), while in other cases such internal

image determinants may be on nonimmunoglobulin structures on lymphocytes or any other cell.

These self molecules that share epitopes with the tumor antigen may be molecularly different from the tumor antigen since antigenic determinants of even different molecular structures can be bound by the same immune receptor with similar affinity (Richards *et al.*, 1975; Volanakis and Kearney, 1981). The complete restriction of an antigen to a tumor is quite difficult to prove, and there are several tumor antigens that were originally thought to be tumor-specific but were later found on a number of mature, embryonic, or regenerating normal host cells. These antigens may also occur in small amounts in the serum of normal individuals. Examples of such tumor-associated antigens are idiotypic antigens on the surface of malignant B cells, carcino-embryonic antigens, and other differentiation antigens. Previous studies have shown that active or passive immunity to such clonal, differentiation, or embryonic antigens can serve as target antigen for immunotherapy (Lynch *et al.*, 1972; Coggin *et al.*, 1971; Bernstein *et al.*, 1980). However, the immune protection that immunity to such antigens provides is usually weak, possibly because of the presence of normal cross-reacting molecules in the body to which the host is tolerant.

It would be important to be able to increase the immunogenicity of such antigens. Studies on immune responses to myeloma proteins provide an example of how elimination of a normal internal image antigen can affect the response to a tumor antigen. Myeloma proteins on the surface of myeloma cells can serve as tumor transplantation antigens (Lynch *et al.*, 1972), although the protective effect of the immunization greatly depends on the immunogenicity of their idiotypes. The degree of this immunogenicity seems to be inversely related to the natural abundance of an idiotype (Sakato *et al.*, 1977). For example, BALB/c mice are usually unresponsive to the T15 myeloma protein, because this idiotype is the main idiotype stimulated by phosphorylcholine-containing antigens in these mice (Sher and Cohn, 1972) and occurs in significant levels in the serum of unimmunized mice raised in a conventional environment. The failure of responsiveness seems to result from the abundance of this T15 idiotype in these BALB/c mice and is not the result of a dominant *Ir* gene (Sakato and Eisen, 1975). Also, BALB/c mice which are germ free or are suppressed with antiidiotypic antibodies as neonates do not express the T15 idiotype in their normal or immune serum but readily produce antiidiotypic antibodies and T cells when immunized with the T15 myeloma protein (Sakato *et al.*, 1977). Furthermore, mice suppressed with anti-T15-idiotypic antiserum can have autogenous antiidiotypic suppressor T cells even without previous immunization with the idiotype (DuClos and Kim, 1977; Schreiber *et al.*, 1977; Bottomly *et al.*, 1978). An explanation for this might be that the antiidiotypic antibodies eliminated

idiotypic lymphocytes and thereby changed the preexisting balance of the idiotypic and antiidiotypic clones in the host. In this way, the relative size of the anti-T15 clone might be specifically increased. Analogous to this example, it is possible that passive administration of antibodies which are specific for determinants on tumor antigens can increase the immunogenicity of these antigens. Such antibodies may suppress lymphocyte clones which express idiotypic determinants representing internal images of the antigenic determinants on the tumor antigen and this in turn may lead to the induction of stronger immune responsiveness of the host to the otherwise weak tumor antigens.

C. Rescue by Elimination of Antigen-Specific Suppression

Immunological resistance against a tumor can be dependent on the balance between the activation of tumor-specific effector T cells on one hand and tumor-specific suppressor T cells on the other hand (Naor, 1979). Such suppressor T cells (Gershon *et al.*, 1974) play a critical role in several tumor systems and may be induced either by the tumor (Gershon *et al.*, 1974; Fujimoto *et al.*, 1976) or by the carcinogen, such as UV light (Fisher and Kripke, 1977; Daynes and Spellman, 1977). Furthermore, suppressor cells may appear "spontaneously," at least in some strains of mice, during aging (for review, see Makinodan and Kay, 1979). Antigen-specific suppressor T cells may also be produced when tumor cells are inoculated along with antibody (Gershon *et al.*, 1974). These suppressor T cells may block the maturation of cytolytic T cells or of helper T cells needed for the production of antigen-specific effector cells, but suppressor T cells usually do not suppress mature tumor-specific effector cells (e.g., Kripke, 1981).

Because suppressor T cells and tumor-specific effector cells seem to react with different determinants or antigens on the tumor cell (Kripke, 1981; Greene, 1980), they probably have different idiotypes. Therefore, it should be possible to eliminate or circumvent the effect of tumor-specific T suppressor cells by two different approaches: specific stimulation of the effector T cells or specific elimination of the suppressor T cells. The first approach is related to the finding that antiidiotypic antibodies can stimulate cytolytic or DTH-mediating effector T cells directly (see Section IV,B). Since antigen is not required for such stimulation, suppressor T cells may not have the antigen bridge needed to interfere with the induction of effector cells. Also, once the effector T cells are mature, they are usually resistant to the suppressor T cells and could then eliminate the tumor without such interference. In the second approach, one might attempt to eliminate the suppressor T cells by antiidiotypic immunity rather then to circumvent their action. For this approach, one could either passively administer suppressive

antiidiotypic reagents specific for the idiotypes of the suppressor T cells or actively immunize the tumor-bearing host with the tumor-specific suppressor T cells.

Experiments related to this latter approach have been reported by Tilken *et al.* (1981). Normal mice were repeatedly immunized with spleen cells from tumor-bearing mice in Freund's adjuvant, and then challenged with tumor cells. The results of a single experiment showed that this tumor challenge was rejected by some of the mice (3 out of 18) previously immunized with spleen cells of tumor-bearing individuals, while none of the control mice (0 out of 10) that had been immunized with adjuvant alone rejected the tumor challenge. Furthermore, the average tumor weights were slightly smaller in the mice previously immunized with the spleen cells of tumor-bearing mice, although there was some overlap between the two groups. If antiidiotypic immunity was indeed induced, one does not know whether it was exclusively directed against tumor-specific suppressor cells or also against tumor-specific effector cells. For example, the immunizing spleen cells might have contained the precursor of tumor-specific effector T cells as well as suppressor T cells, and both types of T cells may therefore have been suppressed. This is particularly important since autoantiidiotypic immunity induced by effector T cells has been shown to result in loss of tumor resistance (Flood *et al.*, 1980a, 1981). Therefore, the induction of suppression of both effector and suppressor T cells may be responsible for the limited success of the above experiment, and a much clearer effect might have been obtained if pure suppressor T cell populations had been used for the induction of suppressive antiidiotypic immunity.

D. Rescue by Using Stimulatory Idiotype-Specific Probes

At present, there is no evidence showing that stimulatory antiidiotypic probes can be used for the rescue of tumor-specific immune responses, although such a strategy holds considerable promise. For several reasons, this approach might be highly advantageous for manipulating immune responses under conditions where antigen-specific suppressor cells are present. First, antiidiotypic probes could stimulate the induction of effector T cells without inducing suppressor T cells. This could occur, as previously mentioned, because suppressor T cells and effector T cells probably express different idiotypes. Second, already existent antigen-specific suppressor cells would not be able to interfere with the induction of effector T cells because antiidiotypic antibodies can stimulate effector T cells directly in the absence of antigen and therefore do not provide, as antigen does, a bridge for the action of suppressor T cells.

The direct stimulation of tumor-specific effector cells with antiidiotypic

probes may also be quite useful in situations where the tumor-specific clonotypes are not sufficiently stimulated because of a "defective immune environment." Such a defective environment may exist in aged mice (Flood *et al.*, 1981). We have shown that such mice are immunologically unresponsive to a tumor *in vivo* but their cells *in vitro* can be made to mount tumor-specific immune responses under particular culture conditions (Urban and Schreiber, unpublished results). Thus, "clonal exhaustion" (Askonas and Williamson, 1972) may not be responsible for the age-dependent loss of the response tumor-specific lymphocyte clones, and there is a potential to recover responses in old animals by restoring a proper environment, possibly by stimulation with idiotype-specific probes. This may not only restore the necessary threshold required for an effective immune response to the tumor but may also circumvent the negative effects of suppressor mechanisms possibly existing in the old host. In any event, rescuing defective tumor resistance in old individuals is an important task. As long as we can not rescue the immune response of older individuals (middle age to elderly) to very immunogenic tumors(e.g., UV-induced regressor tumors in mice), it will be difficult to achieve effective immunity to other less immunogenic tumors.

VI. Idiotype on Lymphoid Tumors and Idiotype-Specific Regulation

Lynch *et al.* (1972) found that the immune response to idiotypic determinants on a myeloma protein specifically suppressed growth of transplanted myeloma cells. This finding was interesting to immunologists for several reasons. First, surface immunoglobulin, and thus surface idiotype, is present on various human B cell-derived malignancies such as chronic lymphocytic leukemia, B cell lymphomas (including Burkitt lymphomas), and myelomas; therefore surface immunoglobulin may be clinically important as a tumor antigen (Lampson and Levy, 1979). Second, since there is detailed information on the origin, function, and molecular structure of surface immunoglobulin, this can be a useful, well-defined model tumor antigen on experimental tumors. Third, since malignant lymphoid cells may still be susceptible to certain regulatory signals from normal lymphoid cells, the expression of idiotype by B cell tumors may be used as a model for studying B cell differentiation or B–T cell regulatory interactions (for review, see Abbas, 1982).

Using this tumor model, it was shown that passive administration of anti-idiotypic antibodies could suppress the growth of idiotype-bearing myeloma cells *in vivo* (Chen *et al.*, 1976; Beatty *et al.*, 1976) and *in vitro* in the presence of complement or normal spleen cells (Schreiber and Leibson, 1978; Mahony *et al.*, 1981). Antiidiotypic antibodies were also cytotoxic to

leukemic B cells and B lymphoma cells *in vitro* (Sugai *et al.*, 1974; Stevenson *et al.*, 1977a; Lanier *et al.*, 1980; Hatzubai *et al.*, 1981) and when administered *in vivo* (Stevenson *et al.*, 1977b; Haughton *et al.*, 1978). Antiidiotypic antibodies have been coupled to the toxin ricin to increase their therapeutic effectiveness *in vitro* and *in vivo* (Korlick *et al.*, 1980). Monoclonal antiidiotypic antibody has been used effectively *in vivo* in a patient with advanced B cell lymphoma (Miller *et al.*, 1982). Idiotype-specific antibodies can also be used to follow the clinical course and effect of therapy by measuring serum levels of the idiotype produced by the tumor (Haughton *et al.*, 1978; Brown *et al.*, 1980) and to identify the malignant cells in a patient that belong to a particular tumor (Hurley *et al.*, 1978; Van Acker *et al.*, 1979), including their earliest stages (Kubagawa *et al.*, 1979).

Chemotherapy and antiidiotypic immunotherapy have been successfully combined *in vivo* (Lanier *et al.*, 1979). However, the therapeutic usefulness of the antiidiotypic probes may be affected by the concurrent use of chemotherapeutic agents. For example, while pretreatment of tumor cells *in vitro* with physiologic doses of chemotherapeutic agents killed most of the tumor cells, the few tumor cells that escaped drug treatment showed a marked difference in their susceptibility to subsequent treatment with antiidiotypic antibody (Leibson *et al.*, 1978; Shapiro *et al.*, 1982). The effectiveness of the antiidiotypic agents may also be dictated by certain features of the tumor cell (Taupier *et al.*, 1983). For example, the magnitude of the observed effect of antiidiotypic antibody on idiotype-bearing tumor cells was positively influenced by the amount of surface idiotype and negatively affected by the amount of idiotype secreted (Schreiber and Leibson, 1978; Leibson *et al.*, 1979). Furthermore, effectiveness depends on the isotype of the antiidiotypic antibody. Isologous murine antiidiotypic antibodies often belonging predominantly to the IgG_1 class do not effectively fix complement or attract effector cells, and it is thus not surprising that isologous antiidiotypic antisera are often not effective *in vivo* or *in vitro* (Sugai *et al.*, 1974; Frikke *et al.*, 1977; Flood *et al.*, 1980b).

Despite a number of studies, it is still unclear by what mechanism mice immunized with idiotype acquire transplantation resistance to idiotype-bearing malignant B cells. It has been shown that the level of circulating antiidiotypic antibodies does not correlate well with the level of transplantation resistance, and this may be related to the isotype of the antibody as mentioned previously (Frikke *et al.*, 1977). Several other lines of evidence suggest that T cells are required for transplantation resistance to myeloma growth in mice immunized with myeloma protein. In particular, postimmunization thymectomy abrogated the resistance to tumor growth *in vivo* (Daley *et al.*, 1978), and noncytolytic T cells purified on idiotype-coated plates could specifically, though only temporarily, affect the growth of the

relevant myeloma cells *in vitro* (Flood *et al.*, 1980b). These T cells did not exert cytolytic effects, although other investigators have suggested that cytolytic T cells specific for idiotypic or allotypic determinants on surface immunoglobulin do exist (Sugai *et al.*, 1974; Snodgrass *et al.*, 1981a,b; Forman *et al.*, 1981). Thus, several idiotype-specific antibody or T cell-mediated mechanisms may be involved in the generation of idiotype-specific transplantation resistance to malignant B cells.

Carrier-specific helper or suppressor T lymphocytes can stimulate or suppress the proliferation of Ig secretion of myeloma cells *in vivo* or *in vitro* in the presence of hapten carrier conjugates (for review, see Lynch *et al.*, 1979). Antiidiotypic suppressor T cells have also been found to suppress secretion by myeloma cells *in vivo* (Rohrer *et al.*, 1979) and *in vitro* (Abbas *et al.*, 1980). It is also possible that antiidiotypic helper T cells stimulate secretion of myeloma cells, but this has not been demonstrated. Although such immunologic regulation of lymphoid tumor cells suggests an attractive way for studying idiotype–antiidiotype interactions (for review, see Abbas, 1982), there are some major problems. For example, monoclonal antiidiotypic antibodies of the IgG_1 class, even at large doses, have little measurable effect on myeloma cells *in vivo* or *in vitro*. However, when given to neonates even at minute doses, they cause a life-long clonal elimination of the relevant normal B cell clone (Kearney *et al.*, 1981). There are also certain problems in using the idiotype on malignant B cells as an example of a tumor antigen. For example, regulatory helper and suppressor T cells may directly modulate the proliferation of tumor cells that are of lymphoid origin, in contrast to nonlymphoid tumor cells which do not normally accept regulatory signals from lymphocytes. Instead, the growth of nonlymphoid tumor cells may be affected only indirectly by regulatory T cells because they regulate the activity of tumor-specific effector T cells.

VII. Problems and Challenges of Idiotype-Specific Regulation of Tumor Immunity

A. Complexity of Idiotype-Specific Interactions

It is now firmly established that idiotypes and antiidiotypes can coexist in the immunologic repertoire of a single individual (for review, see Jerne, 1974; Eichmann, 1978; Urbain, 1979). Antiidiotypic immunity has been induced by immunization with purified antibodies (Sirisinha and Eisen, 1971; Yakulis *et al.*, 1972) or with T lymphoblasts (Andersson *et al.*, 1976, 1977; Aguet *et al.*, 1978; Binz and Wigzell, 1978; Krammer, 1978; Flood *et al.*, 1980a). Such immunity may either specifically suppress or stimulate

immune responses to a given antigen (for review, see Eichmann, 1978). This difference in type of effect appears to be at least partially due to the fact that antiidiotypic T cells or antibodies exist in different functional subclasses (Cantor and Boyse, 1977). Thus, antiidiotypic T lymphocytes that mediate helper effects (Woodland and Cantor, 1978; Eichmann *et al.*, 1978), suppressor effects (Eichmann, 1975; Owen *et al.*, 1977; Owen and Nisonoff, 1978; Hetzelberger and Eichmann, 1978; Bona *et al.*, 1979; Rohrer *et al.*, 1979; Flood *et al.*, 1980b), and cytotoxic effects (Andersson *et al.*, 1976; Binz and Wigzell, 1978; Flood *et al.*, 1980a) have now been described. Many of these elements may play a role in physiological immune regulation or may be useful for effective manipulation of the host's immune response.

There are two major questions remaining unanswered: (1) How are different functional subclasses of antiidiotypic B and T cells preferentially induced, and (2) How do the different functional subclasses of B and T cells expressing complementary idiotypes interact in a way that results in meaningful positive or negative feedback regulation? Very little is known about possible restrictions in the interactions between functionally different or similar complementary lymphocyte subsets, and it is unclear which determinants or surface molecules are used by the complementary lymphocytes to allow selective and meaningful interaction. Although it has been suggested that the effects of an interaction between complementary clones may be unidirectional (Lindemann, 1979), it is more likely that they are bidirectional in the sense that either clone may regulate or suppress the other with an outcome of the interaction simply depending on the time sequence of the response of the clones (Rowley *et al.*, 1976) or their functional subclasses (Lamb and Feldmann, 1982; Ertl *et al.*, 1982). Depending upon which types of interactions between the complementary clones lead to their induction and effective regulation, approaches for experimentally or therapeutically eliminating suppressor cell clones and for inducing or suppressing immune responses must necessarily be different. For example, in a case where an individual is unresponsive to a tumor antigen because of dominance of a preexisting antiidiotypic suppressor cell clone, pretreatment with cytolytic tumor-specific antibodies could be sufficient to eliminate the suppressive clonotype; such a reagent is certainly easier to obtain than an anti-(anti-idiotypic) reagent. Similar approaches may also be used to control the induction of certain functional subclasses of antiidiotypic T cells or antibodies.

B. Complexity of Tumor-Specific Antigens and Idiotypes

At present very little information is available about the possible complexity of tumor-specific antigens or idiotypes on the tumor-specific lymphocytes responding to these antigens. Such information would be quite useful for

developing effective strategies for immunotherapy, including the use of anti-idiotypic probes. Resistance of normal young mice to transplantation of the UV-induced 1591 tumor was shown to be dependent upon a unique tumor-specific antigen and the immune response to it. Thus, the tumor grows progressively either from the loss of this antigen, as is the case with host-selected progressor variants (Urban, 1982a,b; Urban and Schreiber, 1983), or as a result of idiotype-specific suppression of the tumor-specific immune response to this antigen (Flood, 1980a, 1981).

Wortzel *et al.* (1983a) have generated a syngeneic cloned cytolytic T cell line specific for a 1591 tumor-specific antigen, termed the *A antigen*, which is present on the parental tumor but absent on the host-selected progressor variants. The investigators then selected variants *in vitro* with the anti-A T cell line for variants that had lost the A antigen and used these variants as immunogen to determine if other antigens remained on their surface. A retained antigen was found, termed the *B antigen* to which a second T cell line (anti-B) was generated. By continuing this process of successive immunization and selection, a C antigen and then a D antigen were identified (Fig. 4 and 5) (Wortzel *et al.*, 1983b). All of these four antigens were uniquely specific for the 1591 tumor (Fig. 6) and were expressed simultaneously on the parental tumor cell, as evidenced by the complete elimination of the parental tumor with any one of the four different T cell reactivities (Fig. 4). To demonstrate the independence of these antigens, variants were also selected that expressed different combinations of these antigens. In every instance of selection, only the appropriate antigen was lost, i.e., the other antigens present before selection were not lost, and the expression of other antigens like H2 D or K was not significantly altered by the selections (Fig. 4). The T cell-selected variants showed remarkable heritable stability during prolonged passages in culture or repeated passage in congenic *nude* mice. The frequency of the variants was found to be $<10^{-4}$ in a 2-month-old cloned population and too low to be detected in a freshly cloned population; the simultaneous loss of two antigens from one cell was also too low to be measured.

At present, the genetic origins of the multiple unique tumor-specific antigens that apparently can be expressed on a single malignant cell are obscure. It is unlikely, however, that they present normal histocompatibility antigens which are recognized by the host because of genetic drift from the strain of origin of the tumor studied. This is improbable because the antigen specific T cell lines failed to react with several other tumors that had been induced by the same agent, at the same time, in the same stock of mice as the tumor analyzed (Wortzel *et al.*, 1983a). A similar multiplicity of independent unique tumor antigens has not yet been reported for other tumors, but it is interesting that chemical mutagenesis of malignant cells has been shown to

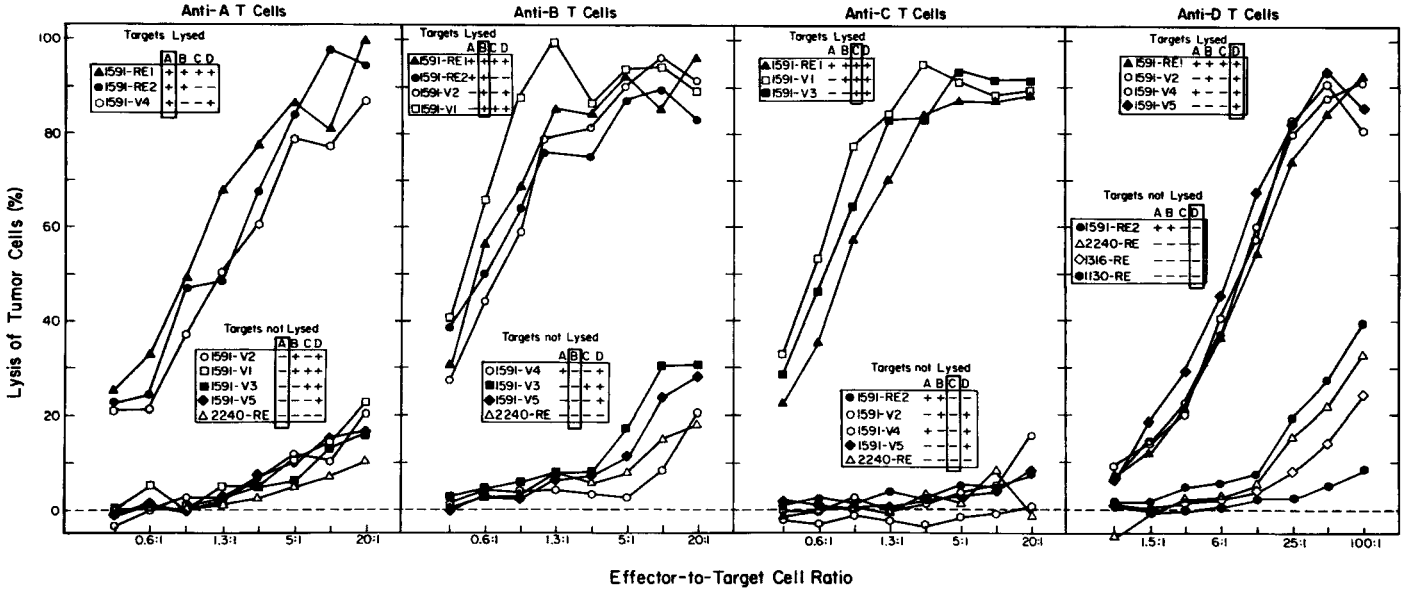

FIG. 4. Multiplicity and independence of tumor-specific antigens demonstrated by cytolytic T cells and immunoselected variants. Tumor-specific cytolytic T cells were generated in mixed lymphocyte-tumor cell cultures from spleen cells of tumor-immune syngeneic mice. The anti-A, anti-B, and anti-C T cells were derived as long-term T cell lines, and these T cells were used for variant selection *in vitro*. The cytolytic activity of the T cells was measured in a ^{51}Cr-release assay. (Reprinted from Wortzel *et al.*, 1983b, with permission.)

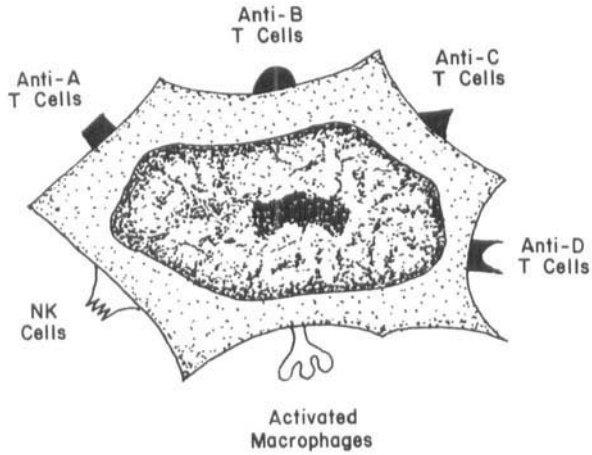

FIG. 5. Scheme of independent target sites on an UV-induced fibrosarcoma. The different black symbols represent multiple T cell-recognized antigens identified by T cell lines. These antigens are lost independently of each other upon variant selection (see Fig. 5) (see Wortzel *et al.*, 1983b, 1984). Furthermore, the macrophage-recognized and NK cell-recognized target sites are independent of the T cell-recognized antigens (see Urban and Schreiber, 1983; Urban *et al.*, 1982a).

introduce multiple independent tumor antigens into a poorly immunogenic tumor (Maryanski and Boon, 1982). Of the multiple unique tumor-specific antigens just described, two are involved in the rejection of the tumor by the normal host. Thus, variants that had lost these two antigens failed to be regularly rejected, in contrast to the parental tumor (Wortzel *et al.*, 1984).

It is curious that although these multiple antigens are simultaneously expressed on the tumor cell, they are not all recognized by the host at the same time, i.e., some antigens are recognized while others are not (Urban *et al.*, 1984). This "hierarchy" in the host response to these multiple tumor antigens may have several important consequences: First, tumor progression may be more likely since, for immune escape, loss of only one antigen may be needed; this event probably occurs more frequently than the simultaneous loss of two or more antigens. Second, such hierarchy allows for the retention of tumor-specific antigens on the progressor variants so that these retained tumor-specific target structures should make the progressively growing tumor still susceptible to immunotherapy. Finally, multiple independent tumor-specific antigens may be retained by progressor tumors, and therefore, a combination of immunologic probes directed simultaneously against all of these target antigens should minimize the possibility for the

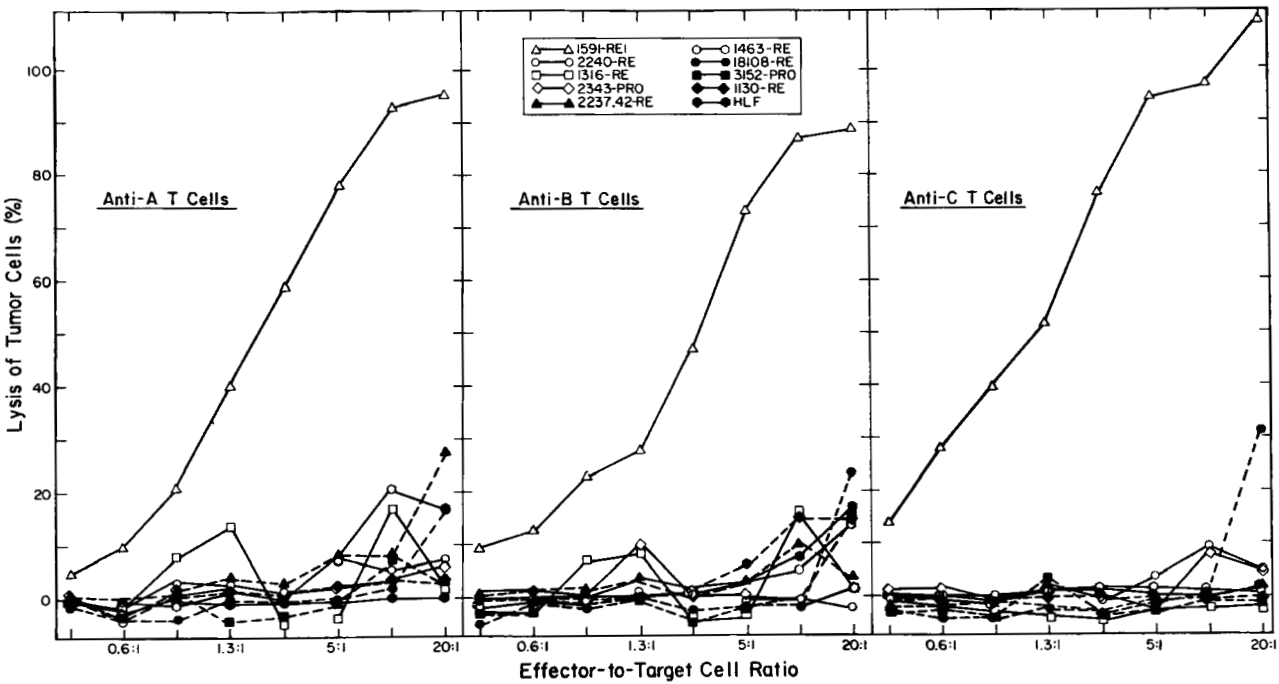

FIG. 6. Unique specificity of different tumor-reactive T cells for the UV-induced 1591 tumor. The cytolytic activity of the cloned anti-A, anti-B, and anti-C T cell lines was determined in a ^{51}Cr-release assay using other syngeneic UV-induced or MCA-induced tumors or normal heart–lung fibroblasts (HLF) as control targets. The tumor specificity of anti-D T cells is shown in Fig. 4 (far right panel). (Reprinted from Wortzel *et al.*, 1983b with permission.)

tumor to escape immune destruction. This strategy would be analogous to using combinations of chemotherapeutic agents with different target sites. Thus, the finding of multiple independent tumor-specific antigens may make it possible to develop much more efficient and effective immunotherapy.

C. Conclusions

Experimental studies have shown that immune responses to tumor-specific antigens (TSA) can be specifically suppressed or stimulated by the interaction between complementary lymphocyte clones. The relative balance between each tumor-specific clone and its complementary antiidiotypic clones determines the outcome of growth of malignant cells. Several conditions may occur.

1. The anti-TSA clone is dominant over suppressive complementary antiidiotypic clones, in which case the tumor expressing such TSA is strongly immunogenic and will be rejected.
2. Suppressive antiidiotypic complementary clones may dominate over the anti-TSA clone which leads to suppression of the anti-TSA response and permits tumor growth. Such suppression may either be preexisting, induced during tumor development and growth, or be induced exogenously by experimental and therapeutic manipulations.
3. A natural abundance of complementary lymphocyte clones expressing an idiotype that resembles the TSA (internal image clone) may cause a lack of immunogenicity of the TSA and on unresponsiveness to the tumor.
4. Suppression of preexisting antiidiotypic and/or the internal image lymphocyte clones, by anti-(antiidiotypic) or idiotypic immunity may lead to reversion of immune suppression and result in rejection of a tumor otherwise considered to be poorly immunogenic.
5. Simultaneous activation of lymphocyte clones specific for multiple independent TSA on a tumor may prevent immune escape of the tumor.

Despite enormous research, an understanding of how the positive and negative feedback loops of the network influence immune responses in general or to tumors is incomplete. Nevertheless, available experimental evidence clearly suggests that idiotypic immune regulation will enable us to devise means for specifically increasing immune responses to weakly immunogenic tumors, to prevent immune escape of tumors, and to circumvent many of the immunosuppressive mechanisms which exist in tumor-bearing hosts. Thus, it is likely that idiotype-specific reagents, procedures, and principles will become indispensable tools for suppressing growth of cancers.

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CHROMOSOMAL LOCATION OF IMMUNOGLOBULIN GENES: PARTIAL MAPPING OF THESE GENES IN THE RABBIT AND COMPARISON WITH Ig GENES CARRYING CHROMOSOMES OF MAN AND MOUSE

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I. Introduction

The mapping of human genes has progressed considerably since the discovery of human chromosome segregation in hybrids between human and mouse fibroblast cells (Weiss and Green, 1967). The human X chromosome

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is now the most densely mapped chromosome, with about 120 loci identified, many of which have also been found on the X chromosome of other mammals (Human Gene Mapping, 1973, 1974, 1975, 1977, 1979, 1981) as predicted by S. Ohno (1969). It remains difficult to establish segmental similarities among autosomes of different species by the single criterion of gene position, since at the present time few localized marker genes are available for comparison. Other approaches, such as chromosome-banding comparison, have been successfully used for tracing phylogenic relationships among mammals. Among primates for example, patterns of chromosomal evolution are well documented (DeGrouchy et al. 1978; Dutrillaux, 1979), but even in this case few mapped genes have been assigned to homoeologous[1] chromosomes.

Several publications have dealt with chromosome localization of immunoglobulin genes in humans and other mammals (Croce *et al.*, 1979; D'Estachio *et al.*, 1981; Hengartner *et al.*, 1978; Meo *et al.*, 1980; McBride *et al.*, 1981; Malcolm *et al.*, 1982; Erikson *et al.*, 1981). We were involved in the mapping of immunoglobulin genes in the rabbit (Buttin *et al.*, 1978; Medrano *et al.*, 1979; Buttin *et al.*, 1979) and also in comparative cytogenetics of primates (Dutrillaux, 1979) and of mammals in general (Dutrillaux *et al.*, 1982). With the accumulation of data, a certain agreement appeared between the results obtained by the methods using gene mapping and those using chromosome banding. Due to the special characteristics of Ig genes, it was of interest to look for carrier chromosomes and determine if they have a high degree of conservatism. Also because of the possible implication of Ig genes and chromosomal rearrangements in some neoplastic diseases, comparative cytogenetics could bring interesting information in the case of rearrangements of equivalent chromosome segments.

Since 1981, knowledge of the chromosomal position of Ig genes in humans has been enriched with new results. The localization of these genes was obscured for some time with contradictory reports (see Section III), but chromosomes 14, 2, and 22 have been conclusively associated with heavy chain (IGH) genes, kappa light chain (IGK) genes, and lambda light chain (IGL) genes, respectively. In addition, some chromosomal comparisons for IGL genes carrying chromosomes in man and mouse have been proposed (Francke *et al.* 1982). In this article we present our findings on the mapping of immunoglobulin genes in the rabbit (Section IV) and on the equivalents of Ig genes carrying chromosomes in the karyotype of the presumed ancestor of all primates (Section V). These findings are waiting for the development of the mapping of multiple syntenic genes before they can be considered as a solidly documented evolution of the chromosomal regions carrying Ig genes

[1]Homoeologous, see footnote *a* of Table III.

in mammals. Our work presents different degrees of difficulty; it is relatively simple for the chromosomes carrying IGH genes because a complete chromosome is equivalent in the three species studied, and it is uncertain for the chromosomes carrying IGL genes because small chromosomal segments are compared. These initial efforts of relating Ig gene mapping and chromosomal comparisons may present some guidelines for further progress in this field.

II. Methods of Mapping Ig Genes

Two main approaches were successful in mapping Ig genes. The first resulted from the applications of techniques for the *in vitro* production of antibodies (the methods to be reviewed here in detail). The second, which used recombinant DNA for detecting Ig genes in nonantibody producing cells, has been reviewed (Ruddle, 1981); no further technical description will be given here, but the contribution of both methods will be considered in the next section.

A. *In Vitro* Production of Immunoglobulins: Lymphomas, Myelomas, and Plasmacytomas

The first attempts to produce Ig *in vitro* started more than 15 years ago with the culture of lymphoblastoid cell lines established from healthy and diseased patients. First reports of Iwakata and Grace (1964), Epstein and Barr (1964), and Pulvertaft (1964) demonstrated that some established lymphoid cell lines could produce *in vitro* light and heavy determinants of immunoglobulins detectable by fluorescent antibody reagents (Takahashi *et al.*, 1969). One special feature of these permanent B lymphocytes established *in vitro* was the transformation by Epstein–Barr virus (EBV) (Klein *et al.*, 1968). Human patients carrying EBV were found more suitable for establishing lymphoid cell lines, and most of these lines continued producing viral antigens after they had been adapted permanently to *in vitro* growth (Jondal and Klein, 1973).

The synthesis of immunoglobulins was not modified in a number of lymphoid cell lines that were made resistant to such drugs as 5-bromodeoxyuridine and 8-azaguanine, and this property could be used for selection of hybrid cells in HAT medium (Littlefield, 1964). After more than 10 years of work with human lymphoid cells, much progress had been made in the characterization of B lymphocyte cell lines and Burkitt lymphoma cell lines (reviewed by Nilsson and Klein, 1982). Some of them are considered as alternatives to hybridomas for producing human antibodies *in vitro* (Steinitz *et al.*, 1977, 1980), and a few of them have been used for the mapping of Ig

genes in humans, but the production of monoclonal antibodies is known to be highly dependent on a few mouse and rat plasmacytoma lines.

The murine B lymphocyte permanent lines which have been described (Raschke, 1980) have similar characteristics to those of lymphoid human cells. Some of them were Abelson murine leukemia virus (AMuLV)-transformed cells, and others were derived from murine lymphomas induced with carcinogens or mineral oils. However, B lymphocyte tumors in mice are rare as compared with murine plasmacytomas, which can be easily induced experimentally (Potter, 1972).

Murine myelomas or plasmacytomas are an optimal source for isolating large amounts of homogeneous heavy and light chain peptides of immunoglobulins and their corresponding mRNA (Kuehl, 1977). By definition, plasma cells represent the last step of B lymphocyte differentiation, but some immature forms of B lymphocytes can also be present in pristane-induced plasmacytomas and myelomas. For unknown reasons, only two strains of mice, BALB/c and NZB, are highly susceptible to plasmacytoma induction (Potter, 1972; Warner, 1975). Viral genes may be required for establishing murine plasmacytoma lines because the incidence of tumors in pristane-primed animals increases when infected with AMuLV, although the induced tumors do not show the presence of viral antigens (Potter *et al.*, 1973). The presence of type C virus is easily detected in all three murine plasmacytoma lines currently used as the immortal cell partner for the production of hybridomas: X-63 (Köhler and Milstein, 1975), NS-1 (Köhler and Milstein, 1976), and SP_2O (Shulman *et al.*, 1978). Figure 1 shows these viral particles in two of the cell lines studied by electron microscopy. However, the relationship between these particles and the immortality of myeloma cells has not been established (J. Peries, personal communication).

B. Intraspecies Hybridomas

The fusion of mouse myeloma cells and normal spleen cells from immunized mice started soon after the work of Potter (1972) on the induction of plasmacytomas. Cotton and Milstein (1973) described the first drug-resistant $HPRT^-$ [2] murine plasmacytoma line used for making a hybrid rat and mouse myeloma cells. Soon after, Köhler and Milstein (1975) described stable mouse–mouse hybridomas obtained from lymphocytes of immunized mice. Murine hybridomas grew permanently in cultures and induced tumors when injected in syngeneic mice. A high titer of antibodies was found in the ascitic fluid and in the serum of hybridoma-bearing mice, indicating that the production of a particular antibody induced during the process of

[2]See Table III for loci symbols.

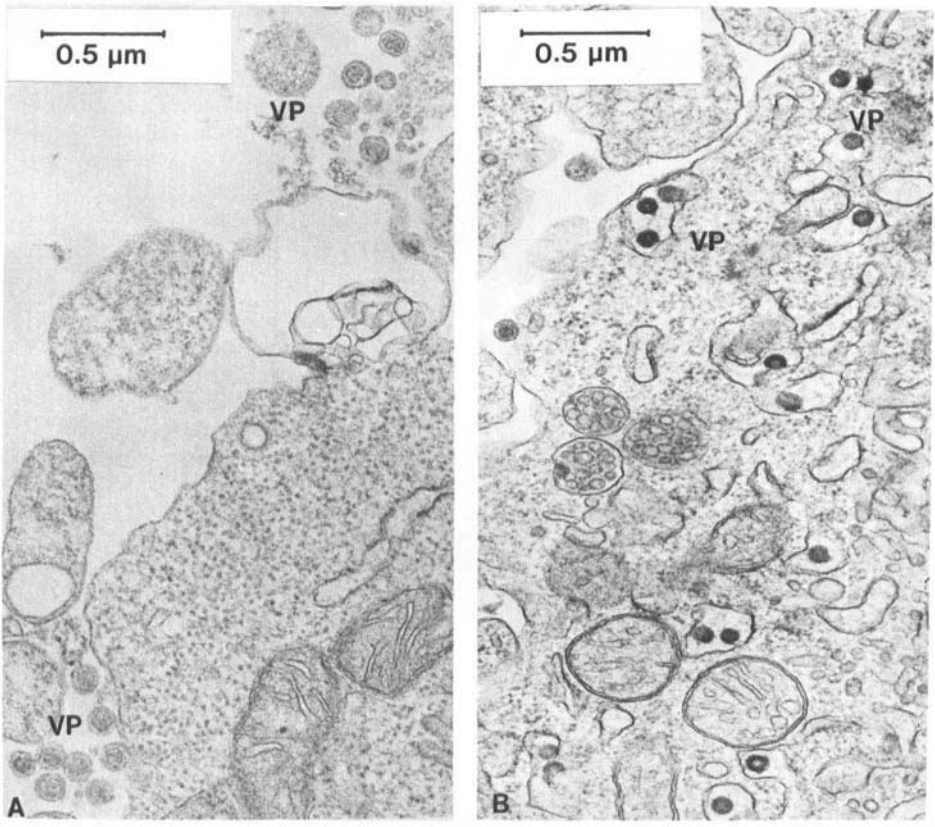

FIG. 1. Electron micrographs showing NS-1 (A) and SP_2O (B) murine plasmacytoma cell lines. Viral particles (VP) are observed either in the intercellular (A) or intracellular (B) spaces. The photographs were obtained by G. Salazar, INSERM U-212, Fondation A. de Rothschild, Paris.

immunization can be immortalized by the hybridoma technology. Hybridomas can be cloned, and each clone produces a single antibody, thus increasing the quality of antibody reagents. Since 1975, the production of hybridoma antibodies has been extended to many types of antigens (Kennett *et al.*, 1980). Even when nonpurified or poorly characterized antigens are taken from human diseases such as melanoma, it has been possible to immunize mice with a melanoma antigen (Medrano *et al.*, 1983) and to produce hybridoma antibodies potentially useful for better purification of the antigen. An increasing number of reports describe the application of monoclonal antibodies in medical diagnosis and the loading of certain monoclonal anti-

bodies with cytotoxic drugs as a highly specific treatment against disease (*Immunological Reviews*, 1982).

Human monoclonal antibodies should be more convenient for medical purposes, but unfortunately this new technology remains based on a few mouse and rat plasmacytoma cell lines. Data on human myeloma lines are almost limited to the report of Olsson and Kaplan (1980), which describes the first human–human hybridomas producing monoclonal antibodies oriented toward 2,4-dinitrophenol. The human myeloma line in this experiment was line U-266, first described by Nilsson *et al.* (1970). A U-266 derivative resistant to 8-azaguanine became sensitive to HAT medium, grew faster in tissue cultures, and continued to produce IgE(λ). Hybridomas derived from U-266 and human spleen lymphoid cells from patients with Hodgkin's disease produced ϵ, γ, and λ chains and new light chains. Thus, the era of human monoclonal hybridomas seems to have already begun.

C. Interspecies Hybridomas

In their first studies of the fusion with drug-marked myeloma lines, Cotton and Milstein (1973) obtained hybrids between mouse and rat myeloma cells which produced mouse heavy and light chains and rat light chains. The instability of these hybridomas was reported soon afterward: after several passages in tissue cultures a loss of antibodies was observed, which was correlated to the loss of rat chromosomes. However, mouse–rat hybridomas probably remained the most stable interspecies hybridomas described, growing like tumors in rats and irradiated mice and producing large amounts of monoclonal antibodies (McKearn *et al.*, 1980). Chromosomal losses are random in intraspecies hybridomas and preferential in interspecies hybridomas. This latter characteristic was used for Ig gene mapping in human, rabbit, and nonmuridae species for which the hybridomas that had been obtained by fusion with murine plasmacytoma lines apparently had preferential segregation of nonmuridae chromosomes.

Mouse–human hybridomas were obtained in several laboratories by fusion of human B lymphocytes or lymphoid lines with one of the drug-marked murine plasmacytoma cell lines. This is exemplified by the results of Levy and Dilley (1980) in which 57% of the hybridomas formed between human B leukemia cells and mouse myeloma line X-63 secreted large amounts of human immunoglobulin light chains. Table I summarizes the work on mouse–human hybridomas used for the mapping of Ig genes in man. The conclusions on chromosomal assignment which are often conflicting are commented on in Section III. Some of the possible sources of error in the methodology used are (1) lack of stability of mouse–human hybridomas, (2) the use in some cases of non-Ig-producing cell lines, (3) the lack of sensitivity

TABLE I

MOUSE–HUMAN HYBRIDOMAS USED FOR THE MAPPING OF Ig GENES IN MAN

References	Parental human cells	Parental mouse cells	Detection of human immunoglobulins	Human chromosome assignment
Croce *et al.* (1979)	Peripheral lymphocytes; GM 607 and GM 1056 lymphoblastoid cells; GM 1500 myeloma cells.	X-63	Immunoprecipitation with rabbit antihuman μ, α, γ, κ, or λ chain specific antisera	Chromosome 14 for γ and μ chains
Erikson *et al.* (1981)	Peripheral lymphocytes; GM 1056 and GM 923 lymphoblastoid cells	X-63	PAGE–SDS and immunoprecipitation	Chromosome 22 for λ chain
Jacot-Guillarmod *et al.* (1979)	Human lymphocytes	Plasmacytoma line	IgM, λ	Chromosome 15 for μ chain
Jacot-Guillarmod *et al.* (1980)	B lymphocytes	Plasmacytoma line	IgM (μ chain)	Chromosome 15 for μ chain
Kucherlapati *et al.* (1979)	B lymphocytes from chronic lymphocytic leukemia patients	NS-1	RIA, PAGE–SDS, indirect immunofluorescence	Chromosome 6 or 11 or both for μ and λ
Levy *et al.* (1978)	B lymphocytes from six leukemia patients	X-63 or NS-1	RIA for Ig κ, λ, and μ chains	Chromosome 6 or 11
Smith and Hirschhorn (1978)	ODY, RPMI 1788, NB60, NB103, and NB114 lymphoid lines	RAG cell line	RIA, rabbit anti human IgG	Chromosome 6 for heavy chains
Smith *et al.* (1981)	Peripheral lymphocytes	NS-1	[^{35}S]Methionine labeling and immunoprecipitation	Chromosome 14 for heavy chains
Wang *et al.* (1980)	Peripheral B lymphocytes	NS-1/P3	μ, λ, κ	Chromosome 11 for κ light chain

in the immunoprecipitation or in the radioimmunoassay for the detection of human-specific immunoglobulins, (4) the lack of stable subclones, and (5) the inability of selecting hybridomas with a single human chromosome. The "allelic exclusion" (see Section VII), which postulates that half of the Ig genes carrying chromosomes exist in a silent state, also seems to be responsible for errors of assignment.

Mouse–rabbit hybridomas were first described by Köhler and Schulman (1978) as secreting incomplete immunoglobulin molecules. Soon after, Buttin *et al.* (1978) reported mouse–rabbit hybridomas obtained by the fusion of B lymphocytes from rabbits with a1/a1,b4/b4 allotypic genotypes. When these rabbits were preimmunized with β-galactosidase, some of the hybridomas expressed rabbit a1 and b4 allotypes and β-galactosidase antibodies. Rabbit chromosomes were detected in these hybridomas by the superposition of Hoechst 33258 fluorochrome staining and G-banding (Medrano *et al.*, 1979). The analysis of rabbit chromosomes segregation in these hybridomas was pursued for several years, and some of the new results obtained are presented in the next section. Yarmush *et al.* (1980) also described mouse–rabbit hybridomas in which secretion of rabbit immunoglobulins was stable and subcloned but in which no rabbit chromosomes were seen.

III. Chromosomal Localization of Ig Genes

Antibody molecules consist of four polypeptide chains held together by disulfide bridges and noncovalent forces. Two of these chains are called heavy because they have 230–240 more amino acids than the other two, which are called light chains (Gally, 1973). Two types of light chains, κ and λ, and nine types of heavy chains, α_1, α_2, γ_1, γ_2, γ_3, γ_4, δ, ϵ, and μ have been described in humans. Long before the chromosomal assignment of these genes became possible, the relationship between the three gene families was investigated in the species in which adequate markers were available, that is in man (Natvig and Kunkel, 1973), in rabbit (Kindt, 1975), and in mouse (Mage *et al.*, 1973). It was concluded at that time that the genes for κ light chain are not linked to the genes for λ light chain nor to any of the genes for heavy chains. Some quantitative estimate of the length of these three clusters was attempted by the classic technique of genetic crossing-over among linked alleles, and a length of 10 cM was suggested for the IgH family (Weigert and Potter, 1977). Data now exist on direct chromosomal assignment for Ig genes. They are obtained by the use of chromosomal segregation of intra- and interspecies hybridomas, by nucleic acid hybridization, and by linkage with previously assigned genes. Additional but indirect information

obtained by other techniques such as comparative cytogenetics and comparative gene mapping is presented in the next section.

A. Localization of Ig Genes in Mouse

In the report of Hengartner *et al.* (1978), the B lymphocytes of two newly described feral mouse populations CB and CD, homozygous for several metacentric chromosomes derived by different Robertsonian translocations, were fused with the myeloma cell line X-63-Ag8. The derived hybridoma subclones were studied for expression of immunoglobulins and karyotype composition. Metacentric chromosomes formed by Robertsonian translocations of chromosomes 6 and 13(13/6), 12 and 14(14/12), 6 and 7(7/6), 10 and 12(12/10) were found in hybridomas producing γ_1 heavy and κ light radiolabeled chains, and some of the derived subclones allowed the authors to propose the assignment of the genes for Igκ and those for heavy chains to chromosomes 6 and 12, respectively.

This assignment could not be conclusive because some discrepancies between chromosomal loss and Ig chain expression were arbitrarily explained by the unverified assumption that allelic exclusion in somatic cell hybrids was operative. The assignment of the IgH gene cluster to mouse chromosome 12 was established soon after (Meo *et al.*, 1980) by segregational analysis in a strain of mice carrying a reciprocal translocation between chromosomes 5 and 12. The breakpoint of chromosome 12 was in band 12F1, giving rise to a long (12^5) and a short (5^{12}) marker chromosome. By crosses of females with translocation (T(5;12)31H and BALB/c males which were homozygous for Ig heavy (H) chains and for prealbumin genes, (Igh-1^a/Pre-1^a genotype), it was concluded that Igh is located in close proximity to band 12F1 of murine chromosome 12 and proximal to the prealbumin locus. Taylor *et al.* (1975) had previously demonstrated that Igh-1 and Pre-1 genes were syntenic in the mouse. Weigert and Potter (1977) had also found that the locus for κ light chain variable region (V_κ) is linked to the gene Ly 2.3 located on chromosome 6 of the mouse. This result was rejected by Valbuena *et al.* (1978) who found by nucleic acid hybridization techniques no correlation between the presence of mouse κ chain DNA sequences in a somatic cell hybrid and the presence of mouse chromosome 6. However, the assignment of mouse κ light chain genes to chromosome 6 was further proposed by Swan *et al.* (1979) using molecular hybridization with two radioactive DNA probes that were cloned from the light chain mRNA purified from mouse myeloma MOPC-41. DNA extracted from a panel of fibroblast-derived interspecies somatic cell hybrids of known karyotype was cleaved by *Eco*RI restriction endonuclease and hybridized with nick-translated DNA probes.

Chromosome 6 was the only mouse chromosome present in four positive lines and absent from the lines which did not exhibit hybridization.

The localization of λ genes in mouse chromosome 16 has been reported by D'Eustachio *et al.* (1981), also by use of nucleic acid hybridization techniques for detecting DNA sequences of λ_1 and λ_2 chains in interspecies somatic cell hybrids. Among a panel of hybrid cell lines tested for mouse λ chain genes, six possessed the full set of mouse λ DNA fragments and five showed no mouse λ DNA fragments. The only mouse chromosome present in all lines containing mouse λ chain genes and absent from all lines lacking these genes was chromosome 16.

One independent set of hybrids made between mouse fibroblasts carrying Searle's translocation T(X;16)16H and chinese hamster cells (Francke *et al.*, 1982) allowed localization of murine λ light chain genes to region cen→B5 of mouse chromosome 16. This result was obtained from blots of electrophoretically separated DNA fragments which showed the presence of Igλ cDNA genes when a translocated chromosome 16^X containing the proximal half of chromosome 16 was present. This work also discounted the possibility that mouse chromosome 15 was carrying Igλ genes, which could be postulated by similarities between mouse chromosome 15 and human chromosome 22 (see Sections IV and V) and by gene mapping comparison of these two chromosomes.

D'Eustachio *et al.* (1980) have also used DNA reassociation techniques for confirming the position of structural genes for murine immunoglobulin heavy chains in chromosome 12. These studies on cells which do not express Ig genes are undoubtedly of great significance. Nick-translated radioactive probes of Ig genes are now available in several laboratories. It is expected, but has not been demonstrated, that by using nucleic acid hybridization which reacts separately with heavy and light chain genes, a certain degree of cross-reactivity should be found among Ig genes carrying chromosomes with similar banding patterns.

B. Localization of Ig Genes in Rabbits

Only the mouse–rabbit hybridomas obtained by the filter fusion technique of Buttin *et al.* (1978) seem to have adequate rabbit chromosome retention. Among 55 hybridomas of independent origin analyzed 3–4 weeks after the fusion experiment, for rabbit b4 and a1 allotype expression (Cazenave, 1977; Heidmann *et al.*, 1981) and for rabbit chromosomes retained, 9 hybridomas expressed the allotype of κ chain constant region (C_κ), 2 hybridomas expressed the allotype of heavy chain variable region (V_H), 1 expressed both allotypes, and the rest expressed no rabbit allotypes (see Table II).

TABLE II

ORIGIN OF MOUSE–RABBIT HYBRIDOMAS RECOVERED AFTER CELL FUSION ON MEMBRANE FILTERS[a]

Rabbit parental lymph node cells	Mouse parental P3/X63 Ag-8	Selective medium	Hybridomas recovered (clones/total wells)	Hybrid colony described
Immunized once with 1 mg peroxydase; 2×10^6 cells	2×10^6 cells	HAT and H-aza	4/48 and 5/48	21.6.F1(19)
Immunized once with 500 μg β-galactosidase; 4×10^6 cells	2×10^6 cells	H-aza	10/96	19.1.F2(24)
Immunized once with 500 μg β-galactosidase; 4×10^6 cells	2×10^6 cells	H-aza	10/96	19.1.F2(26)
Immunized once with 500 μg β-galactosidase; 2×10^6 cells	2×10^6 cells	H-aza	1/48	19.1.F5(50)
Immunized once with 500 μg β-galactosidase; 4×10^6 cells	2×10^6 cells	H-aza	1/48	19.1.F6(60)
Immunized twice with 500 μg β-galactosidase; 2×10^6 cells	2×10^6 cells	H-aza	7/48	28.2.F1(6)
Immunized twice with 500 μg β-galactosidase; 2×10^6 cells	2×10^6 cells	H-aza	4/48	28.2.F4(42)
Immunized twice with 500 μg β-galactosidase; 5×10^6 cells	3×10^6 cells	H-aza	7/48	28.2.F5(56)
Immunized twice with 500 μg β-galactosidase; 5×10^6 cells	2×10^6 cells	H-aza	4/48	2.3.F1(3)
Immunized twice with 500 μg β-galactosidase; 5×10^6 cells	2×10^6 cells	H-aza	2/48	2.3.F5(53)

[a] These hybridomas were selected by L. Phalente, laboratory of Dr. Buttin, Institut de Recherches en Biologie Moléculaire, 2 Place de Jussieu, Paris.

Most of the hybridomas producing rabbit allotypes were subcloned. Hybridoma 19.F2(26) gave in a first subcloning (Fig. 2), with some hybridomas having only a few rabbit chromosomes but still expressing rabbit allotype b4. Subpopulations of 19.1.F2(26) after passage in new born hamster also expressed allotype b4, but no expression of allotype was obtained and no rabbit chromosomes were retained except X after passage in nude mice. The proportion of rabbit chromosomes retained varied greatly from subclone to subclone, being highest in subclone 19.1.F2(26) with about 15%

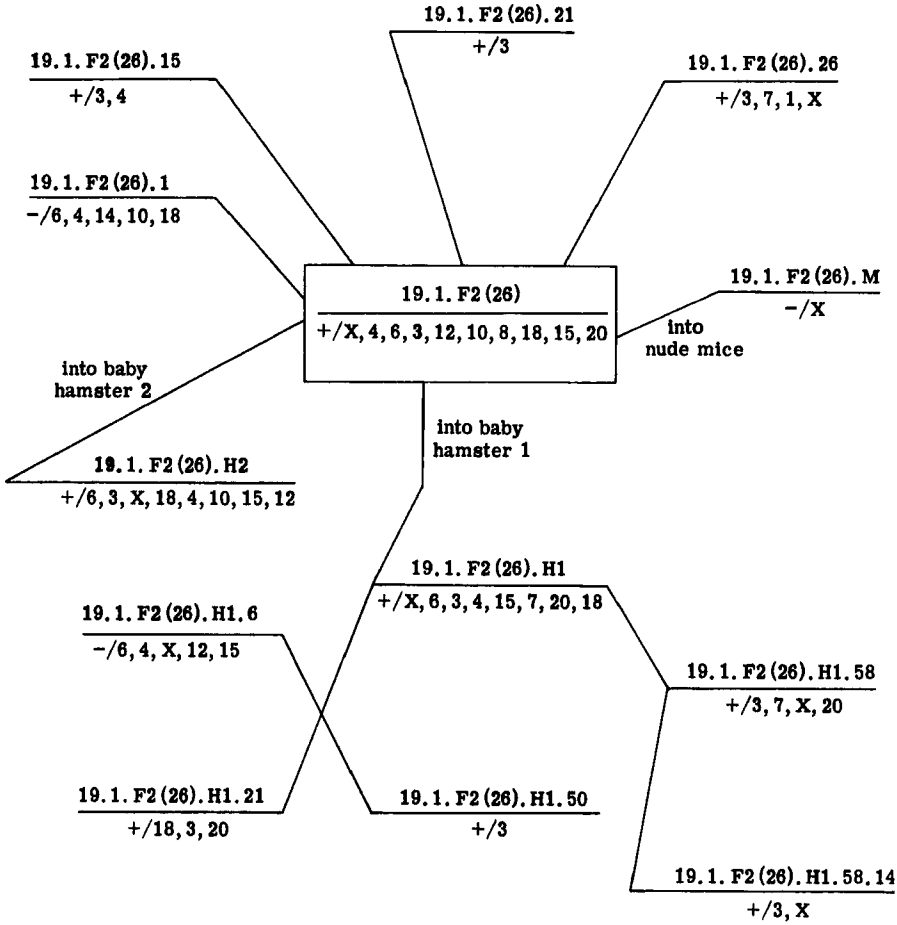

FIG. 2. Subclones and subpopulations of hybridoma 19.1.F2(26) studied for rabbit Igκ chain secretion and for rabbit chromosomes retained. Above the lines are hybridoma nomenclature. Below the lines, the presence (+) or absence (−) of b4 allotype is indicated and rabbit chromosomes are identified.

of metaphases having rabbit chromosome 3, sometimes in two copies, with no other rabbit chromosome. Figure 3 shows one of these metaphases photographed after staining with Hoechst 33258 and after G-banding. Other results which relate rabbit chromosome 3 to the expression of allotype for κ light chain have been published elsewhere (Buttin *et al.*, 1979; Medrano and Dutrillaux, 1982).

A similar approach was attempted for the mapping of rabbit allotype a1 representative of heavy chains. In this particular case, three hybridomas expressing allotype a1 were used, and none of them gave subclones retaining rabbit chromosomes. In the first karyotype of 28.2.F5(56), 28.2.F1(6), and 2.3.F5(53), chromosomes X, 16 and 6 were always retained (Fig. 4). Chromosomes X and 6 were often present in hybridomas expressing allotype b4 but were also in hybridomas negative for rabbit Ig production, such as 21.6.F1(19). Only the Y rabbit chromosome was never observed in the mouse–rabbit hybridomas studied. It was concluded that rabbit chromosome 16 was the best candidate for carrying Ig genes for heavy chains.

Expecting that, by allelic exclusion, some hybridomas with silent or nonfunctional Ig genes carrying chromosomes should appear, we analyzed karyotypes of hybridomas that were positive for rabbit allotypes, and thus all had to have functional chromosomes. However, in some particular clones like 19.1.F5(50), a silent chromosome seems to have been present but no further analysis was undertaken because of the instability of these hybridomas.

No attempts for localizing λ chain genes in rabbits have been published. The only information available is indirect: banding similarities exist between mouse chromosome 16 and the distal part of rabbit chromosome 14, but this chromosomal comparison is uncertain due to the fact that small chromosomal segments are compared (see next section).

C. Localization of Ig Genes in Man

The report of Levy *et al.* (1978) described hybridomas obtained by the fusion of human B leukemia cells obtained from six patients and murine myeloma cells X-63 or NS-1. Only human chromosomes 6 and 11 were found in more than 70% of three subclones secreting human Igκ or λ chains and μ heavy chains as detected by radioimmunoassay. These results conflict with other assignments of Ig genes in man described by Smith *et al.* (1976) who proposed the localization of genes for heavy chain immunoglobulin production to chromosome 2. They also conflict with another report of the same group (Smith and Hirschhorn, 1978) relating genes for heavy chain immunoglobulins to chromosome 6. In addition, Croce *et al.* (1979) proposed chromosome 14 for the localization of Ig heavy chain genes. The linkage

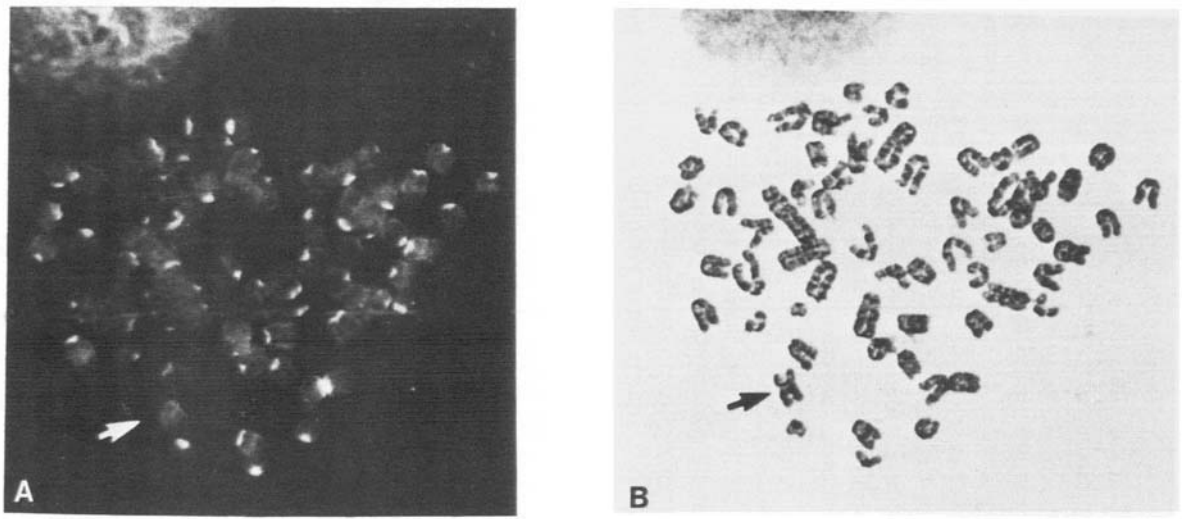

FIG. 3. Metaphase of hybridoma 19.1.F2(26).26 showing Hoechst 33258 juxtacentromeric fluorescence (A) and G-banding (B). Rabbit chromosome 3 is identified by the absence of juxtacentromeric fluorescence and by three major G-bands in the long arms and two G-bands in the short arms. Pale fluorescence all along the chromosome and long arms that show more intense fluorescence than short arms are also characteristic of this chromosome.

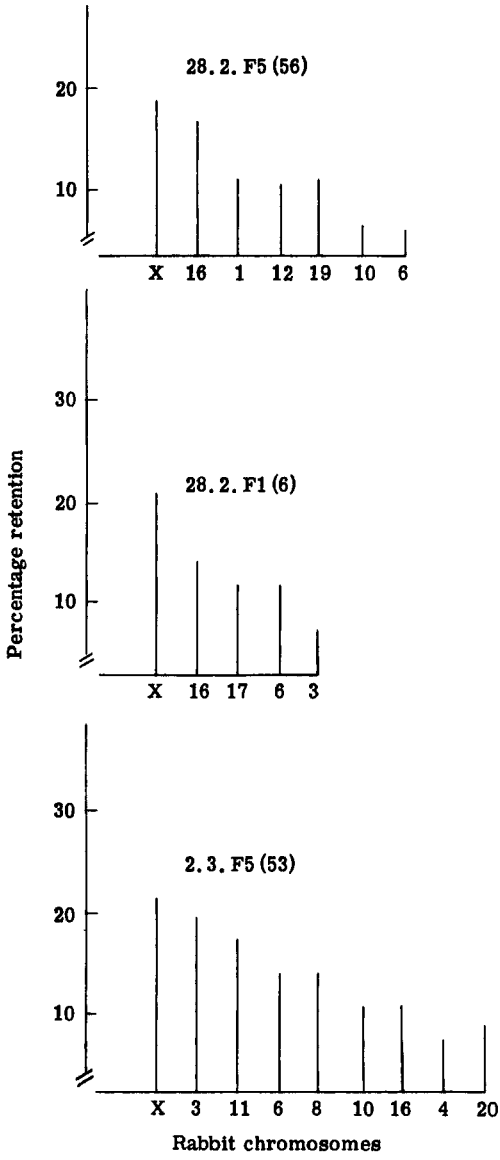

FIG. 4. Estimated frequency of rabbit chromosomes in mouse–rabbit hybridomas expressing the allotype a1 of Ig heavy chains. Fifty metaphases were photographed and studied (trypsin or viokase banding, Hoechst fluorescence) for each hybridoma. Chromosomes observed in less than three metaphases are not considered.

between HLA and Ig genes was excluded by the analysis of lod scores (Namboodiri *et al.*, 1979). Also, at the Gene Mapping Conference in Oslo (1981) four reports confirmed the localization of Ig heavy chain genes in human chromosome 14.

In the original report by Croce *et al.* (1979), hybridomas were obtained by fusion of mouse myeloma line X-63 with human peripheral lymphocytes or human lymphoblastoid or myeloid cell lines. The human Ig chains secreted by some of the hybridomas were immunoprecipitated with rabbit antihuman μ, α, or γ heavy chains antisera and analyzed by polyacrylamide gel electrophoresis. As expected, when allelic exclusion is operative, two types of subclones are found: those having human chromosome 14 and expressing human heavy chain genes (V_HC_H+/14+) on those having chromosome 14 and not expressing human heavy chain genes (V_HC_H−/14+). Hybridomas with chromosome 14 as the single human chromosome expressing human heavy chain genes were also found, which strengthened the assignment to this chromosome.

The claim for a synteny (Jacot-Guillarmod *et al.*, 1979, 1980) between human IgM genes and β_2-microglobulin, clearly assigned to chromosome 15, can be definitely rejected, although it is possible that HSA 15 may be coding for additional complexity in the extracellular secretion of heavy chains. Contradictory assignments for Ig light chain genes were proposed at the 4th International Congress of Immunology (Martinis *et al.* 1980; Wang *et al.*, 1980), contradicting a previous assignment of κ light chain genes to human chromosomes 7 or 11 (Human Gene Mapping, 1979). Malcolm *et al.* (1981) determined the localization of a $V_κ$ locus on the short arm of chromosome 2. This conclusion was reached after *in situ* hybridization of a cDNA probe, H_k101λ Ch4A, on spread metaphase chromosomes. In two separate hybridization experiments a total of 105 prebanded T lymphocytes mitosis were analyzed by autoradiography, and five slides were found with a significant excess of hybridization on chromosome 2. This assignment was confirmed by McBride *et al.* (1982) using hybrid cells of human and mouse cell lines and ^{32}P-labeled human $C_κ$ probes. DNA from hybrid cell lines was digested with *Eco*RI restriction nuclease, fractionated by agarose gel electrophoresis, transferred to nitrocellulose, hybridized with human $C_κ$probe, and visualized by autoradiography. The human chromosomes present in each hybrid cell line were determined from starch gel electrophoresis. Eight human–rodent hybrid cell lines contained the human κ constant region gene cluster, and the markers of human chromosome 2 long arm (IDH-S) and short arm (MDH-S and ACP1) segregated concordantly in all cell lines except two.

Another well-documented study of Malcolm *et al.* (1982) on the *in situ* hybridization of two cDNA κ light chain probes and normal human diploid

lymphocytes confirmed the localization of κ light chain variable-region genes to the short arm of chromosome 2. Moreover, chromosomal preparations of stimulated lymphocytes from a balanced translocation carrier 46 XXT(2;16)(q13;q22), which contained one copy of normal chromosome 2 and 16, one abnormal 2/16, and one abnormal 16/2, showed grain value distributions concordant with a position of V_κ genes above the break point q13.

The assignment of human λ chain genes has been proposed by Erikson *et al.* (1981) to human chromosome 22. These authors preselected mouse–human hybridomas that expressed λ chains, and did not find any clone having human chromosome 22 and nonexpressing human λ chain. Moreover the loss of human chromosome 22 resulted in the inability to produce human λ chain. These results were confirmed by McBride *et al.* (1982) using a group of hybrid cell fibroblast lines and a ^{32}P-labeled human C_λ probe. Six of the human–rodent hybrid cell lines contained the human λ constant-region gene cluster, as demonstrated by hybridization with a C_λ probe. There were no discordancies between the segregation of C_λ and chromosome 22.

Other confirming results on the mapping of human Ig genes have been presented by Cox *et al.* (1982) and by Kirsch *et al.* (1982).

The various proposals for chromosomal localization of Ig genes in man are summarized in Fig. 5. It indicates that chromosome 14 is the only candidate for carrying Ig heavy chain genes (IGHA1, IGHA2, IGHD, IGHE, IGHF, IGHG1, IGHG2, IGHG3, IGHG4, IGHM, and IGHV), although chromosomes 2, 6, and 15 have also been proposed. For κ light chain genes (IGKC and IGKV), chromosomes, 2, 7, 11, and 21 have been proposed, but chromosome 2 is now considered to be the only candidate. For λ chain genes (IGLC and IGLV), chromosomes 22, 21, 7, and 11 have been proposed, but chromosome 22 seems to be the best candidate. We shall now present the comparative cytogenetic approach to show if these assignments fit with the results obtained by chromosomal-banding comparison.

IV. Comparative Cytogenetics of Ig Genes Carrying Chromosomes

A chromosomal comparison of more than 100 species of mammals, mainly primates, has been carried out over the past 10 years (Dutrillaux, 1979; Dutrillaux *et al.*, 1982). It was found that euchromatic bands (nonvariable R and Q bands) were probably identical in all primates, with the differences of karyotype resulting from balanced chromosomal rearrangements. The analysis of these rearrangements lead to the karyotypic reconstruction of the phylogeny of primates. On the other hand, the presence of identical nonrearranged chromosomes in different species lead to a progressive reconstruction of an ancestral karyotype for all primates (Fig. 6), and it will be used as a reference for comparison of the presumed Ig genes carrying chro-

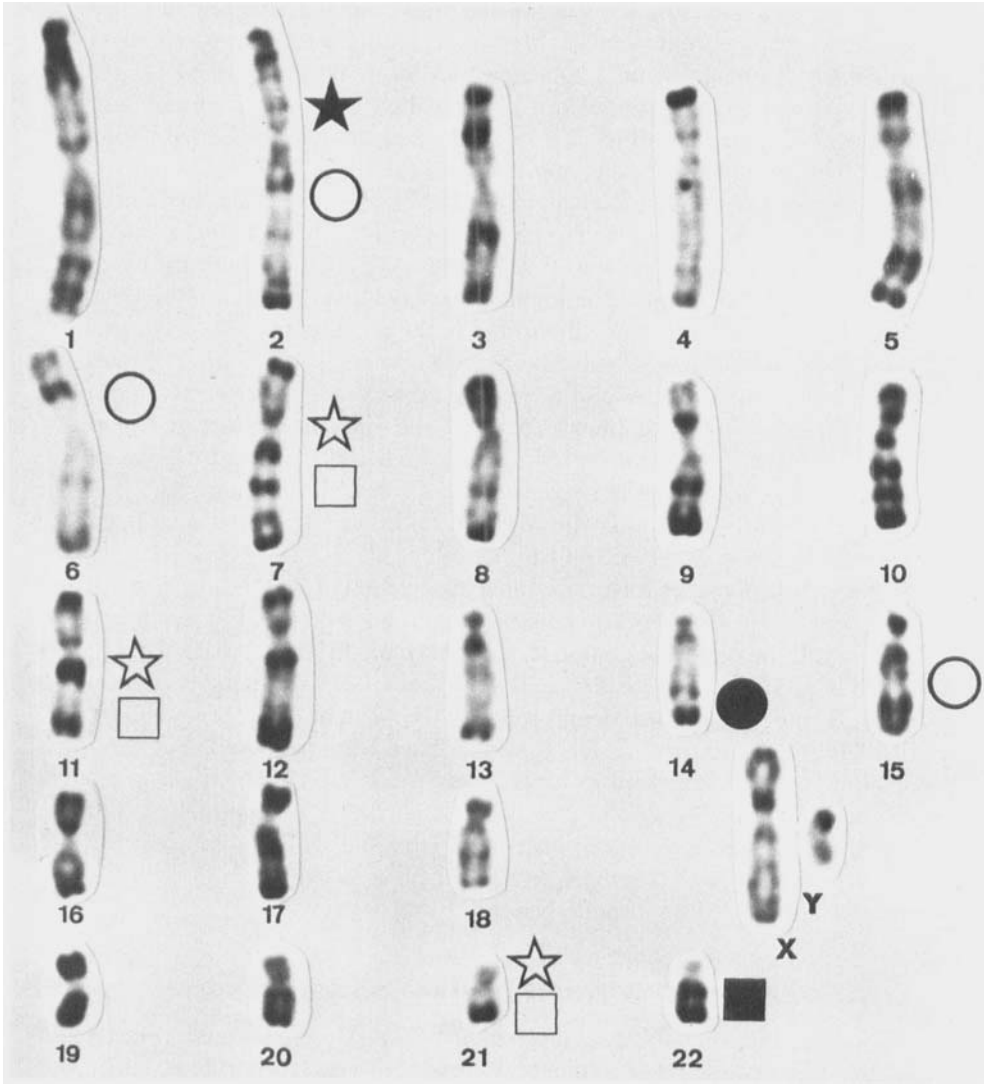

FIG. 5. Distribution on a human R-banded karyotype of the various localizations proposed for the Ig genes. Circles, heavy chain genes. Stars, κ light chain genes. Squares, λ light chain genes. Black symbols correspond to confirmed assignments.

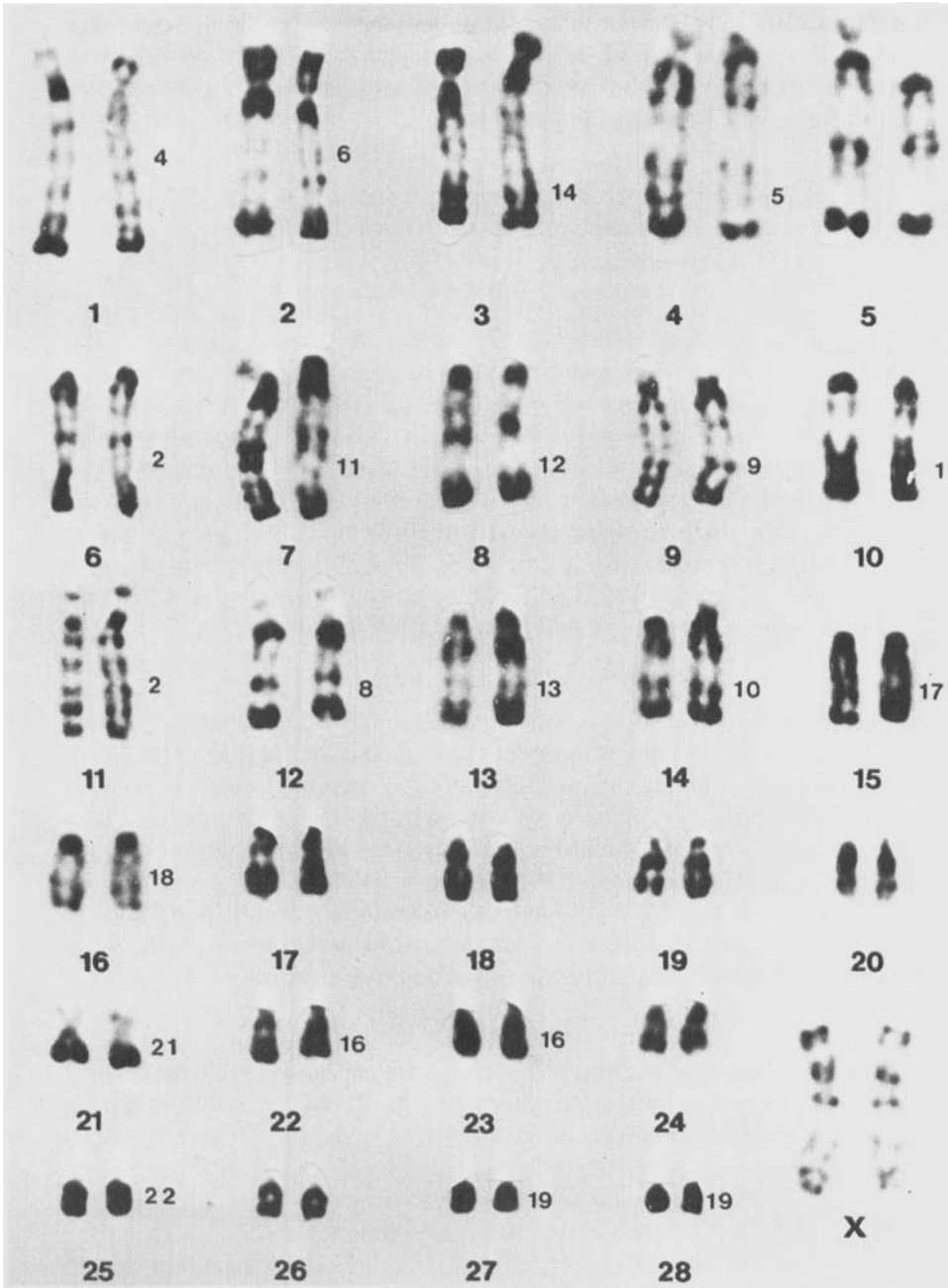

FIG. 6. Reconstructed ancestral karyotype of the primates. For details see Dutrillaux *et al.* (1982).

mosomes of man with those of rabbit and mouse. In addition to chromosomes HSA[3] 2, 14, and 22, on which Ig genes are definitively assigned, chromosomes HSA 8 and 21 will also be considered since Ig genes were localized on their presumed homoeologies, either in rabbit or in mouse.

A. The Equivalent of Human Chromosomes 2, 14, and 22 in the Karyotype of the Presumed Ancestor of all Primates

1. Evolution of Human Chromosome 2

Human chromosome 2 (HSA 2) corresponds to two elements in all the other primate species. These chromosomes were acrocentric in the primitive and more recent primates. The orangutan still possesses the acrocentrics. One of them, corresponding to the long arm of HSA 2, underwent a pericentric inversion and is still observed in the gorilla and chimpanzee. The other element, corresponding to all of the short arm and to the proximal part of the long arm of HSA 2, more recently underwent an inversion forming an element still observed in the chimpanzee. Finally the two elements were translocated and gave HSA 2 (Fig. 7). The element corresponding to HSA 2p is chromosome 11 of the reconstituted ancestral karyotype (Fig. 6).

2. Evolution of Human Chromosome 14

Except in Pongidae species (orangutan, gorilla, and chimpanzee), which possess the same element as man, the equivalent of human chromosome 14 (HSA 14) corresponds to the distal part of the long arm of a submetacentric chromosome observed in most primate species. Gene mapping (Human Gene Mapping 5, 1981) indicates that this large element possesses genes assigned both to chromosome HSA 14 and to HSA 15. The presumed ancestral aspect of HSA 14 corresponds to chromosome 5 in Fig. 6. A fission of this ancestral chromosome 5 probably occurred in a common ancestor to Pongidae and Hominidae after the separation of Cercopithecoids.

3. Evolution of Human Chromosome 22

This chromosome is found acrocentric in many species as well as man or is translocated onto various other chromosomes. It was probably originally acrocentric (chromosome 25 of Fig. 6).

[3]HSA, MMU, and OCU indicate the chromosomes of man (*Homo sapiens*), mouse (*Mus musculus*), and rabbit (*Oryctolagus cuniculus*), respectively.

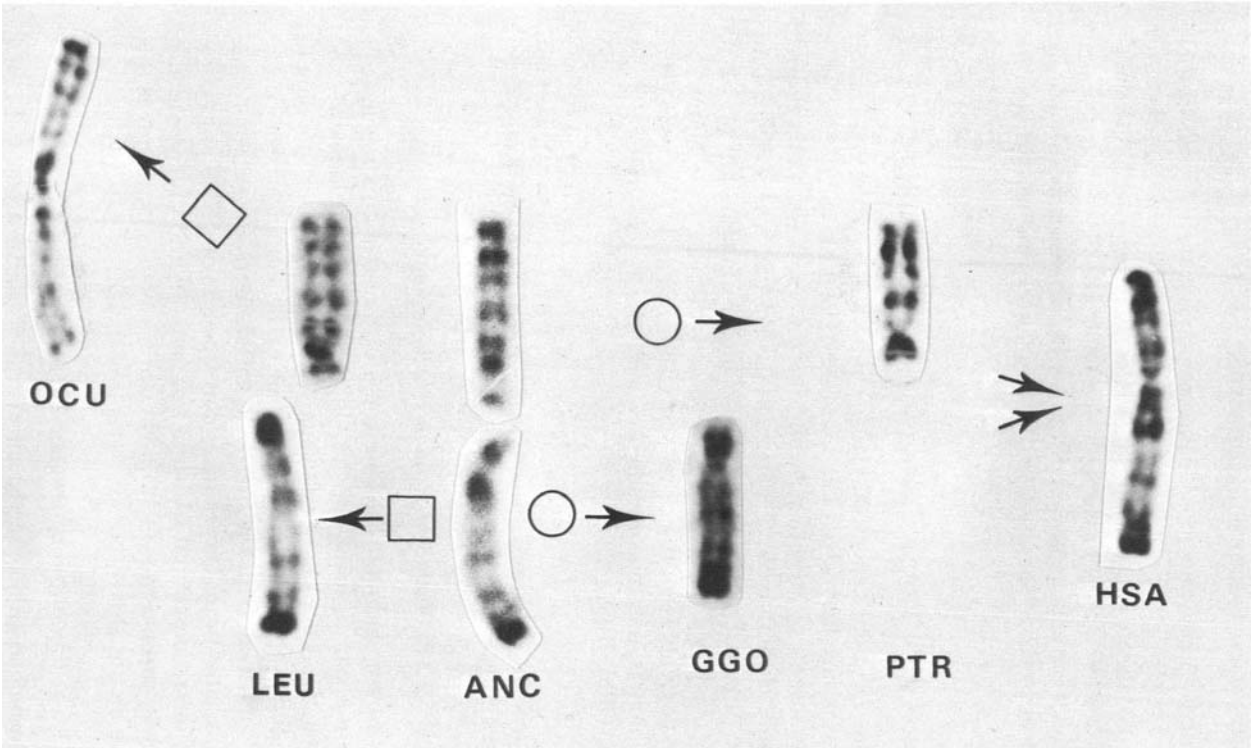

FIG. 7. Evolution of the chromosomes equivalent to HSA 2. From the ancestoral (ANC) chromosomes 6 and 11 (Fig. 6), an inversion (white circle) occurs on ANC 6 before giving gorilla (GGO); another occurs on ANC 11 before giving chimpanzee (PTR). The fusion of the two inverted chromosomes gives HSA 2. From the same ancestral chromosomes, a translocation (white square) occurs on ANC 6 before giving the hare (LEU) and the rabbit (OCU); another occurs on chromosome ANC 11 before giving the rabbit.

B. Comparison with Rabbit Chromosomes

Chromosome HSA 2p is ancestral 11. It corresponds to the long arm of rabbit chromosome 2 (OCU 2). The acrocentric form is still observed in the hare, closely related to the rabbit. Chromosome HSA 14 is ancestral 5 (distal part of long arm). It corresponds to chromosome OCU 16. This last has undergone a complex rearrangement which cannot be reconstituted accurately. Chromosome HSA 22 is ancestral 25. It may correspond to chromosome OCU 21.

C. Comparison with Mouse Chromosomes

Chromosome HSA 2p is ancestral 11. No chromosomes correspond to this element in the mouse karyotype. Chromosome HSA 14 is the distal part of ancestral chromosome 5. Most or possibly all of chromosome HSA 14 corresponds to mouse chromosome 12. Chromosome HSA 22 is ancestral 25. An analogy of the proximal part of HSA 22 with the proximal part of MMU 16 has been proposed by Francke *et al.* (1982). By the use of R-banding we propose an analogy between the distal part of HSA 22 and the distal part of MMU 15, which is not in contradiction with the results presented by Francke *et al.* (1981, 1982).

D. Comparison of Human Chromosomes 8 and 21 with Ig Genes Carrying Chromosomes in Other Species

1. Evolution of Human Chromosome 8

An element corresponding to the long arm is observed as an acrocentric or as a chromosome arm in many species. In most Catarrhini monkeys, the same element as HSA 8 is observed. Thus, an acrocentric corresponding to the long arm, and probably one corresponding to the short arm, were translocated in a common ancestor to Catarrhini, and the derivative chromosome remained stable afterward. The element corresponding to HSA 8q is chromosome 16 of the reconstituted ancestral karyotype (Fig. 6). It corresponds to the long arm of chromosome OCU 3. No chromosome corresponds to this element in the mouse karyotype.

2. Evolution of Human Chromosome 21

This chromosome is similar to that of man in Pongidae species. In new world monkeys, it is often found acrocentric, but its proximal G-band is slightly longer. In many monkey species, this small chromosome is found translocated onto various other chromosomes, confirming that it was proba-

bly a small acrocentric originally. This chromosome corresponds to the distal segment of a large chromosome found in many prosimian species and in many nonprimate mammals. It would correspond to rabbit chromosome 14 and, in an acrocentric form, to chromosome 2 of the ancestral karyotype (distal segment). It may correspond to the distal part of chromosome OCU 14 and to the distal part of chromosome MMU 16.

The assignment of Ig genes to human, mouse, and rabbit chromosomes discussed in Section III, allows some comparative cytogenetics among these three species. For the chromosomes carrying the genes for heavy chains, a good correspondence exists (Fig. 8) between chromosomes MMU 12, HSA 14, and OCU 16. The mapping of κ light chain genes in chromosome MMU 6 has been confirmed by several laboratories, but this chromosome does not show a sufficient degree of similarity with chromosomes of the other two species. The κ light chain genes have also been mapped in chromosome HSA 2p, and homoeology is clearly observed between the short arm of chromosome HSA 2 and the long arm of chromosome OCU 2. However, κ light chain genes had not been mapped in OCU 2 (as expected by comparison with HSA 2) but rather in OCU 3. As presented in Fig. 10, homoeology is clearly observed between the long arm of chromosome OCU 3 and the long arm of chromosome HSA 8. The banding analogies between MMU 6 and either HSA 2p or 8q are difficult to demonstrate, making the comparative cytogenetics difficult for this chromosome.

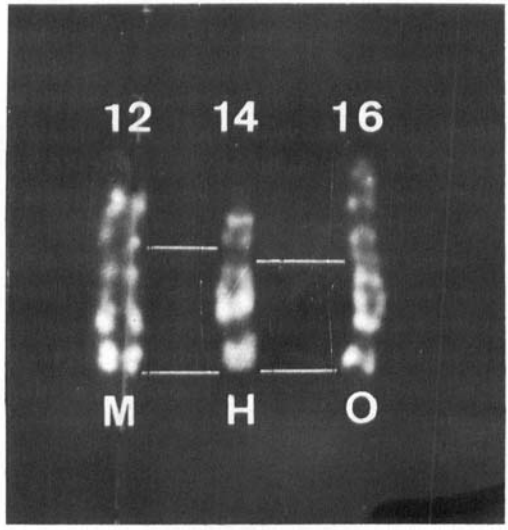

FIG. 8. Comparison between chromosomes HSA 14, MMU 12, and OCU 16. R-banding after BUdR substitution. The white lines indicate segments presumably homoeologous. M, *Mus musculus*; H, Homo sapiens; O, *Oryctolagus cuniculus*.

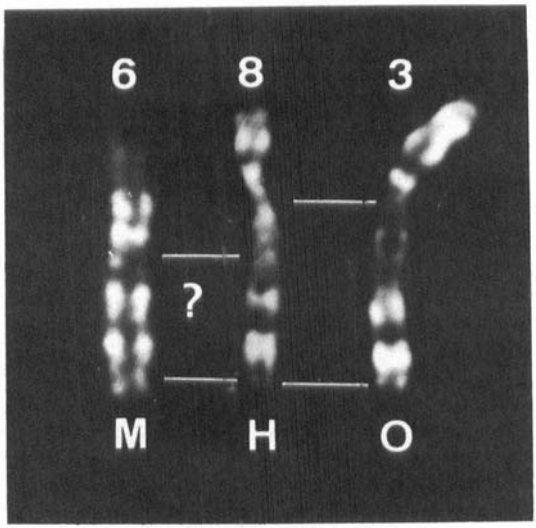

FIG. 9. Comparison between chromosomes HSA 8q, MMU 6, and OCU 3q. See Fig. 8 for legend.

Francke *et al.* (1982) proposed that a distal part of MMU 16 is equivalent to the distal part of HSA 21 and that the proximal part of MMU 16, which is carrying λ chain genes, is equivalent to the proximal part of HSA 22, also carrying these genes. As indicated in Fig. 11 we observed homoeologies among the distal parts of MMU 16, HSA 21, and OCU 14 which probably do not correspond to the position of λ chain genes. We also observed homoeologies between the distal parts of MMU 15, HSA 22, and OCU 21 which do not correspond to the position of λ chain genes. We cannot predict which chromosomal segment of the rabbit is carrying these genes, but ancestral chromosome 25 and chromosome OCU 21 possesses segments in common with HSA 22.

V. Comparative Gene Mapping

At the time of being revised by the Gene Mapping Conference in Edinburgh (Human Gene Mapping 5, 1979), the mammalian gene maps were being developed in man (345 loci mapped) and mice (438 loci mapped). Gene mapping in primates and domestic animals is a rapidly growing field, and it is expected to play a major role in reconstructing chromosome evolution.

Many genes may be assigned to the same single chromosomal band because an enormous difference in magnitude exists between the 1.8-kilobase

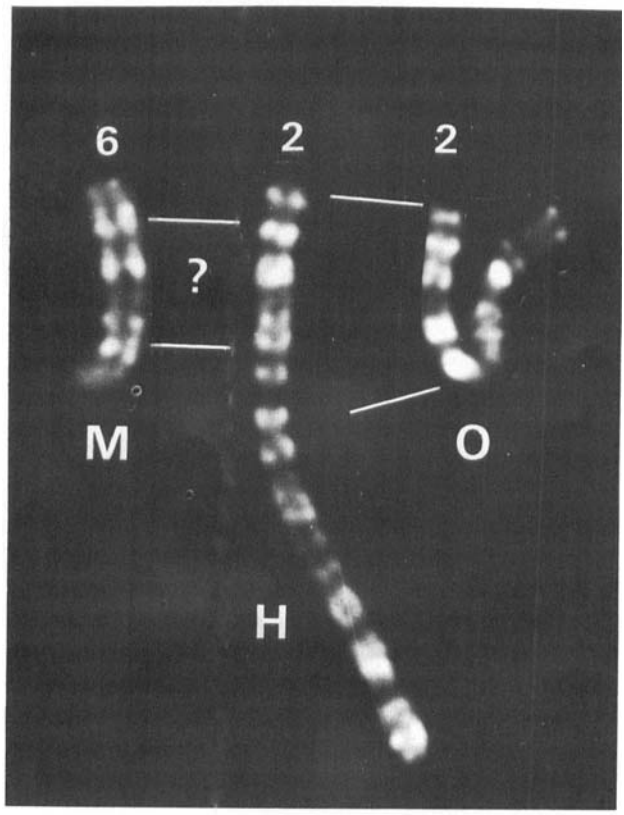

FIG. 10. Comparison between chromosomes HSA 2p, MMU 6, and OCU 2q. See Fig. 8 for legend.

average length of genes and the 7.1-megabase average length of chromosomal bands (see calculations later). This gap is far from being filled, and the attempts to relate cytogenetic similarities to gene mapping are incomplete. In the optimal case of chromosomal X, gene mapping has begun to confirm the cytogenetic data showing that intrachromosomal changes have occurred.

A. Comparative Gene Mapping for Human, Mouse, and Rabbit Chromosomes Except Ig Genes Carrying Chromosomes

Comparison of the human and mouse gene maps shows more than 60, probably identical, loci mapped in both species. Because these loci are dispersed over many chromosomes of each species, with an average of 1 or 2 genes per chromosome, they give insufficient information for claiming chromosomal identity (Dalton *et al.*, 1981; Shows *et al.*, 1982; Francke, 1982).

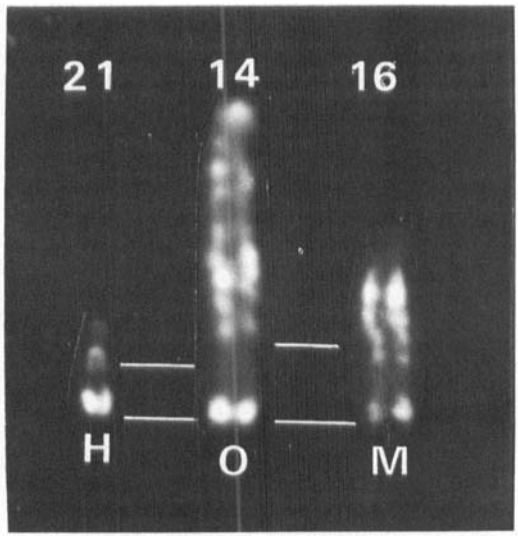

FIG. 11. Comparison between chromosomes HSA 21, MMU 16, and OCU 14. See Fig. 8 for legend.

Chromosome X is well conserved in mammals as observed either by the position of X-linked genes or by cytogenetic banding. Some of the loci found in common between man and mouse are *G6PD*, *HPRT*, *GLA*, and *PGK* (Human Gene Mapping 6, 1981), although the order of these loci in reference to the centromere is different (Francke and Taggart, 1980). These loci also have been found in rabbit X chromosome, but their sequence remains unknown (Echard and Gillois, 1979; Cianfriglia *et al.*, 1979).

Table III lists the chromosomal fragments which seem equivalent in man and mouse when studied by gene position or by cytogenetic banding. As listed in Table III *PGD*, *ENO1*, *AK2*, *PGM1*, *GDH*, and*FUCA* form a syntenic group of genes located in human chromosome 1p and mouse chromosome 4, and these chromosomal fragments display marked banding similarities. In rabbits, this chromosomal fragment is also easily identified (chromosome 13), although the synteny for *ENO1*, *PGD*, and *PGM1* has not been found (Echard *et al.*, 1981). The *TK1* and *GLK* loci have been located in human chromosome 17q and mouse chromosome 11 (Human Gene Mapping 5, 1979), and these chromosomes also display marked banding similarities. No similar loci have yet been mapped in rabbit, but we assume by banding comparison that OCU 19 is the corresponding chromosome. Shows *et al.* (1982) described a few other syntenic genes in man and mouse not listed in Table III, but for many of them chromosomal banding similarities are uncertain.

TABLE III

CHROMOSOMAL SEGMENTS OF MAN AND MOUSE IDENTICAL WHEN STUDIED BY GENE POSITION (EXCEPT Ig GENES) AND BY CHROMOSOMAL BANDING

Human chromosomes	Homoeologous in mouse[a]	Identical loci found[b]
X	MMU X	*G6PD, HPRT, GLA, PGK (Gpdx, Hprt, Ags, Pgk-1)*
1p	MMU 4	*PGD, ENO1, AK2, PGM1, GDH, FUCA (Pgd, Eno-1, Ak-2, Pgm-2, Gpd-1, Fuca)*
17p	MMU 11	*TK1, GALK (Tk-1, Glk)*
21	MMU 16 distal	*SOD1, IFRC, PRGS (Sod-1, Ifrc, Prgs)*
22	MMU 15 distal	*DIA1, ARSA (Dia-1, As-1)*
13	MMU 14 distal	*ESD (Es-10)*
Other human–mouse conserved syntenic groups for which similarities in chromosomal banding are uncertain (see Francke, 1982)		
6p	MMU 17	*HLA, GLO1 (H-2, Glo-1)*
11p	MMU 7	*LDHA, HBB (Ldh-1, Hbb)*
4	MMU 5	*PGM2, PEPS, ALB (Pgm-1, Pep-7, Alb-1)*
7	MMU 5	*ASL, GUSB, MDH2 (As1, Gus, Mod-2)*
10	MMU 10	*HK1, PP (Hk-1, Pyp-1)*
12p	MMU 6	*GAPD, TPI (Gapd, Tpi)*
15	MMU 9	*MPI, PK3 (Mpi-1, Pk-3)*
19	MMU 7	*PEPD, GPI (Pep-4, Gpi-1)*

[a] Homoeologous designates the residual homology of originally completely homologous chromosomes (Rieger *et al.*, 1976, "Glossary of Genetics and Cytogenetics, " 4th ed. Springer-Verlag, Berlin and New York).

[b] Symbols for genes as in Standardized Human Nomenclature (Human Gene Mapping 6, 1981). The corresponding nomenclature of the mouse (*Mouse News Lett.* **66**, 6–34, 1982) is indicated in parentheses.

Two years after the description of a high number of chromosome similarities between man and rabbit (Dutrillaux *et al.*, 1979), gene mapping generally confirmed the identity of certain chromosomal fragments. Soulié *et al.* (1981) have found the locus *LDHA* on chromosome OCU 1, which was described as equivalent to HSA 11 and which also carries this locus. These authors found the syntenic group *LDHB-TPI* on chromosome OCU 4, considered to be equivalent to HSA12. We also found the *NP* locus on chromosome OCU 16, considered to be equivalent to HSA 14, and the *MDH2* locus on chromosome OCU 15, considered to be equivalent to HSA 7. Echard *et al.* (1981) have found the loci *LDHB* and *TPI* syntenic in rabbits (these syntenic genes seem to be located within the homeologous region of HSA 12 and OCU 4), but the *PEPB* locus was not found syntenic in rabbits, although

it is in man. In conclusion, these few examples show that most of the chromosomal similarities proposed by banding comparisons agree with and may anticipate results from gene mapping studies. However, some discrepancies indicate rearrangements involving small segments which are beyond the resolving power of cytogenetics.

B. Comparative Mapping on Presumed Ig Genes Carrying Chromosomes

The different approaches in man, mouse, and rabbit gave indications that several chromosomes of these species might be carriers of Ig genes. The chromosomes equivalent to HSA 2 (short arm), HSA 14, and HSA 22 are presented for the three species, and the arguments for considering whether the assignment of Ig genes is correct are also discussed.

1. Chromosomes Equivalent to HSA 22

This chromosome has been demonstrated by Erikson *et al.* (1981) and McBride *et al.* (1982) to be the site of λ chain genes. As indicated previously, banding similarities exist between the distal part of human chromosome 22 and the distal part of mouse chromosome 15, and this may also be true for rabbit chromosome 21. Furthermore, the loci of *DIA1* and *ARSA* have been mapped in human chromosome 22 and in mouse chromosome 15 (Franche *et al.*, 1981). Thus the banding and gene mapping (Franche *et al.*, 1982) are good arguments for considering that human chromosome 22 and the distal part of mouse chromosome 15 have other genes in common. If the hypothesis that the assignment of λ chain genes to the proximal half of HSA 22 and to the region between the centromere and band B5 of MMU 16 is correct, we are not able to predict the type of rearrangements that occurred from the ancestor karyotype (chromosome 25) nor the equivalent chromosomal segment of the rabbit.

2. Chromosomes Presumed Equivalent to HSA 2p

The κ chain genes of Ig had been assigned by Malcolm *et al.* (1982a) to band 2p11→2p21 of chromosome HSA 2. This result has been confirmed by McBride *et al.* (1982). Banding similarities exist between the short arm of this chromosome and the long arm of rabbit chromosome 2, but no similarities could be found in any of the mouse chromosomes. Genes mapped in human chromosome 2 are listed in Table IV. One of them, *ACP1*, located at region p23 has also been found in mouse chromosome 12 to which Ig heavy chain genes have been assigned (Hengartner *et al.* 1979; Meo *et al.* 1980). The loci *MDH1* was assigned to rabbit chromosome 2 (Soulié and De-Grouchy, 1982), and this confirms the homoeologies between HSA 2p and

TABLE IV
LOCI FOUND ON HUMAN, MOUSE, AND RABBIT CHROMOSOMES CARRYING Ig GENES

Chromosome		Loci
Human chromosome	14	*RNR, NP, WARS, EMP195, CKBB, PGFT, PFGS, ESAT, D14S1, D14S2, SPH1, PI, EBV, IGHA1, IGHA2, IGHD, IGHE, IGHF, IGHG1, IGHG2, IGHG3, IGHG4, IGHM, IGHV*
Mouse chromosome	12	*rRNA, Acp-1, Pre-1, Igh-1, Igh-V*
Rabbit chromosome	16	*RNR, NP, IGHV*
Human chromosome	2	*RPE, AHH, ANI, ACP1, MDH1, GLAT, IF1, IGAS, ACEE, ADCP2, IDH1, FS, UGP2, POC, IGKC, IGKV, JK, D2S1*
Mouse chromosome	6	*Gapd, Tpi-1, Igk-C, Igk-V*
Rabbit chromosome	3	*IGKC*
Human chromosome	22	*RNR, CES, DGS, GLB2, CML, ARSA, DIA1, ACO2, S16, IGLC, IGLV, NAGA*
Mouse chromosome	16	*rRNA, Igl-C, Igl-V, Sod-1, Ifrc*

OCU 2q. Because the Igκ chain genes are located on the short arm of human chromosome 2, we should expect that these genes were found on the long arm of rabbit chromosome 2. However, these genes were assigned to rabbit chromosome 3 (Buttin *et al.*, 1979), whose long arms possess a banding pattern very similar to the long arms of chromosome HSA 8, but not to chromosome HSA 2. No banding analogies could be found between HSA 2p and any of the mouse chromosomes, indicating marked rearrangements.

Thus only gene mapping arguments can be considered. Among the genes mapped on chromosome HSA2p, *ACP1* is on MMU 12, and the κ chain genes are assigned to MMU 6. These genes are assumed to be located in HSA 2p, and the fact that they are split into two different chromosomes in the mouse may simply indicate that rearrangements with breaking points between the two loci has occurred.

3. *Chromosomes Presumed Equivalent to HSA 14*

There is no doubt that chromosome 14 is the place for Ig heavy chain genes in man (Croce *et al.*, 1979; Cook *et al.*, 1981; Solomon *et al.*, 1981; Smith *et al.*, 1981; Cox *et al.*, 1982). The homoeology of this chromosome and mouse chromosome 12 and rabbit chromosome 16 have been shown in Fig. 8, and the arguments for assigning Ig genes to these chromosomes have been discussed in Section III. Table IV lists the loci mapped in human chromosome 14. The gene coding for nucleoside phosphorylase (NP) has been located in HSA 14q13 (Aitken and Fergusson-Smith, 1978). It has also been located in the presumed homoeologous chromosome OCU 16 (Soulié

et al., 1981). Other loci found in human chromosome 14 have not been studied in other species, but large homoeologous segments are expected.

In addition to the results discussed in the previous paragraphs, the fact that human chromosomes 8 and 14 were associated with the nuclear anchorage of certain viruses (Yamamoto *et al.*, 1978; Kucherlapati *et al.*, 1978) must be considered. Although this may be restricted only to certain clones (Steplewsky *et al.*, 1979), the information is sufficiently interesting to stimulate further work. Finally, it is tempting to search for analogies between the reports describing nonrandom abnormalities of mouse chromosomes 15, 12, and 6 after the induction of plasmacytomas (Ohno *et al.*, 1979; Wiener *et al.*, 1980) and specific breaks of human chromosomes 8 and 14 in some neoplasias. This is reviewed in Section VI. The involvement of HSA 2p in certain lymphomas will also be considered.

VI. Ig Genes Carrying Chromosomes and Neoplasia

As discussed in Section III, the localization of Ig genes is well established in mouse and in man, but in rabbit it is still preliminary. However chromosomal comparison with rabbit chromosomes appears to be an indirect but helpful basis for studying chromosomes carrying Ig genes. For this reason, five human chromosomes are considered. The first is chromosome HSA 2p, which is associated to κ chain genes by *in situ* hybridization and by Southern blot hybridization. Second is HSA 14, which is associated to heavy chain genes by chromosomal segregation in hybridomas and by direct *in situ* hybridization. Third is HSA22, which is associated to λ chain genes by chromosomal segregation in hybridomas and by DNA hybridization procedures. In addition, HSA 8q is studied in this section because of its homoeology with the presumed carrier of κ chain genes in rabbit, and HSA 21 is also considered because it has a terminal region equivalent to MMU 16 which is carrying λ chain genes.

Two sets of information are available concerning the relationship between cancer and chromosomes. One is given by the spontaneous cancers observed in man, the other by the induced tumors in the mouse. Let us consider the latter initially, as it represents a homogeneous system for demonstrating the relationship between tumoral processes and the development of gross rearrangements affecting the Ig genes carrying chromosomes.

A. Murine Tumors

T cell leukemias and tumors have been induced in mice by leukemogenic viruses (Wiener et al., 1978a), X radiation (Chang *et al.*, 1977), and chemical carcinogens (Wiener *et al.*, 1978c). Cytogenetic studies on this material have

shown striking correlation with trisomy of mouse chromosome 15. Spontaneous leukemia in the AKR mice is also associated with trisomy of mouse chromosome 15 (Dofuku *et al.*, 1975), and this trisomy is also observed in murine leukemia cell lines (Hagemeijer *et al.*, 1982). Cytogenetic studies on pristane-induced mouse plasmacytomas have also been reported (Ohno *et al.*, 1979; Shepard *et al.*, 1978; Voss *et al.*, 1980) which show the frequent breakage of chromosomes 12 and 6 and also the deletions of chromosomes 15 and 18. The breaking points have been localized at band F2 of chromosome 12 and less accurately at band C of chromosome 6. Furthermore, chromosome 15 is broken at the distal region when it is involved in the reciprocal translocation T(6;15) or T(12;15) associated with pristane-induced plasmacytomas and with MuLV transformation.

Cytogenetic studies on IgA(λ)-producing murine plasmacytomas have also been published (Wiener *et al.*, 1980), confirming the translocation T(12;15) but not the T(6;15). It was concluded that the latter might be restricted to κ-chain-producing plasmacytomas. These authors have also studied Abelson murine leukemia virus-induced tumors. Trisomy of chromosome 15 followed viral transformation (Spira *et al.*, 1979; Wiener *et al.*, 1978a,b,c). The study of translocations showed that the relevant region of chromosome 15 was the distal part of this chromosome.

The reasons for the major involvement of mouse chromosome 15 has been clarified. A DNA sequence that generates rearranged immunoglobulin heavy chain constant-region genes, NIARD (Harris *et al.*, 1982a), has been located on chromosome 15 in the mouse germ line (Harris *et al.*, 1982b; Calame *et al.*, 1982). The NIARD sequence is the *myc* oncogene locus, which is found recombined with the α heavy chain locus in most of the murine plasmacytomas (Shen-Ong *et al.*, 1982). Rearrangement occurs at the left side of the *myc* gene, the rest of the gene remaining intact. The *myc* gene has also been mapped in human chromosome 8q24 (Dalla-Favera *et al.*, 1982; Neel *et al.*, 1982; Taub *et al.*, 1982). These results clearly indicate equivalent translocation processes for chromosomes MMU 15 and HSA 8q and for MMU 12 and HSA 14 in lymphomas and plasmacytomas. Equivalent translocation processes are also expected for chromosomes MMU 6 and HSA 2p and for MMU 16 and HSA 22. Not every one of these predictions agrees equally well with the established chromosomal similarities; this may be explained by the low resolution of chromosomal comparisons. No similarities are observed between HSA 8q and MMU 15, and the distal part of MMU 15 is equivalent to the distal part of HSA 22 as shown by gene mapping and chromosomal banding. We do not find a sufficient degree of similarity between the chromosomal region carrying IGL genes in man and mouse, i.e., the proximal part of HSA 22 and the proximal part of MMU 16. Another point that remains obscure is that no alterations of mouse chromosome 16

have been observed in λ-producing murine plasmacytomas, although the λ chain genes were assigned to this chromosome (D'Eustachio *et al.*, 1981).

B. Involvement of Chromosomes 2, 8, 14, 21, and 22 in Human Neoplasia

The reviews of Harnden and Taylor (1979) and Mitelman and Levan (1981) showed nonrandom involvement of human chromosome abnormalities in neoplasia. Chromosomes 14, 8, and 22 are the most frequently affected in cancers, and chromosome 21 has also clearly been associated with leukemias (Rowley, 1980). On the contrary, chromosome 2 is infrequently involved in lymphomas or in other types of cancers, but it has been related to some specific translocations in Burkitt lymphoma cell lines, with a breaking point at the locus for IGK genes. Thus all the chromosomes carrying Ig genes may be affected in tumors, but to various amounts. This involvement, which is discussed in the next section, seems highly dependent on the type of tumor.

1. Involvement of HSA 14 and HSA 8 in Neoplasia

There is a general agreement on the existence of a specific translocation t(8;14) in Burkitt's lymphoma (review in Mitelman, 1981). Cytogenetic studies of this disease have shown a marker 14q+ and a marker 8q− usually associated that is interpreted as a reciprocal translocation t(8;14) (q24:q32) (Zech *et al.*, 1976; Manolova *et al.*, 1979). Philips (1975) and Mark (1975) suggested that a correlation exists between the appearance of marker 14q+ and the beginning of B lymphocyte proliferation. This translocation is also found in other lymphomas such as Hodgkin's disease, but is associated with other chromosomal abnormalities and with nonrandom chromosomal breakages. In addition to its exchanges with chromosome 14, other abnormalities of chromosome 8 have been described. Table V lists the structural aberrations of chromosomes 14 and 8 in most of the myeloproliferative and lymphoproliferative disorders. Breakpoints on these chromosomes have been found at band 14q32 and 8q24 in cases of reciprocal translocation t(8;14). Other minor breakpoints described are 14q12 and 14q14 in acute myeloid leukemia and 8q22 in chronic and acute myeloid leukemias. A direct relationship between translocation t(8;14) in Burkitt's lymphoma and immunoglobulin V_H genes has been demonstrated by Erikson *et al.* (1982), which showed in Daudi cell lines that the 8q− chromosome contained V_H genes but no C_H genes. Therefore, a breakpoint in chromosome 14 had occurred which separated C_H and V_H region genes.

2. Involvement of HSA 2p in Neoplasia

This chromosome is usually considered to be randomly involved in human neoplasia. However, the existence of certain cases of Burkitt's lymphomas in

TABLE V
FREQUENCY OF INVOLVEMENT OF HUMAN CHROMOSOMES 2, 8, 14, 21, AND 22 IN MYELOPROLIFERATIVE AND LYMPHOPROLIFERATIVE DISORDERS[a]

Syndromes	Number of structural aberrations per chromosome				
	2	8	14	21	22
Chronic myeloid leukemia (CML), 361 cases[b]	7	165	16	28	162
Acute myeloid leukemia (AML), 496 cases	22	184	19	116	56
Polycythemia vera (PV), 86 cases	2	22	2	4	3
Burkitt's lymphoma (BL), 26 cases	3	17	24	—	3
Myeloproliferative disorders (MD), 278 cases	13	65	6	37	10
Malignant lymphoma (ML), 105 cases	25	33	69	15	16
Acute lymphocytic leukemia (ALL), 156 cases	12	33	38	35	40
Chronic lymphocytic leukemia (CLL), 30 cases	6	3	11	3	1
Monoclonal gammopathies (MG), 23 cases	—	6	11	3	4

[a] Extracted from Mitelman and Levan (1981).
[b] Translocation t(9,22) excepted.

which translocations t(2;22) and t(2;8) occurred has been pointed out. In the cases of reciprocal translocation t(2;8) described by Van den Berghe *et al.* (1979), by Miyoshi *et al.* (1979), and by Bernheim *et al.* (1980), a breakpoint was found near 2p13→2p11, i.e., very close to the IGK locus. Moreover, Lenoir *et al.* (1982) have found a direct relationship between expression of immunoglobulin light chains and specific type of translocation: BL cells with t(8;22) express λ chains, whereas those with t(2;8) express κ chains.

Translocation t(2;14) has been reported by Sessarego *et al.* (1977) in the CML syndrome. Complex translocations involving chromosomes 2 and 22 have been reported by Hayata *et al.* (1980), Smadja *et al.* (1980), and Van den Akker *et al.* (1980), but the breakpoints usually occurred in the long arm of chromosome 2.

3. *Involvement of HSA 21 in Neoplasia*

Most of the myeloproliferative and lymphoproliferative disorders present a significant increase in abnormalities of HSA 21 (Table V). Translocation t(8;21) frequently results from breaks at bands 8q22 and 21q22 in acute

nonlymphocytic leukemia (Rowley and Testa, 1982; Rowley, 1980). Infrequent translocations t(21;22) have also been reported by Bottura and Couthinho (1974) and by Cimino *et al.* (1979).

4. Involvement of HSA 22 in Neoplasia

The occurrence of a translocation of chromosome 22 which has been called Philadelphia chromosome is a well-known observation in chronic myeloid leukemia. In most of the cases the distal part of chromosome 22 is translocated onto chromosome 9 (Nowell and Hungerford, 1960), but other sites of translocation have been described (Mitelman and Levan, 1981). Myeloid and lymphoid leukemias also display a high number of aberrations in HSA 22 involving band q13 in most of the cases (Rowley and Testa, 1982).

Several conclusions can be derived from these observations. A relationship exists between Ig gene positions and chromosomal rearrangements in malignant lymphoid proliferations for IGM and IGK genes in man and mouse. A major explanation for the translocation observed in mouse chromosome 15 and in human chromosome 8 is derived from the presence of *myc* oncogenes in these chromosomes (Harris *et al.*, 1982b; Calame *et al.*, 1982; Shen-Ong *et al.*, 1982; Dalla-Favera *et al.*, 1982b; Neel *et al.*, 1982; Taub *et al.*, 1982). The existence of oncogenes in some of these chromosomes highly rearranged in neoplasia was initially proposed by G. Klein (1981) (Klein and Lenoir, 1982). Another oncogene, c-*mos*, also maps to human chromosome 8 (Prakash *et al.*, 1982; Neel *et al.*, 1982), and other chromosomes such as HSA 15 and HSA 9 (Heisterkamp *et al.*, 1982), HSA 15 and HSA 6 (Dalla-Favera *et al.*, 1982), HSA 11, (McBride *et al.*, 1982) and HSA 22 (Swan *et al.*, 1982) also carry oncogenes. Discrepancies also exist: no specific involvement of chromosome MMU 16 has been described, although it is carrying IGL genes. HSA 21 does not carry Ig genes or oncogenes and is frequently rearranged in lymphoid and myeloid leukemias. Banding comparison of chromosomes HSA 21, HSA 8, and MMU 15, indicate partial homoeology with chromosomes that do carry Ig genes in other species. Indeed this may reflect errors or limitations in chromosome banding comparison or in gene mapping. Another interpretation could be that similar sequences of bases exist in DNA of these chromosomes that would favor the exchanges leading to rearrangements, a part of them being related to cancer formation, another part to chromosome evolution.

VII. Rearrangements of Ig Genes

The sequencing of DNA and mRNA from Ig genes created an adequate degree of resolution for establishing the number of genes and gene fragments involved in the production of immunoglobulins. The hypothesis of

Dreyer and Bennet (1965) that the genes for variable and constant regions are separately encoded in the genome was confirmed by restriction analysis of DNA in mature and stem cell lymphocytes (Hozumi and Tonegawa, 1976). Multiple rearrangements which have been observed are reviewed (Adams *et al.*, 1981; Honjo *et al.*, 1981). They allow the definition of several families of linked Ig genes in the mouse as follows:

1. A family of κ light chain genes composed of a group of variable region genes (IGKV), a single gene for constant region (IGKC), and from one to five joining segments (J_κ) close to the IGKC locus. All these gene segments are syntenic.
2. A family of λ genes composed of multiple IGLV genes, from one to four IGLC genes, and four functional J_λ joinings, all syntenic and located on another chromosome.
3. A family of heavy chain genes composed of multiple genes for the variable region IGHV, single copy genes for the constant region situated next to each other in the order 5′-C_μ-C_δ-$C_{\gamma 3}$-$C_{\gamma 1}$-$C_{\gamma 2B}$-$C_{\gamma 2A}$-C_ϵ-C_α-3′ (Honjo *et al.* (1982), and the so-called J and D fragments in number of four or five.

The formation of light chains requires a single recombination event for fusing V and C genes (Max *et al.*, 1979; Sakano *et al.*, 1979), but the formation of a heavy chain requires two different recombination events for fusing V, D, and J elements (Early *et al.*, 1980; Sakano *et al.*, 1980). In addition to these rearrangements for bringing together different encoding regions, rearrangements are perhaps required to explain allelic exclusion and isotypic exclusion.

A. Productive Rearrangements

According to Davies *et al.* (1980), productive (or normal) rearrangements are the recombination events bringing the encoding Ig variable and constant regions within a single transcription unit. In the mouse, the family of genes for λ light chain is reduced to two variable region genes (IGLV), three constant region genes (IGLC1, IGLC2, and IGLC3), one pseudogene (IGLC4) close to IGLC2, and three functional J_λ elements (Miller *et al.*, 1982) residing 1.2 kilobases apart from the 5′ end of the C_λ genes (Blomberg *et al.*, 1981; Miller *et al.*, 1981). In man, a much larger V_λ gene repertoire was described (Hieter *et al.*, 1981a). The family of κ light chain genes of the mouse comprises between 90 and 320 variable-region genes (IGKV genes), one IGKC gene, and four functional J_κ elements (Bentley and Rabbits, 1981; Adams *et al.*, 1981). In domestic rabbits four forms of the constant region of κ light chain were found (Oudin, 1960; Dubiski and Muller, 1967). Joining

V–J segments requires a single deletion of the intervening DNA with two conserved sequences at the 3′ end of the V segment and the 5′ end of the J segment (Max *et al.*, 1979; Sakano *et al.*, 1979).

The formation of an active gene for a heavy chain also requires a selection among the different genes that compose the IGHC family. The mechanism of selection among the eight different IGHC genes (heavy chain switch) is only partially understood (Rogers and Wall, 1981). In addition, the formation of an active κ gene represents a certain commitment which forces other alleles to remain silent. Mature B cells or plasma cells present the phenomena of allelic exclusion and isotypic exclusion. The allelic exclusion refers to the fact that these cells transcribe an immunoglobulin polypeptide from a maternal allele or from a paternal allele but never from both. Isotypic exclusion refers to the observation that when κ genes are expressed, λ genes remain silent or vice versa (except in rare cases) (Froland and Natvig, 1972; Melcher *et al.*, 1975). This seems to be a normal step of B lymphocyte differentiation, because the excluded allele either remains in the germ line form or is deleted or rearranged in an aberrant fashion (Max *et al.*, 1980; Altenburger *et al.*, 1980; Perry *et al.*, 1980). The type of rearrangements which lead to the nonexpression of λ chain genes in favor of κ chain genes may be considered as abnormal or as a nonproductive rearrangement (Hieter *et al.*, 1981b).

It has been shown in mouse splenic B cells that (1) nonproductive rearrangements of κ genes occur frequently; (2) λ_1 genes are rarely rearranged in κ-expressing cell lines, but κ genes are often rearranged in λ-producing cell lines; and (3) rearrangements usually occur in both heavy chain alleles (Coleclough *et al.*, 1981). We concluded that aberrant rearrangements significantly contribute to allelic exclusion. Similar results have been published by other groups (Wilson *et al.*, 1979; Steinmetz *et al.*, 1979; Cory *et al.*, 1980; Bernard *et al.*, 1981). A theory for explaining allelic and isotypic exclusion for immunoglobulin genes has been proposed by Wabl and Steinberg (1982) and is based on two postulates: (1) the existence of a heavy chain binding protein (BiP) which may be displaced from H chain by light chain, and (2) the elimination of those cells that productively rearranged both alleles at the H chain locus by the toxicity of an excess of H chain.

B. Nonproductive Rearrangements

Nonproductive or abnormal rearrangements are defined as those creating nonfunctional genes (Early and Hood, 1981). These rearrangements have been observed on both diploid and heteroploid cells expressing and nonexpressing Ig genes. Among the abnormal rearrangements, various possibilities are possible: (1) nonsense sequences and null genes at the V/J

recombination region (Max *et al.*, 1980; Altenburger*et al.*, 1980); (2) abnormal heavy chain switch processes (Seidman and Leder, 1980); (3) recombination at a pseudo J site with no proper RNA processing signal (Perry *et al.*, 1980; Choi *et al.*, 1980); (4) recombination with errors in joining that create translational stop codons (Altenburger *et al.*, 1980; Max *et al.*, 1980); (5) recombination extinguishing transcription after selection of a nonfunctional V region (Perry *et al.*, 1980); and (6) abnormal recombination events among V_H and D_H and among D_H and J_H. The rearrangements at the D and J loci are specially frequent (Bernard *et al.*, 1981; Eckhardt *et al.*, 1982; Zuñiga *et al.*, 1982).

Among the unexpected rearrangements related to Ig genes are the following: (1) the existence of one cloned functional IgE gene with four constant-region domains and one pseudogene that has lost the first two coding domains and the 5′-flanking sequences found adjacent to the functional gene(Max *et al.*, 1982); (2) the existence of one cloned ϵ-like gene which is processed and has moved from human chromosome 14 to human chromosome 9 (Battey *et al.*, 1982); (3) the existence of nonimmunoglobulin associated rearranged DNA sequences (NIARD) which have been mapped in mouse chromosome 15 and may move to chromosome 12 in the reciprocal translocation T(12;15) observed in murine plasmacytomas (Harris *et al.*, 1982b; Calame *et al.*, 1982).

The fact that λ genes remain in the germ line configuration in λ-producing cells while κ genes are rearranged or deleted in κ-producing cells (Hieter et al. 1981b) seems to indicate that κ gene rearrangements precede those of λ genes. In addition, μ chain synthesis might well precede that of light chains (Levi and Cooper, 1980; Maki *et al.*, 1980). These data are consistent with a cascade of gene rearrangements (Korsmeyer *et al.*, 1981): that of heavy chain genes preceding that of the light chain genes and that of κ light chain genes preceding that of λ.

There are few studies that directly relate Ig gene rearrangements to the structural changes affecting chromosomes, such as inversions or translocations (Erikson *et al.*, 1982; Harris *et al.*, 1982b; Calame *et al.*, 1982). The observations discussed in the previous paragraphs allow one to postulate that if Ig gene rearrangements are associated with chromosomal changes, the chromosome carrying Ig heavy chain genes should be more frequently rearranged than those carrying the light chain genes. Furthermore, the chromosome carrying Ig λ chain genes would often be silent or would have the original germ line configuration. In addition, the most likely regions for pairing and recombination are the V/J joining regions or the D/J joining regions. Pairing can occur either within the same DNA strand by looping out or between sister chromatids, as proposed by Obata *et al.* (1981). These changes which affect a given region of a single chromosome (intrachromoso-

mal changes) do not exactly correspond to most of the rearrangements described in lymphoproliferative and myeloproliferative disorders (Section VI), which are translocations (interchromosomal changes), but intrachromosomal changes may have been missed in many instances (Aurias *et al.*, 1980).

C. Chromosomal Length of Ig Genes

The number of antibody genes, i.e., the antibody repertoire, has been estimated as 10^6 different types of immunoglobulins for mammalian species (Williamson, 1976). This number is arrived at mainly by combinatorial association of a few hundred different IGKV, IGLV, or IGHV genes, a few copies of the corresponding constant-region genes, four or five diverse intervening sequences (D genes) for the heavy chain, and four or five joining segments for each one of the three families J_κ, J_λ, and J_H. The antibody repertoire also includes the length of intercalating fragments and the deletions that occur, but this length remains mostly undetermined. The extent of the contribution of somatic mutations to antibody diversity also remains undetermined.

Well-banded human karyotypes do not exhibit more than 1000 bands per haploid set of chromosomes, being three to four times less in routine banding. The conclusion from the estimated 7×10^9 bp of human diploid genome (Saunders *et al.*, 1972) or from the total haploid length in recombination units, established in 3000 cM per human genome (Yunis *et al.*, 1978), is that 7×10^6 bp or 3 cM is the average length of a cytogenetic band.

Our chromosomal comparisons of Ig genes carrying chromosomes allow us to speculate on the limits of chromosome homoeologies in man, mouse, and rabbit that presumably include Ig genes and many other unknown genes. We have compared these homoeologies in many other mammals. Our main conclusion is compatible with the idea that a single chromosome band or 3 cM is largely sufficient for containing all syntenic Ig gene segments. This can be summarized as follows:

1. The family of λ genes may be located within a single chromosome band in man and in mouse. This single band may contain other genes. The family of λ genes in man, however, is particularly complex (Bernard *et al.*, 1981) and is assigned to one of the two smaller chromosomes of man.

2. The family of κ chain genes in mouse comprises between 90 and 320 variable-region (IGKV) genes, one IGKC gene, and four functional J_κ elements (Adams *et al.*, 1981; Bentley and Rabbits, 1981). These genes have been located in chromosome 6 of the mouse and in segment 2p11→2p21 of man. No homoeologies have been found between these two elements nor

they have they been found with rabbit chromosome 3 to which IGKC genes have also been assigned.

3. The family of heavy chain genes have been located in proximity to band 12F1 of the mouse (Meo *et al.*, 1980) and in band 14q32.3 of man (Cox *et al.*, 1982). Homoeologies between these two elements and rabbit chromosome 16 extend most of the chromosome length. Other identical genes not related to heavy chains of immunoglobulins have been found in these three chromosomes. Breaking points at band 14q32, which correspond with the exact position of Ig heavy chain genes, have frequently been described in human neoplasia and especially in Burkitt's lymphoma (Erikson *et al.*, 1982).

VIII. Concluding Remarks

The chromosomal localization of Ig genes in man and mouse has been firmly established. Our own results with mouse–rabbit hybridomas indicate that it is possible to localize Ig genes in rabbit chromosomes, although these hybridomas are unstable and do not allow correlations to be made between enzyme gel electrophoresis of biochemical markers and chromosomal segregation. The instability of nonmurine chromosomes seems to be the main reason why the mapping of Ig genes in man has been long obscured with conflicting reports.

Rabbit chromosomes are specially suitable for comparison with human chromosomes. Banding similarities for Ig heavy chain genes carrying chromosomes in the three species examined in this article extend to most of the chromosome. However, the comparison of the small chromosomal segments carrying λ chain genes in man and mouse present some difficulties which have been resolved by studying certain translocations. No equivalent chromosomal segment for λ chain genes could be found in the rabbit. Discrepancies have been found in the comparison of the chromosomes carrying κ chain genes in man and rabbit, showing limitations of this approach even if high resolution banding and reconstructed ancestral karyotypes are used. The homoeologies that can be establish for HSA 8q with κ chain genes carrying chromosomes in the rabbit may represent additional information for understanding the involvement of Ig genes in neoplastic diseases.

Of great interest is the nonrandom involvement in cancer of the chromosomes carrying Ig genes, but further work is required to construct a molecular model before general statement is possible. Pristane-induced murine plasmacytomas showed specific rearrangements of chromosomes carrying heavy and κ light chain genes. However, human chromosome 2, which has been confirmed as being the site of κ light chain genes and is translocated with break points at the position of these genes in some Burkitt's lymphoma

cell lines, is not primarily involved in the myeloproliferative and lymphoproliferative disorders listed in Table V.

The progress in this field, and particularly the techniques of nucleic acid blotting and hybridization between radioactive probes of Ig genes and DNA from hybrid fibroblast cells of known karyotype, leads us to believe that the topics reviewed here will be better documented in the future. Until then, the present data on the position of Ig genes in man, mouse, and rabbit can be summarized as follows:

1. Genes for Ig heavy chains can be definitively assigned to the homoeologous region of human chromosome 14, mouse chromosome 12, and rabbit chromosome 16. The mapping of IGH genes in these three species is in agreement with chromosome banding similarities, and the genes can confidently be assigned to a specific band located at the distal part of these chromosomes.
2. Genes for κ light chain have been assigned to mouse chromosome 6, to human chromosome 2p, and to rabbit chromosome 3. The longest part of this rabbit chromosome is very similar to the long arm of human chromosome 8. No chromosome banding similarities could be found for human chromosome 2 in any of the mouse chromosomes.
3. Genes for λ light chain have been assigned to mouse chromosome 16 and to human chromosome 22 but not yet to any rabbit chromosome. These genes are located within a very small equivalent chromosomal region in these two species, and we are unable to predict any equivalent region in the rabbit, but it may be located in chromosome 25 of the ancestral karyotype.

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