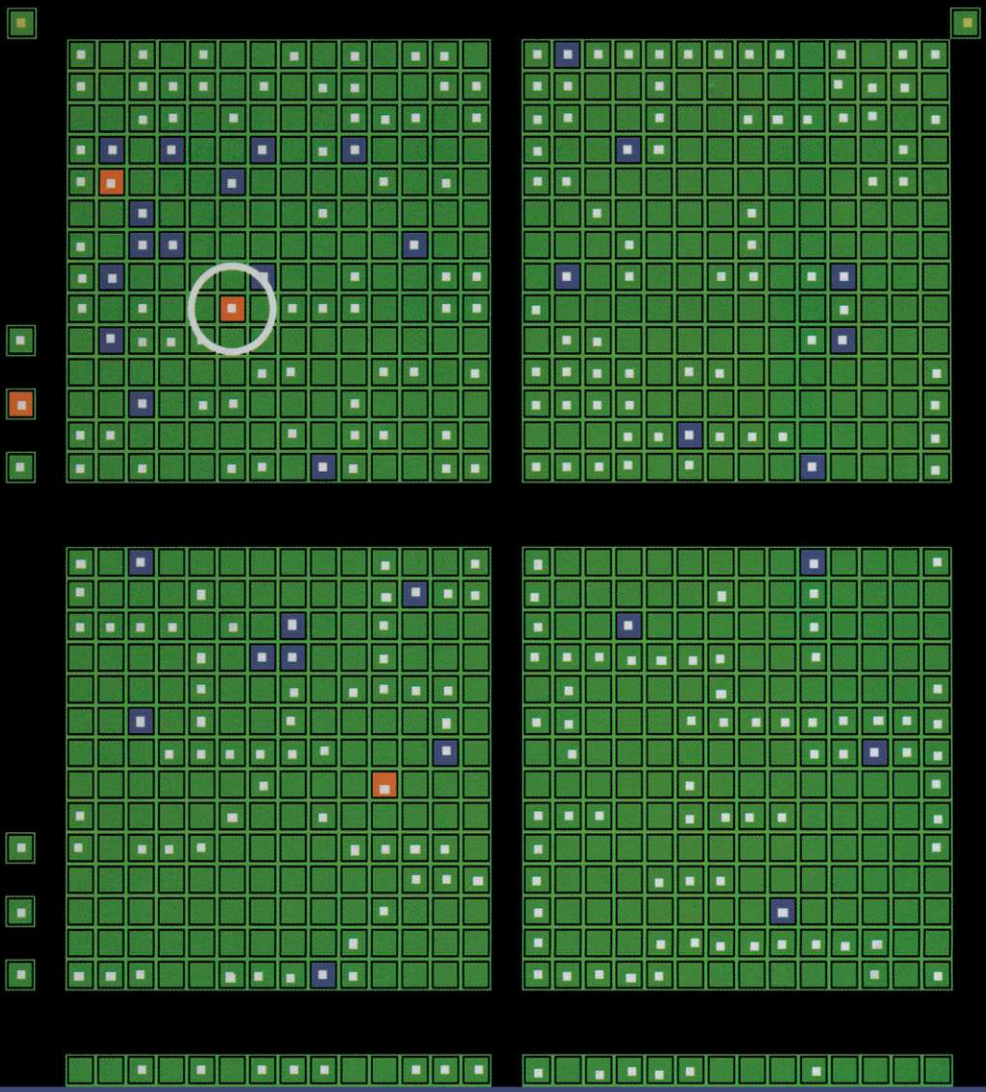

ALUMINIUM AND ALZHEIMER'S DISEASE

THE SCIENCE THAT DESCRIBES THE LINK

Christopher Exley, Editor

ELSEVIER

Aluminium and Alzheimer's Disease

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The cover illustration is a DNA array (Clonetech Corp. Palo Alto, CA, USA) showing how exposure of explanted human brain neural cells in primary culture to 100 nM aluminium chloride influenced gene expression. Each white square represents an expressed gene. A white square bordered by a blue square shows down-regulation of this gene at least 3-fold relative to brain cells not exposed to aluminium. A white square bordered by a red square shows up-regulation of this gene by at least 3-fold relative to brain cells not exposed to aluminium. The up-regulated gene that has been encircled is the gene for the amyloid precursor protein. (Thanks to Walter Lukiw for supplying this information.)

Aluminium and Alzheimer's Disease
The Science that Describes the Link

Edited by

Christopher Exley
Birchall Centre for Inorganic Chemistry and Materials Science,
School of Chemistry and Physics,
Keele University,
Staffordshire, ST5 5BG, UK

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PREFACE

Why is Research into Aluminium and Life Important?

Christopher Exley *

Birchall Centre for Inorganic Chemistry and Materials Science, School of Chemistry and Physics, Keele University, Keele, Staffordshire, ST5 5BG, UK

* Corresponding author, email: cha38@keele.ac.uk

Abbreviations: Al, aluminium; AD, Alzheimer's disease

Summary

The versatility of aluminium chemistry will ensure its burgeoning use in the future in all manner of applications. This very same chemistry will also ensure its increased biological availability in all biota including Man. We do not know enough about the biological chemistry of a chronic exposure to aluminium to be able to predict its impact on human health. The scientific evidence linking aluminium with Alzheimer's disease is as strong as it is for any other single aetiological agent. If we can identify where aluminium fits into the aetiology or pathogenesis of diseases such as Alzheimer's we may be able to accurately assess the risk to human health posed by biologically available aluminium.

Personal Perspective

The natural abundance of aluminium (Al) and the development at the end of the nineteenth century of its electrolytic refinement have conspired to make Al the most widely used and diversely applied metal of our age. Behind many of its successful applications is an extremely versatile chemistry which will lend itself to the continued and varied use of Al in the future (Atwood & Yearwood, 2000). Man-made alterations in the environment, such as the acidification of land by, for example, the impact of acidic deposition or the extensive use of intensive agriculture, and Man's use of Al in everyday life are increasing its abundance in the biosphere relative to the lithosphere and, concomitantly, its biological availability or potential to participate in the processes of life and, ultimately, evolution (Exley & Birchall, 1992). Therefore, it is important that we understand the biological chemistry of Al and, in particular, in its relation to human health.

The widespread use of products made from or containing Al is ensuring the omnipresence of Al in our bodies. It is unlikely that Al is absent from any organ, tissue, body fluid or even cell in our body. New research continues to document our burgeoning exposure

to Al (for example, Linebarger et al., 1999; Roider & Drasch, 1999; Mora et al., 1999; Kristjansson et al., 2000; Dawson et al., 2000; Schlesinger et al., 2000) and, in some cases, has linked the observed burden of Al to human health (for example, Hovatta et al., 1998; Burge et al., 2000; Riihimäki et al., 2000; Shanklin et al., 2000). Every year new research highlights aspects of the biological chemistry of Al which might help to explain much of its known toxicity (for recent examples see; Toninello et al., 2000; Vieira et al., 2000; Jankowska et al., 2000; González-Revaldería et al., 2000; Berg et al., 2000; Verstraeten & Oteiza, 2000; Tanino et al., 2000; Zatta et al., 2000; Zatta et al., 2000; Struys-Ponsar et al., 2000; Lévesque et al., 2000; Smans et al., 2000; Hong et al., 2000; Mahieu et al., 2000). However, the emergence of Al as an environmental toxin has not yet received serious recognition in human toxicology. It is intriguing that the advent of the recognition that the most abundant metal in the lithosphere is inimical to life has not been sufficient by itself to arouse the precautionary principle in the same way as has recently been the case in Europe with, for example, transmissible encephalopathies? It is clear that many equate the omnipresence of Al with a benign influence on health. It is reasoned that Al toxicity can only be as the result of an acute exposure to the metal and that an event of this kind will be extremely rare in the general population. It is true that Man's systemic absorption of Al is limited and that this will reduce the likelihood of an acute exposure. However, it is a coincidence of Al chemistry that the systemic absorption of Al is low. There is no evidence that the exclusion of Al from the body is the result of evolutionary pressure. Likewise there is not an element specific reaction to the presence of systemic Al. There is no homeostatic control of the concentration of Al in either the intracellular or the extracellular environment. Al is a silent visitor to our bodies and its transport and fate are governed by a large number of Trojan Horse-like molecules. We are fortunate that some of these molecules facilitate the removal of Al from the body via the kidney. However, other molecules actually contribute towards an increase in the body burden of Al by delivering it to more permanent body stores such as bone and, the brain.

The brain is an obvious target for chronic Al intoxication. The longevity of neurones identifies them as sinks for systemic Al. The uptake of Al into the brain is at least an order of magnitude more efficient than its release (Yokel et al., 2000) and this ensures an increase in neuronal Al with age. There can be no dispute over the presence, and indeed accumulation, of Al in the human brain. The task now is to identify the biological chemistry of the brain Al burden and how it may be influenced by brain biochemistry and physiology in health and disease.

The research summarised in this book in concert with the informed opinions of the authors have provided the most up to date account of the science that describes the link between Al and Alzheimer's disease (AD). The subject has been reviewed from the perspective of delineating what we think we know about Al (Historical Perspective), including exposure, human disease, animal models of disease, toxicokinetics and cell biochemistry, and then using this information to think about what we do not know about Al and AD (Informed Opinion). Certainly Al is not inert in the body. Wherever it is found it will be biologically available. The question is whether the biological reactivity of Al is sufficient to influence essential biochemical processes or physiological functions in any particular environment. In the brain, during an acute exposure to Al, for example, as has occurred in dialysis encephalopathy, the concentration of biologically available Al

will be both high and persistent and normally robust biochemistry will be irreversibly altered within a very short timeframe. However, when the brain is subjected to a chronic exposure to Al (similar, perhaps, to our everyday exposure to the metal) the concentration of biologically available Al in the brain will be low, though it will increase with age and it will persist throughout the lifetime of the individual. The ability of the brain to cope with this persistent challenge will reflect individual differences in brain physiology and brain uptake and accumulation of Al. It will be this balance which will dictate whether or not Al will influence brain function and not simply the brain burden of the metal. In AD there is a selective and progressive loss of cognitive function which precedes neuronal loss and the formation of characteristic pathology. Al has been implicated in each stage of the disease and it is only a red mist of controversy and not any decision based upon sound scientific principles that has blinded some individuals and organisations to its possible role in the aetiology of the disease. We have not been able to conclude a specific role for Al in AD. However, neither have we concluded that Al is a benign influence in the disease. We hope that this book will act as a stimulus to much needed further research into the biological chemistry of Al and the link between Al and AD.

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Contents

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CHAPTER 1

Aluminium Induced Disease in Subjects with and without Renal Failure — Does It Help Us Understand the Role of Aluminium in Alzheimer's Disease?

Paul Altmann *

Oxford Kidney Unit, The Churchill, Oxford Radcliffe Hospitals, Oxford OX3 7LJ, UK

* Corresponding author, email: paul.altmann@orh.nhs.uk

Abbreviations: μg/l – micrograms per litre; mg/l – milligrams per litre; μg/g – micrograms per gram; ms – milliseconds; g – gram; P – probability; r – regression; n – number; % – percent; F–P difference – difference between flash and pattern stimulated visual evoked potential; IQ – intelligence quotient; DHPR – dihydropteridine reductase; HPA – hyperphenylalaninaemia

Summary

The neurotoxicity of aluminium is well described. It poses a very significant risk for patients suffering from renal failure who cannot excrete the element well. They may develop an encephalopathy due to aluminium neurotoxicity which is similar in several ways to Alzheimer's disease. In the latter condition there is animal and human evidence for the role of aluminium in its genesis. However, given that there is very good evidence for a genetically determined abnormality of amyloid deposition in the brain of such patients, perhaps Alzheimer's disease is a condition whose genesis depends on both the environmental and the genetic factors being present.

Historical Perspective

There is little doubt that in the experimental setting aluminium is toxic to hundreds of cellular processes both in man and animals. It is interesting, nevertheless, how much controversy surrounds its role in human disease. What is not controversial is that under certain conditions aluminium poisoning may occur and that its most alarming effects are as a result of potent neurotoxicity.

Aluminium is the third most abundant element in the earth's crust, of which it comprises up to 13%, existing naturally in complex compounds such as silicates and oxides. In the late 19th century the manufacture of metallic aluminium became a practical possibility, and since then it has been widely used in industry. In a paper by von Döllken in 1897, reference was made to the earliest publication (by Orfila in 1814) discussing the potential toxic effects of aluminium salts on animals (von Döllken, 1897). Behavioural

changes and irritability were noted following parenteral administration. von Dölken also commented on work performed in 1887 by Siem in which detailed animal studies found quite profound neurological changes following the administration of aluminium. In the same article von Döllken confirmed these findings and one of his concluding comments was that the changes induced were clinically similar to human dementia. The earliest reference to aluminium in an English language medical journal was in the Lancet of 1913 where its successful use in the kitchen was discussed (Anonymous, 1913b; Anonymous, 1913a). The authors did not investigate potential toxicity.

Aluminium Toxicity in Subjects with Normal Kidneys

Industrial exposure

The first description of the toxic effects of aluminium on man was not until 1921. Spofforth reported the case of a 46 year old metal worker who had been dipping red-hot metal articles into concentrated nitric acid using an aluminium holder and developed "loss of memory, tremor, jerking movements and impaired coordination" (Spofforth, 1921). Large amounts of aluminium were found in his urine, although no details were given of the methodology used. The report did not discuss the subsequent course of his illness. In 1957 Campbell and colleagues reviewed 503 references to "aluminium in the environment of man" (Campbell et al., 1957). Except for cases of industrial poisoning due to inhalation of aluminium dust, they dismissed the potential toxicity of this element to man. In 1974 the same group reviewed the field once more, but again concluded that there was "still no need for concern by the public or producers of aluminium or its products concerning hazards to human health" (Sorenson et al., 1974).

Since the case of encephalopathy reported by Spofforth in 1921 several other cases of industrial poisoning with aluminium have been reported, but only many years later. Pulmonary fibrosis and granulomata were found in aluminium workers but encephalopathy associated with the inhalation of aluminium dust was not reported again until 1962 (McLaughlin et al., 1962). A 49 year old man developed a rapidly progressive encephalopathy associated with epileptiform seizures. The earliest symptoms started three years before his death with an intermittent deficit of short-term memory and difficulty with speech. There followed increasing muscle twitching and speech difficulty. The electroencephalogram was abnormal with generalised slowing and spike activity. Other investigations were all normal except for mild anaemia; renal function had been normal and he died after the onset of bronchopneumonia following further deterioration of his cerebral state. No measurements of serum aluminium concentration were made during life or at post-mortem, but very high levels of aluminium were found in all the tissues examined, including the brain (480 μg/g dry weight compared with less than 0.6 μg/g in normals). The lungs showed severe fibrosis associated with areas of stainable aluminium. No comment was made on the bone histology. In 1985 came a further report of neurological disorders (incoordination, tremor, cognitive defects) in three patients who had worked for over 12 years in the same aluminium smelting plant (Longstreth et al., 1985), although no conclusive evidence for the role of aluminium was documented. A type of pneumonia

has also been described (Herbert et al., 1982) and an increased incidence of carcinoma of the lung in Norway (Andersen et al., 1982). Accidents involving single subjects exposed to aluminium in medical applications have also occurred (Hantson et al., 1994; Renard et al., 1994). Rifat and colleagues reported a large study in which miners exposed to aluminium for therapeutic purposes (in the form of McIntyre powder to prevent silicotic lung disease) developed impaired cognitive function which was worse the longer the exposure to the agent (Rifat et al., 1990). More recently White and colleagues described neuropsychological changes in aluminium smelting plant workers (White et al., 1992) confirming earlier worries about this type of exposure. In addition, one study reported an incident where source water had been contaminated by effluent from an aluminium die-casting plant (Kilburn & Warshaw, 1993). Contaminants included a variety of organic chemicals, and although it is quite possible that large amounts of aluminium may have been present also this was not discussed. Residents adjoining the plant did develop quite significant psychomotor difficulties including loss of short-term memory.

Aluminium accumulation in parenteral nutrition

Patients on long-term parenteral nutrition may develop bone pain and osteomalacia. Contamination of parenteral solutions by aluminium may be an important factor in the genesis of this condition, as, in spite of normal renal function, these patients may accumulate large amounts of aluminium in various tissues (Klein et al., 1982a; Klein et al., 1982b; Vargas et al., 1986). High levels of serum, urine and bone aluminium were reported, with deposition of aluminium at the mineralisation front of bone and osteomalacic changes. None had any evidence of encephalopathy. Children may be particularly susceptible, although the reason for this is not clear (Ott et al., 1983; Sedman et al., 1985; McGraw et al., 1986; Bishop et al., 1989).

Aluminium and Chronic Renal Failure

After the discovery that aluminium caused severe bone disease and often fatal encephalopathy in dialysis patients, the exposure of such patients to aluminium was greatly reduced and these conditions rapidly disappeared, except for the occasional sporadic case. Ackrill and colleagues had shown that desferrioxamine was an effective chelating agent which could be useful in the treatment of both aluminium induced brain (Ackrill et al., 1980) and bone disease (Ackrill et al., 1982; Ackrill & Day, 1985), although since then the significant toxicity of this chelating agent has meant that much smaller doses are now used and it may take many months to see resolution of disease (De Broe et al., 1993). Subsequently I became interested in the potential for persistent toxic effects from the relatively low levels of aluminium exposure, as experienced by the great majority of dialysis patients world-wide. Could such levels be regarded as 'safe' or were they still associated with insidious low grade toxic effects? Below I have reviewed the effects of aluminium on cerebral metabolism and function in such patients followed by a brief review of the known effects on bone and parathyroid metabolism, and haemopoiesis in renal failure patients.

Aluminium encephalopathy

The potential of aluminium as a major cause of neurological disease was not realised until approximately 15 years after the emergence, in the early 1960's, of haemodialysis as a successful treatment for patients with end stage renal disease. Neurological complications of renal failure such as uraemic encephalopathy, convulsions, neuromuscular irritability and peripheral nerve damage improve with effective dialysis treatment, or in the case of 'dialysis disequilibrium', occur during dialysis (Peterson & Swanson, 1964; Kiley & Hines, 1965; Jebsen et al., 1967; Tyler, 1968).

Alfrey and colleagues (1972) described a fatal encephalopathy of chronic haemodialysis patients quite distinct from previous reports, arising after 3–7 years' treatment. Five patients were reported whose first symptom was a speech abnormality. The speech became slow and deliberate with a stuttering quality, associated with difficulty in naming objects. There followed increasing tremor, muscle twitching, disturbance of movement, memory loss, concentration defects, personality changes and intermittent psychosis. These symptoms were often worse during or immediately after dialysis. The severe encephalopathy proceeded, in spite of a number of therapeutic manoeuvres, including intensive dialysis, to coma and death after 6–7 months. Only one patient survived, following successful renal transplantation. Laboratory findings were unhelpful: lumbar puncture was normal; electroencephalography was non-specifically abnormal; post mortem examination of the brain was normal or in a few cases focal oedema was noted. It was concluded that, in the absence of other causes of dementia, the syndrome was a metabolic encephalopathy. The sudden appearance of this encephalopathy only in patients dialysed for more than 3 years led the authors to believe that it could be caused by an unidentified toxic agent. The syndrome of dialysis encephalopathy, which was almost uniformly fatal, was subsequently described in other reports (Mahurkar et al., 1973; Platts et al., 1973; Barratt & Lawrence, 1975; Burks et al., 1976; Nadel & Wilson, 1976).

Chugh and colleagues (1968) proposed that the use of aluminium resins for the treatment of hyperkalaemia in renal failure would be safer than calcium resins, which may lead to hypercalcaemia, as the aluminium would not be absorbed. Shortly after this first use of aluminium as a therapeutic agent, aluminium hydroxide was found to be a very effective dietary phosphate binder and entered standard practice, as by reducing hyperphosphataemia the severe sometimes fatal renal osteodystrophy due to secondary hyperparathyroidism and other factors, could be alleviated or even prevented. However no attempt was made to demonstrate the non-absorbance of aluminium. This prompted the studies reported by Berlyne and colleagues (1970) describing elevated concentrations of aluminium in dialysed as well as non-dialysed uraemic patients. Not all patients investigated had been on oral aluminium-containing medicine but still they had high serum aluminium concentrations. It was proposed that the patients treated by dialysis may have acquired the aluminium from the dialysate. No explanation was offered for the elevated levels in non-dialysed uraemics who apparently had not been on aluminium containing phosphate binders. A variety of techniques for the measurement of aluminium were used and they acknowledged that none were entirely satisfactory, the results obtained being considerably higher than later studies. In the same year Prosser and colleagues (1970) examined the movement of fluoride across the dialyser membrane and into bone,

and a year later the same group (Parsons et al., 1971a; Parsons et al., 1971b) reported increased bone aluminium concentrations in the same subjects. Clarkson and colleagues (1972) confirmed that aluminium could be absorbed from the intestine following ingestion of aluminium hydroxide, and that this caused aluminium deposition in bone.

In 1976 Alfrey and co-workers reported studies suggesting that the syndrome of dialysis encephalopathy (or dialysis dementia), first described four years earlier, could be caused by deposition of aluminium in the brain (Alfrey et al., 1976). They found that brain grey matter aluminium concentrations were significantly higher in patients dying with dialysis encephalopathy (mean 24.98 μg/g) than in those dialysis patients dying from other causes (6.5 μg/g) or non-uraemic subjects (2.18 μg/g), suggesting that the syndrome was due to aluminium intoxication. There was good correlation between length of time on dialysis therapy as well as oral aluminium intake and tissue aluminium levels, but the aluminium content of water used to prepare dialysate was 'negligible'. In the same year Flendrig and colleagues reported the occurrence of dialysis encephalopathy in patients in whom the source of aluminium was dialysate with an extremely high aluminium concentration of 1000 μg/l (Flendrig et al., 1976a; Flendrig et al., 1976b). Further studies confirmed geographical differences in the incidence of dialysis encephalopathy due to either variation in tap water (used to prepare dialysate) aluminium concentrations (Platts & Hislop, 1976) or the method of preparation of the dialysate (Flendrig et al., 1976a; Flendrig et al., 1976b). Platts & Hislop found considerably higher tap water aluminium concentrations of patients dialysing in Sheffield city (mean of 240 μg/l) as compared with those in the rest of the Trent region (mean of 100 μg/l). Eight of the 10 cases of dialysis encephalopathy occurred in the city and 5 of these cases had never been treated with oral aluminium containing phosphate binders. Flendrig described an unfortunate problem with the preparation of dialysate, which until their unit moved to new premises, had used a water boiler containing two aluminium anodes for cathodic corrosion protection. Since then there have been many descriptions of dialysis encephalopathy resulting from aluminium intoxication and all workers agree that the principal sources are dialysate and aluminium phosphate binders, and that the syndrome can be largely prevented by reverse osmosis treatment of water used to prepare dialysate for haemodialysis whenever tap water aluminium concentrations exceed between 50 μg/l (Platts & Anastassiades, 1981) and 80 μg/l (Davison et al., 1982; Sideman & Manor, 1982; Bates et al., 1985).

The role of oral aluminium initially was less certain but then recognised as an important source of the element capable of leading to dialysis encephalopathy, not only whilst on haemodialysis (Dewberry et al., 1980), but also in patients treated by peritoneal dialysis (Smith et al., 1980; Freundlich et al., 1985) and even in those patients with chronic renal failure who are not yet on dialysis (Mehta, 1979; Rotundo et al., 1982; Kaye, 1983; Andreoli et al., 1984; Freundlich et al., 1985). In the cases of dialysis encephalopathy described, serum aluminium concentrations were generally greater than 200 μg/l when measured. It has been proposed that the cause of dialysis encephalopathy is multifactorial (Arieff et al., 1979; Prior et al., 1982), although there is no doubt that aluminium has a central role. The neuropathological features of the condition have been difficult to identify, although 'neurofibrillary tangles' similar, but not identical, to those characteristic of Alzheimer's disease have been described (Brun & Dictor, 1981; Scholtz

et al., 1987; Harrington et al., 1994). The mechanism by which aluminium affects the brain in dialysis encephalopathy has not been fully elucidated.

However, animal and cell culture studies have shown that aluminium inhibits a number of important enzymes in the brain, including cholineacetyltransferase, acetylcholinesterase, ADP-ribosylation, cytosolic and mitochondrial glycolysis, monoamine oxidase (Yates et al., 1980; Crapper McLachlan et al., 1983; Lai & Blass, 1984; Sharp & Rosenberry, 1985; Tsuzuki & Marquis, 1985); neuronal nitric oxide metabolism (Hermenegildo et al., 1999); protein kinase C (Cochran et al., 1990); disregulation (Sternweis & Gilman, 1982; Hughes & Barritt, 1987) and inhibition of adenylate cyclase (Womack & Colowick, 1979); and interference with calmodulin (Siegel et al., 1982; Siegel, 1983; Siegel, 1985). It may also increase blood–brain barrier permeability (Banks & Kastin, 1983), and block neuronal RNA initiation (Sarkander et al., 1983), and disrupt microtubular function which could explain the development of neurofibrillary tangles (Macdonald et al., 1987). A number of studies suggest that neuronal nuclear function (messenger RNA and gene expression processes) are adversely affected (Crapper et al., 1979; Muma et al., 1988; Walker et al., 1989). More recently with the increased understanding of the role of amyloid in the neuropathology of Alzheimer's disease, important interactions between aluminium and beta-amyloid have been found (Exley et al., 1993; Exley et al., 1995; Exley, 1997).

Tetrahydrobiopterin metabolism in haemodialysis patients without overt encephalopathy

Dihydropteridine reductase (DHPR) is an enzyme essential for the regeneration of tetrahydrobiopterin, itself a co-factor necessary for the hydroxylation reactions in the brain leading to the synthesis of tyrosine, dopa, noradrenaline and 5-hydroxytryptophan (Fig. 1). DHPR salvages the unstable quinonoid dihydrobiopterin, converting it back to tetrahydrobiopterin. The maintenance of normal tetrahydrobiopterin levels is more dependent on this regenerative pathway than on de novo synthesis. Deficient phenylalanine hydroxylase leads to classical (Type I) hyperphenylalaninaemia (HPA), also known as

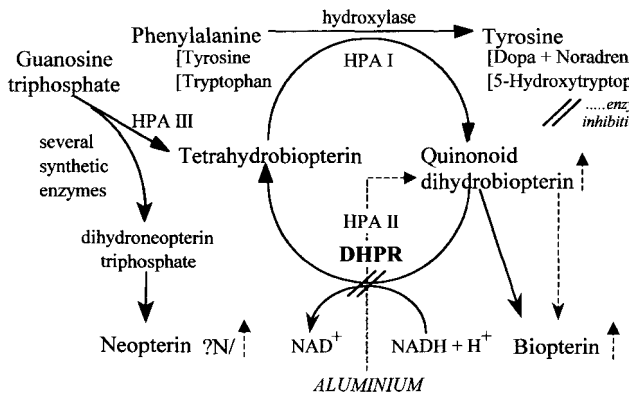

Fig. 1. Tetrahydrobiopterin metabolism and the effect of aluminium: inhibition of dihydropteridine reductase (DHPR) should be associated with a rise of serum biopterin (HPA = hyperphenylalaninaemia)

phenylketonuria. Neurotransmitter abnormalities due to DHPR deficiency are responsible for the neurological dysfunction in Type II hyperphenylalaninaemia, despite the presence of the alternate pathway of tetrahydrobiopterin synthesis from guanosine triphosphate (Kaufman et al., 1975; Rey et al., 1980). The latter pathway is disturbed in Type III HPA (Smith et al., 1975; Danks et al., 1978; Rey et al., 1980). Type II and III HPA do not respond to dietary restriction of phenylalanine, unlike classical phenylketonuria, and are usually rapidly fatal. In Type II HPA the activity of DHPR is reduced in brain, liver, skin, leucocytes and erythrocytes, and serum biopterin derivatives are increased. Erythrocyte DHPR activity can be used diagnostically as it appears to reflect cerebral DHPR activity (Leeming et al., 1984; Jeeps et al., 1986).

When rat, hamster or human brain extract was exposed to levels of aluminium similar to those found in the brains of patients who had died from aluminium encephalopathy, the activity of DHPR was reduced by about 40% (Leeming & Blair, 1979; Dhondt & Bellahsene, 1983; Al-Salihi, 1985). Abnormal concentrations of biopterins were found in the serum and urine of patients with chronic renal failure (Baker et al., 1974; Leeming et al., 1976). Leeming found high levels of biopterin derivatives in their blood, but low urinary levels, concluding that the kidney must participate in their elimination. Dhondt and Vanhille, (1982) confirmed these findings and reported elevated levels of serum neopterin in renal failure (Dhondt & Vanhille, 1982). Brain tetrahydrobiopterin metabolism is inhibited in Alzheimer's disease (Barford et al., 1984; Hamon et al., 1987), and when transferrin (the iron transport protein that also binds aluminium) is added to the Alzheimer's disease brain homogenate some reversal of tetrahydrobiopterin inhibition has been observed (Cowburn & Blair, 1989). Abnormalities in other neurotransmitters have also been identified in uraemic patients: increased levels of tryptophan in cerebrospinal fluid and plasma (Sullivan et al., 1977); reduced levels of γ-aminobutyric acid and choline acetyl-transferase in CSF and brains of uraemic patients (Perry et al., 1985; Sweeney et al., 1985). Furthermore, plasma phenylalanine concentrations are raised (Stepniewski et al., 1985), possibly due to a disorder in tetrahydrobiopterin metabolism and reduced excretion.

My group studied 38 anaemic haemodialysis patients (mean haemoglobin concentration 8.9 g/dl as this was before recombinant human erythropoietin had become available), with a mean serum aluminium concentration of 67.6 μg/l, all of which had serum aluminium concentrations below 200 μg/l, and in 30 they were below 100 μg/l. None had any clinical evidence of encephalopathy (Altmann et al., 1987a; Altmann et al., 1987c; Altmann et al., 1988a; Altmann, 1991). Serum aluminium concentrations below 100 μg/l are generally considered unlikely to cause encephalopathy in haemodialysis patients. Despite only moderate hyperaluminaemia, erythrocyte DHPR activity was inversely related to the serum aluminium concentration. This appeared to be a direct relationship rather than an indirect one involving anaemia as there was no relationship between serum aluminium and haemoglobin. The suggestion that erythrocyte DHPR activity is altered by aluminium was also supported by the effects of a single intravenous injection of the aluminium chelating agent desferrioxamine. Four days after the desferrioxamine, DHPR rose by 105%, and at 28 days its activity was still above pre-desferrioxamine values, although the serum aluminium had, by then, returned to baseline.

As predicted from Fig. 1 serum biopterin concentrations in these haemodialysis patients were markedly elevated and tightly related to DHPR activity. Concentrations of

serum neopterin were also increased but were not correlated with DHPR. This may be due to the fact that dialysis patients may have persistent leucocyte activation from the dialysis membranes (Craddock et al., 1977; Aljama et al., 1978; Arnaout et al., 1985) and activated T lymphocytes release interferon-γ, which in turn activates macrophage guanosine triphosphate cyclohydrolase leading to increased synthesis and excretion of neopterin (Fuchs et al., 1982; Huber et al., 1983; Huber et al., 1984).

Four of our dialysis patients died of non-neurological causes without any clinical evidence of aluminium induced disease. DHPR activity was measured in samples of frontal lobe removed from the brain within 48 hours of death, at autopsy. The small numbers make statistical analysis invalid, but in these few samples DHPR activity was inhibited, moreso the greater the antemortem serum aluminium concentration (Altmann et al., 1987c).

Psychomotor function in haemodialysis patients without overt encephalopathy

Previous studies of psychomotor function in haemodialysis patients have given contradictory results, although impairment has been described after high dialysate aluminium exposure (English et al., 1978; Ackrill et al., 1979) and has been related to duration of dialysis (Gilli & de Bastiani, 1983). Gilli and de Bastiani (1983) found evidence of psychological impairment related to time since starting dialysis and to parathyroid hormone, but not to pre-dialysis blood aluminium, urea, creatinine or haemoglobin concentrations. They suggested that parathyroid hormone is a neurotoxin, but the relation between psychological impairment and the levels of this hormone may have been due to the progressive increase in its concentration behaving as a surrogate for increasing duration of dialysis, and to a reduction in psychomotor performance with age. Savazzi and colleagues found that almost half their patients under 50 years of age who had been haemodialysing for over 10 years had diffuse or frontal cerebral cortical atrophy, and the authors were able to correlate this with mean blood pressure (Savazzi et al., 1985). Despite suggested trends there was no significant correlation with aluminium hydroxide intake, blood lipids or arterial calcification. Jackson and colleagues studied 28 patients who had dialysed for more than 5 and a half years, without overt evidence of aluminium encephalopathy, and had 'low' source water aluminium. The patients had received 2510 g of orally ingested aluminium from phosphate binders (a greater cumulative oral aluminium intake than the patients reported in my studies). They found that the intelligence quotient (IQ) of 5 patients was slightly less than their pre-morbid levels as assessed by the National Adult Reading Test (Jackson et al., 1987). They did not use control data for comparison. A weak negative correlation was found between cumulative oral aluminium intake and cognitive function tests.

Certain tests of cerebral function are known to be quite sensitive to even minor physical brain damage, but a problem in assessing the validity of the test results in an individual is that, although they are monitored by an observer, they depend on the subject's self-motivated response to the set task. In an attempt to reduce the effects of patient-motivated voluntary bias we used a computerised battery of tests (Acker & Acker, 1982) on dialysis patients exposed to low levels of aluminium with no clinical evidence of aluminium intoxication, and demonstrated significant abnormalities in psychomotor function which were related to aluminium status (Altmann et al., 1989; Altmann, 1991).

One of the tests within the battery, the Symbol Digit Coding test, is thought to be one of the more sensitive tests for organic brain disease (as opposed to functional disease such as depression or other mental illnesses) (Lezak, 1976). Such tests have been used extensively in the assessment of cognitive impairment associated with drugs (Acker, 1982; Estrin et al., 1988; Zur & Yule, 1990; Ghouri et al., 1991), toxins (Boey & Jeyaratnam, 1988; Ng et al., 1990; Seeber et al., 1990) and other conditions such as hypoglycaemia (Stevens et al., 1989) or Alzheimer's disease (Emmerson et al., 1989; Pfefferbaum et al., 1990). Nevertheless patient related bias can alter the way in which he/she performs and so even such tests are open to subjective influences. In addition pre-morbid IQ was assessed by means of the National Adult Reading Test, so as to compare pre-morbid IQ with current psychomotor performance as measured by the above battery of tests, and to select IQ and age-matched controls. The pattern of results was consistent with organic brain disease in that the less sensitive tests were performed normally by the haemodialysis patients but the Symbol Digit Coding results were abnormal. In addition, those patients with aluminium deposition at the bone mineralisation front (on bone biopsy) performed far worse than those with no aluminium, although the latter group still performed significantly worse than the control subjects.

Visual evoked potentials in haemodialysis patients without overt encephalopathy

Alteration in visual evoked potentials has been described in Alzheimer's disease: the pattern-stimulated visual evoked potential remains normal, whilst the flash-stimulated visual evoked potential is delayed (Wright et al., 1984; Wright et al., 1986) and this delay may increase as the dementia gets worse (Orwin et al., 1986). In normal subjects, Wright and colleagues (1987) found that the flash-pattern difference increased with age but this did not account for the abnormality in Alzheimer's disease. Visual evoked potentials were measured in 16 haemodialysis patients (10 of whom had also had the above psychomotor tests), with a mean serum aluminium of 63 μg/l, and other determinants of aluminium exposure similar to those described for the whole group. The pattern-stimulated visual evoked potential was within the normal range at 101.8 ms whereas the flash-stimulated visual evoked potential was delayed at 133.4 ms ($P < 0.0001$). The mean difference between the flash and pattern potentials (F–P difference) was 32.7 ms, which was significantly greater than in the control subjects (19.4 ms, $P = 0.0028$) or patients with non-Alzheimer dementia (21.2 ms, $P = 0.05$) but less than that in Alzheimer's disease (48.5 ms, $P = 0.03$).

The F–P difference was related to cumulative oral aluminium ($r = 0.7$, $P = 0.02$), whilst there was an inverse relationship with daily urine volume ($r = -0.7$, $P = 0.02$), a determinant of aluminium accumulation in dialysis patients (Altmann et al., 1987b). The F–P difference was greater in patients with aluminium deposition at the mineralisation front (35.8 ms, $n = 4$) than in those without any aluminium deposition (28.7 ms, $n = 6$) but this did not achieve statistical significance. The latency of the flash visual evoked potential was longer in the patients with aluminium deposition (138.9 vs 129.7 ms, $P = 0.05$). The fact that the F–P difference correlated with Symbol Digit Coding response times ($r = 0.7$, $P = 0.03$) gave additional credence to the abnormalities in psychomotor function in these patients. Following 3 months' desferrioxamine therapy improvements in

the activity of dihydropteridine reductase were associated with improvements in cerebral function assessed by the methods discussed above (Altmann et al., 1989; Altmann, 1991).

Aluminium interactions with bone and the parathyroid glands

A refractory osteomalacic bone disease was first described in patients dialysing in the Newcastle area and became known as "Newcastle Bone Disease" (Schorr, 1968). In such patients, bone pain, fractures and muscle pains and weakness develop in spite of the maintenance of acceptable plasma calcium and phosphate concentrations. The condition is quite distinct from classical renal bone disease (due to disturbances of calcium and phosphate metabolism in chronic renal failure), and does not respond to therapy with vitamin D analogues during which hypercalcaemia may develop more readily.

Parsons and colleagues (1971b) found increased levels of aluminium in the bone of patients with chronic renal failure (up to 2800 μg/g of bone calcium compared with up to 400 μg/g of calcium in normal subjects), the content appearing to be higher in the patients who had been on dialysis for longer. No correlation with the consumption of oral aluminium was found and no comment regarding possible sources was made. The role of tap water contamination by aluminium in the genesis of both fracturing osteomalacia and encephalopathy in haemodialysis patients was confirmed by other workers, once again suggesting that oral aluminium consumption made a negligible contribution, but that duration of dialysis therapy was an important factor (Platts et al., 1977; Ward et al., 1978; Cournot-Witmer et al., 1979; Parkinson et al., 1979). However more recent studies suggest that orally ingested aluminium may be responsible for aluminium associated bone disease occurring in children (Andreoli et al., 1984; Koch et al., 1985).

Aluminium induced bone disease often presents with high blood calcium concentrations (hypercalcaemia) and normal or low plasma alkaline phosphatase (Parkinson et al., 1981; Boyce et al., 1982). The parathyroid glands in the neck are closely involved with the control of bone metabolism. Levels of parathyroid hormone were reported to be normal in patients with aluminium induced bone disease as compared with higher levels in those with osteitis fibrosa, suggesting relative hypoparathyroidism (Andress et al., 1983), but in another study aluminium intoxicated patients also had elevated parathyroid hormone levels (Kraut et al., 1983). Suppression of parathyroid gland function by aluminium may occur experimentally (Morrissey et al., 1983), although at much higher concentrations of aluminium than seen *in vivo* and appears to be transferrin-mediated (Smans et al., 2000). Deposition of aluminium in the bone inhibits bone formation and remodelling, and aluminium can also interfere with the actions on bone of parathyroid hormone and vitamin D (Blumenthal & Posner, 1984; Dunstan et al., 1984; Christoffersen & Christoffersen, 1985; Lieberherr et al., 1987).

My group's studies revealed that even at so-called safe levels of aluminium accumulation in patients with no evidence of overt clinical aluminium toxicity, there was significant blunting of parathyroid responsiveness to a hypocalcaemic or hypercalcaemic stimulus (Altmann, 1991; Altmann, 1993). On bone biopsies, approximately half of the patients had aluminium deposition at the mineralisation front, and this was associated with histological changes of aluminium induced bone disease (Altmann, 1991; Altmann, 1993).

Aluminium induced anaemia

Most patients with end-stage renal disease are anaemic, the exceptions being those with adult polycystic kidney disease or (rarely) who have developed cysts in end-stage kidneys. The origin of this anaemia is certainly multifactorial, although functional erythropoietin deficiency and uraemic toxins dominate (Navarro et al., 1982; Anonymous, 1983). Elliott and colleagues (1978) observed that haemoglobin concentrations fell during the year before dialysis encephalopathy developed and also in 3 of 4 other patients with very high serum aluminium concentrations (greater than 400 μg/l). The anaemia was of the microcytic hypochromic sort (characteristic of iron deficiency) but with normal iron stores (Short et al., 1980; O'Hare & Murnaghan, 1982). A number of mechanisms have been proposed based on earlier studies on the effects of aluminium on haem synthesis (Huber & Frieden, 1970; Berlyne & Yagil, 1973; Meredith et al., 1977; Meredith et al., 1979; Mladenovic, 1988).

At the outset of my group's studies there did not appear to be any relation between aluminium levels and the haemoglobin concentration (these studies were done before recombinant human erythropoietin was available). However, during a three month therapeutic trial of the chelating agent desferrioxamine, there was a very significant rise in the patients' haemoglobin concentrations which was related to aluminium status. These results suggested that aluminium is a far more potent inhibitor of haemoglobin synthesis than previously thought (Altmann et al., 1988b; Altmann, 1991; Altmann, 1993), and subsequent studies have demonstrated that aluminium can blunt the response to erythropoietin.

Sources of aluminium

In the context of renal failure, there is no doubt that aluminium may be absorbed from the gastrointestinal tract and from contaminated dialysis fluids. Studies have shown that dialysis encephalopathy and aluminium induced bone disease were associated with tap water aluminium concentrations above 50–80 μg/l. Kaehny and colleagues (1977) showed that less aluminium was transferred across the haemodialysis membrane with lower dialysate aluminium concentrations, but that there was a net gain even if dialysate aluminium was very low (no concentration quoted). Hodge and colleagues (1981) showed that, irrespective of a mean serum aluminium concentration of 108 μg/l (range: 54–204 μg/l), the transfer of aluminium into the blood-stream was related to the dialysate concentrations of the element. At dialysate concentrations between 5 and 18 μg/l (mean 14 μg/l) aluminium moved from the dialysate into the blood compartment. Only at concentrations below these did aluminium move out of the blood compartment. These findings suggested that most of the aluminium in serum is strongly protein bound, and other studies have confirmed that binding is mainly to transferrin (Trapp, 1983; Cochran et al., 1985). In addition the pH of dialysate solutions may alter the dialysability as the solubility curve (% ionised) of aluminium is U-shaped. At pH 6 the ionic form is at a minimum (the non-ionic forms being complexed molecules), with a steeper rising curve as the pH drops than as it rises (Parkinson et al., 1981).

If water aluminium concentrations and oral aluminium intake from phosphate binders

are fairly constant throughout treatment then there might be a good relationship between either of these variables and serum aluminium concentration. Elliott and colleagues (1978) correlated serum aluminium concentrations (up to 756 μg/l) of 22 haemodialysis patients whose dialysate was prepared from tap water with aluminium concentrations up to 702 μg/l, ($r = 0.84$, $P < 0.001$). However, below 80 μg/l this linear relationship with the serum aluminium levels (of up to 300 μg/l) was not apparent. Boukari and colleagues (1978) investigated 39 patients with serum concentrations up to 165 μg/l, who had dialysed against water aluminium concentrations of 20–60 μg/l. No correlation was calculated between water aluminium and serum levels, but there was a positive correlation between daily oral aluminium intake (from phosphate binders) and serum levels, ($r = 0.52$, $P < 0.001$). Other recognised sources of aluminium leading to intoxication in renal failure are contaminated peritoneal dialysate (Cumming et al., 1982; Rottembourg et al., 1984); the Redy sorbent cartridge (Shapiro et al., 1983); intravenous feeds (Sedman et al., 1985; McGraw et al., 1986); and plasma protein fraction (Maher et al., 1986).

As dialysate exposure has been reduced the significance of oral aluminium intake and urinary excretion through residual renal function in dialysis patients has become a more significant determinant of aluminium accumulation (Altmann et al., 1987; Altmann, 1991). The major source remains aluminium hydroxide containing phosphate binders, but some foods contain quite large amounts of aluminium as a result of processing. Levels of up to 4000 μg have been reported to be present in one serving of food (Lione, 1983) and many proprietary medicines contain aluminium (Lione, 1985). In 1913 studies from the Lancet Laboratory suggested that aluminium was not likely to be leached from aluminium cooking utensils (Anonymous, 1913a). However, later work showed that such cooking utensils do liberate quantities of the element into the food during cooking (Lione, 1983; Lione, 1984; Lione et al., 1984).

In addition, it may be that certain naturally occurring aluminium salts are more readily absorbed from the gastrointestinal tract than the chemical species found in aluminium hydroxide preparations, thereby introducing a further, but as yet immeasurable factor in the accumulation of body aluminium in renal failure. It is clear that other dietary factors as discussed earlier, may also affect absorption.

Of course, when aluminium induced disease was first identified in renal failure patients the worst epidemics of the condition were to be found in areas where the source water for dialysate preparation had far higher aluminium concentrations than other areas. In London where my studies were performed aluminium induced disease was rare and water aluminium was sufficiently low that in the 1980's it was not felt necessary to use reverse osmosis units in the home haemodialysis patients' dialysis installations. However even at the relatively low levels of water aluminium exposure (generally less than 10 μg/l) patients' serum levels varied according to source water concentrations as well as other factors such as oral intake and urinary excretion (Altmann, 1991).

Finally, because the aluminium content of tap water depends both on source water concentrations and the use of aluminium sulphate as a flocculant by water authorities, the aluminium concentrations vary with the seasons. In the summer source water may have higher concentrations of aluminium as a result of acid winter rains leaching more of the element from the ground. In addition in the warmer months when algae proliferate and water turbidity is worse the water authorities add more flocculant to the water during pro-

cessing and these factors lead to higher water aluminium concentrations, reflected, in one study, by higher water and serum levels — winter vs summer water aluminium 4.3 vs 9.3 μg/l ($P < 0.001$) and serum 51.5 vs 63.2 μg/l ($P = 0.011$) respectively (Altmann, 1991).

Water Contamination by Aluminium — The Camelford Incident

On July 6, 1988, 20 tonnes of aluminium sulphate were accidentally emptied into the treated water reservoir at Lowermoor Water Treatment Works, which supplied 20,000 people in the Camelford area of Cornwall. Within a few days local residents and holiday-makers started reporting rashes, gastrointestinal disturbances, and mouth ulcers, although there was much delay in informing the public of the accident. The water was heavily contaminated with aluminium (up to 620 mg/l, the European Union guideline for tap water is less than 0.2 mg/l), copper (up to 22.5 mg/l), lead (up to 0.46 mg/l) and the pH was very low (Clayton, 1989). In the following weeks and months after the event, exposed individuals complained of joint and muscle pains, malaise, fatigue, and impairment of concentration and memory (Clayton, 1989). A number of veterinary reports emerged, most notably that of fish dying from aluminium poisoning in the local rivers following their contamination (Allen & Sansom, 1989). Two years later approximately 400 people were suffering from symptoms that they attributed to the incident. The standardised hospital discharge ratios in the 5 years following the incident were far greater than for other areas of Cornwall, although no single diagnosis prevailed (Owen & Miles, 1995).

Considerable debate ensued as to whether the persisting complaints of memory impairment and difficulty in concentration were due to anxiety or to toxic cerebral damage. However, little systematic study of the clinical effects of the incident was carried out soon after the event. Several early reports of high blood aluminium concentrations have been discounted (Clayton, 1991) as little effort was made to avoid sample contamination (a great problem when measuring concentrations of this element in any water or biological sample), and elevated lead accumulation had also been described (Powell et al., 1995). One study reported psychological changes in 10 affected individuals (McMillan et al., 1993). In two of these patients, bone biopsies done at 6–7 months had demonstrated presence of aluminium which subsequently disappeared at re-biopsy by 19 months after the accident. Eight other patients biopsied 12–17 months after the incident showed no stainable aluminium (McMillan et al., 1993).

My group was asked, almost 3 years after the Camelford incident, to study 55 adults who had problems with poor short term memory and concentration following exposure to the contaminated water, using identical techniques to earlier studies on dialysis patients (Altmann et al., 1989; Altmann, 1991). None of the patients had any relevant past or family history, and in particular we were not made aware of any Alzheimer's disease in their families. None were on any form of psychotropic drug or consuming excessive alcohol. Assessment included psychological tests of anxiety, psychomotor function, and measurement of visual evoked potentials. The nearest (in age) available sibling who had not lived in the area of water contamination since before the incident was asked to attend. Only 15 such siblings were available. To exclude any potential effect of anxiety on the psychomotor tests and visual evoked potentials the subjects' level of anxiety was also

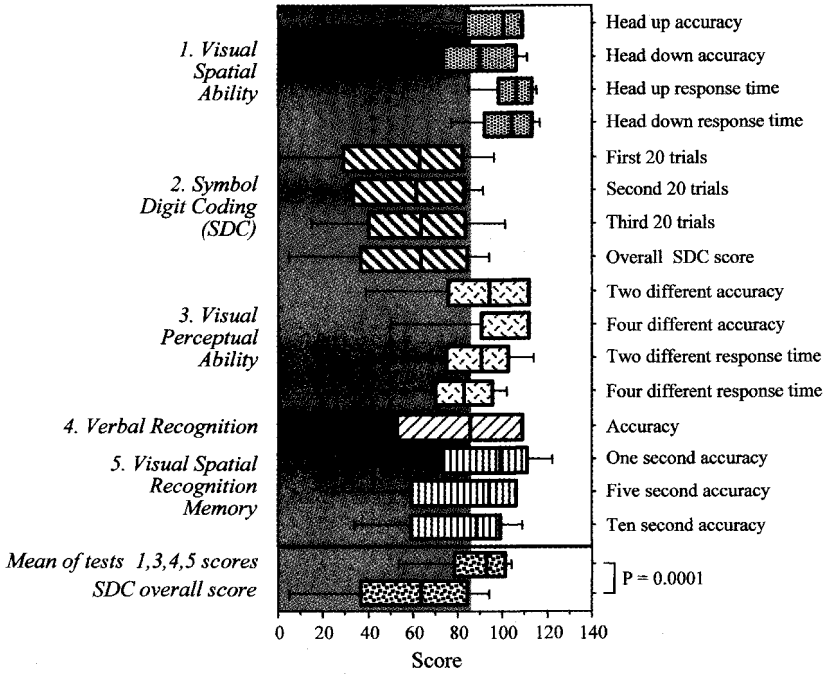

Fig. 2. Summary of BMAPS test results. In all the tests the results are regarded as abnormal (shaded area) if the standardised score is less than 85 (corresponding to 1 standard deviation). The box plots show the 10^{th}, 25^{th}, 50^{th}, 75^{th}, 90^{th} centiles.

assessed. After this study was published there was a fair amount of correspondence, much of it factually incorrect, criticising the design of the study, even though we had raised and dealt with all the concerns about self-selection in the report. Our studies were designed to get as much objective information after the event as possible and of course could not be likened to a prospective intervention study.

The mean pre-morbid IQ as assessed by the National Adult Reading Test was above average at 114.4. Nevertheless the subjects performed very poorly in the Symbol Digit Coding test compared to the other psychomotor tests as shown in the Fig. 2. The mean flash-pattern difference of the Camelford subjects (mean age 41.8 yr) was 27.3 ms. In a group of 42 similar aged unrelated and unmatched control subjects (age 44.1 yr) the flash-pattern difference was 18.6 ms. The difference (8.7 ms) between the two groups was significant ($P = 0.0002$). Whilst the 2.2 yr difference in age between the groups was not significant, analysis of covariance (with case and age variables entered into the model) showed that the difference in flash-pattern differences with age adjustment was a little greater at 9.4 ms ($P < 0.0001$). As in the dialysis patients studied there was a relationship between the Symbol Digit Coding test and the flash-pattern difference. Anxiety levels were quite low and there was no significant effect on any of the measured parameters (Altmann et al., 1999).

The siblings had come from outside the area of pollution, thereby providing both out of area and genetic (phenotypic) control data. Unexposed siblings were chosen as

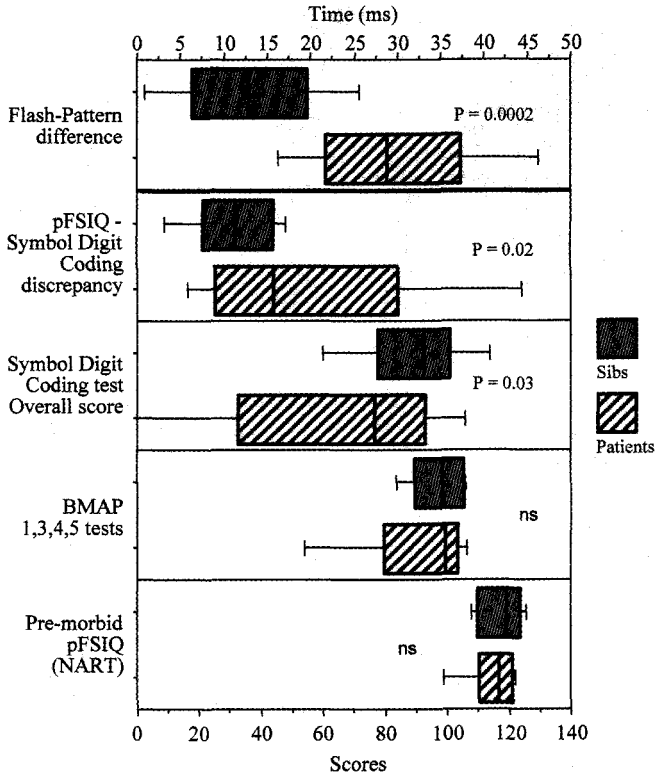

Fig. 3. Sibling control studies: summary of NART assessed pre-morbid IQ (pFSIQ), BMAPS test scores and visual evoked potentials (top panel) in 15 subjects and their siblings (P values refer to paired Students t test). Box plots as in Fig. 2.

controls as this is a standard way of establishing abnormalities which might be acquired rather than inherited. The results are quite striking and added considerable weight to the suggestion that the Camelford subjects had organic brain dysfunction at the time of the study (Fig. 3). Although such controls, out of loyalty, might strive to "give 110%" in the psychological tests, it is noteworthy that the Bexley Maudsley Automated tests and Symbol Digit Coding scores were not above normal and it was the Symbol Digit Coding tests and flash-pattern differences which differentiated the two groups.

Informed Opinion

Aluminium in Chronic Renal Failure

In addition to previous reports of the severe consequences of aluminium intoxication in renal failure patients, the results of my own studies, in a group of dialysis patients exposed to relatively low levels of aluminium without overt cerebral impairment, have

shown that psychomotor function was impaired in these patients and that there was an apparent influence of aluminium on this finding. There were no differences in age or duration of treatment between those patients with or without aluminium deposition at the mineralisation front, suggesting that neither of these factors were responsible for this finding. Apart from aluminium deposition at the mineralisation front other measures of aluminium exposure did not appear to influence the Symbol Digit Coding results. This might be due to the blunting effects of other factors, such as 'uraemic toxins' and social problems, in the patients.

Modest aluminium loading of the patients studied by my group was associated with impaired erythrocyte DHPR activity and this impairment was improved by desferrioxamine treatment. Brain DHPR activity in the small number of autopsy brain specimens studied correlated inversely with serum aluminium, suggesting that aluminium inhibits both erythrocyte DHPR and cerebral DHPR activity with consequent disturbance of neurotransmitter metabolism. The relation between Symbol Digit Coding response times and aluminium deposition in the bone suggests that aluminium does indeed play a role in the impaired psychomotor performance of the patients studied.

These studies make the assumption that erythrocyte DHPR activity does reflect brain DHPR activity. Apart from the results of the very small number of brain samples studied, several other aspects of tetrahydrobiopterin metabolism should be considered:

(i) Neonates with Type II HPA have reduced or absent brain DHPR activity, and the diagnosis may be confirmed by the measurement of erythrocyte DHPR activity which is similarly affected (Leeming et al., 1984; Jeeps et al., 1986). Furthermore, animal studies have confirmed the inhibitory effect of aluminium on brain DHPR (Leeming & Blair, 1979; Dhondt & Bellahsene, 1983; Al-Salihi, 1985). Any effect of aluminium on brain DHPR might be reduced by restricted movement of aluminium across the blood–brain barrier. Conversely, the same restriction could favour trapping of aluminium within the brain and one or both of these processes could explain the marked intercellular and intracellular variations in brain aluminium concentrations (Scholtz et al., 1987). Therefore, although these results suggest that aluminium may inhibit brain DHPR in non-encephalopathic dialysis patients, erythrocyte DHPR activity may not be a quantitative reflection of brain DHPR.

(ii) Concentrations of biopterin derivatives, biopterin and neopterin were considerably raised (as in Type II HPA where it is of diagnostic importance), due to reduced salvaging of quinonoid dihydrobiopterin by DHPR, failure of renal excretion and macrophage activation. The strong relation between biopterin and DHPR activity confirmed inhibition of this enzyme.

(iii) If the relatively low levels of aluminium exposure experienced by these haemodialysis patients is associated with psychomotor disturbances, one must consider the fact that in heterozygous congenital DHPR deficiency erythrocyte DHPR activity is reduced to 46% of normal (Leeming et al., 1987) compared to the patients with the highest aluminium concentration whose erythrocyte DHPR activity was suppressed to only about 70% of normal. In heterozygous congenital DHPR deficiency mental retardation is thought to be uncommon. Moreover, although the haemodialysis patients have less DHPR inhibition than outwardly normal heterozygotes, studies of subtle psychomotor function in heterozygotes are not available. In haemodialysis

patients DHPR is likely to be only one of several cerebral enzymes affected by aluminium, whilst in the congenital DHPR deficiency syndromes DHPR is the only enzyme affected. Also, the severity and results of intra-cerebral DHPR inhibition, which could be incompletely reflected by erythrocyte DHPR, may vary, depending on the known regional variations in aluminium and tetrahydrobiopterin metabolism within the brain. Thus it is not possible to equate the functional results of aluminium exposure with those of congenital DHPR deficiency.

In addition, the possibility that aluminium intoxication was a factor adversely affecting these patients' psychomotor function was supported by the positive correlation between desferrioxamine induced changes in erythrocyte DHPR activity and changes in Symbol Digit Coding response times. The changes in erythrocyte DHPR may have been related causally to the alterations in Symbol Digit Coding response times, as diminished brain DHPR activity would have led to impaired neurotransmitter production. Furthermore, although the improvement in Symbol Digit Coding response times coincident with desferrioxamine treatment in over half the patients could have been due to increasing familiarity with the test procedure, it is noteworthy that the response times deteriorated in four of the five patients whose erythrocyte DHPR activity diminished after desferrioxamine treatment. This may have been due to a transient increase in bio-availability of aluminium as it became mobilised from non-cerebral tissues. It is compatible with reports of exacerbation of acute aluminium encephalopathy after transplantation (Sideman & Manor, 1982) or following instigation of desferrioxamine therapy (Alfrey et al., 1987; Lillevang & Pedersen, 1989; McCauley & Sorkin, 1989). Other metabolic indices were studied to see if these changed over the period of desferrioxamine administration, and if any such change could be related to alterations in Symbol Digit Coding response times — no evidence of either was forthcoming. The patients were receiving regular conventional haemodialysis and their schedules were unchanged throughout the investigation. No attempt was made to determine if more intensive dialysis would alter their psychomotor function.

There was also evidence of a selective delay in flash stimulated visual evoked potentials with preservation of the pattern stimulated visual evoked potentials. The defect (F–P difference) was significantly related to the impairment of psychomotor performance as measured by the pre-desferrioxamine Symbol Digit Coding response times of these patients. The amount of aluminium present at the mineralisation front in bone may best represent the degree of current aluminium exposure to susceptible tissues, in that it is most likely to reflect that part of the body aluminium burden which is bioavailable. The patients without aluminium deposition had both smaller F–P differences but also shorter flash visual evoked potential latencies than those few patients with aluminium deposition. In addition, cumulative oral aluminium intake (which in these patients is the most important determinant of aluminium accumulation) was positively related, and maintained urine flow (which may protect dialysis patients against aluminium accumulation) was negatively related to the visual evoked potential defect. This suggests that the changes in visual evoked potentials are more specifically related to aluminium intoxication than are measurable alterations in psychomotor function. It is also possible that the reduction in psychomotor performance may in part be due to a cognitive deficiency within the optical pathways of the brain.

We were able to demonstrate some reversibility of the sub-clinical effects observed, and even with quite severe aluminium intoxication in dialysis patients chelation therapy with desferrioxamine or renal transplantation can reverse neurotoxicity by mobilising aluminium from the brain. Whether this is of any relevance to patients with Alzheimer's disease is questionable as the type of intoxication in dialysis patients is relatively acute compared to the putative life-long exposure in Alzheimer's disease, and slowly accumulated brain aluminium may be considerably more difficult to mobilise.

Our results raised the possibility that there may be a disturbance of neurotransmitter metabolism in the brains of dialysis patients without clinical evidence of encephalopathy, and that subtle neurotoxicity may affect a large number of patients on dialysis. Emotional lability and occupational difficulties in long-term dialysis patients have usually been regarded as manifestations of general debility and psychological stress, but might also reflect neurological dysfunction due to aluminium accumulation and consequent alteration in neurotransmitter metabolism.

The known causal relationship between severe aluminium intoxication and dialysis dementia does not necessarily mean that there is a similar relationship between mild hyperaluminaemia and psychological impairment in dialysis patients (or other subjects). It is difficult to quantify psychological impairment which although subtle, might be very important in the context of domestic, social and occupational life. Previous studies of psychological function in dialysis patients had not used controls. These results refer to apparently normal regular haemodialysis patients, with only mild or moderate hyperaluminaemia, compared with controls matched for age and for the patients' pre-morbid IQ.

Aluminium in Subjects without Chronic Renal Failure

The role of aluminium as a neurotoxin has interested scientists for more than a century. Following the work of Orfila and Siem, von Döllken confirmed the toxic effect of aluminium on the central nervous system following systemic administration of aluminium salts in animals (von Döllken, 1897). He described the behavioural state induced as being similar to human dementia. Several other studies have suggested that aluminium is neurotoxic (Seibert & Wells, 1929; Scherp & Church, 1937), and in 1942 studies demonstrated the epileptogenic potential of aluminium (Kopeloff et al., 1942). In the search for an animal model for the human dementia process, aluminium was found to cause neurofibrillary degeneration experimentally (Klatzo et al., 1965) following intracerebral injection in rabbits. These degenerative changes were remarkably similar to the neurofibrillary tangles found in Alzheimer's disease (Terry & Peña, 1965). These animal studies on aluminium induced brain pathology, together with the report in 1962 of aluminium induced encephalopathy in an aluminium worker (McLaughlin et al., 1962), prompted the examination of brain aluminium content in patients dying with Alzheimer's disease. Crapper, Krishnan & Dalton (1973) found that aluminium concentrations, approaching those used in experimentally induced neurofibrillary degeneration, were present in certain regions of the brains of affected patients (0.8–11.5 μg/g dry weight compared with up to 2.7 μg/g in normals) (Crapper et al., 1973). Later studies confirmed

these findings (Crapper et al., 1976; Crapper et al., 1978) but remained controversial as it was suggested that this observation was a phenomenon of ageing and not specific to Alzheimer's disease (Crapper et al., 1978). However, Perl & Brody (1980) were able to detect aluminium accumulation specifically in the neurones containing neurofibrillary tangles, the characteristic pathological change in Alzheimer's disease. Perl and colleagues further strengthened the case for the association between aluminium and dementing disorders by making the same observations in the brains of Guamanian Chamorros dying with either amyotrophic lateral sclerosis or Parkinsonism-dementia of Guam (Perl et al., 1982; Perl et al., 1985a; Perl et al., 1985b). These and other islanders in the Pacific lived on an unusually aluminium-rich soil and suffered a previously unexplained high incidence of dementing disorders. Secondary hyperparathyroidism was proposed to be an additional factor in the accumulation of brain aluminium in these Pacific dementias, as dietary calcium deficiency was also present and calcium was deposited with the aluminium in the tangles (Garruto et al., 1984).

The epidemiological phenomenon noted in the Pacific islands has not been found elsewhere, although an unexplained geographical distribution of Alzheimer's disease was noted in Edinburgh (Whalley & Holloway, 1985). More recently, however, there has been an epidemiological link made between drinking water aluminium concentrations and the incidence of Alzheimer's disease in England and Wales (Martyn et al., 1989) as well as in France (Michel et al., 1990). There continues to be controversy, as the accumulation of aluminium could be an epiphenomenon resulting from damage to the blood–brain barrier, and genetic factors have been strongly implicated in both the commoner sporadic form and the rarer familial form of Alzheimer's disease (Delabar et al., 1987; Goate et al., 1989; Rumble et al., 1989). The suggestion that aluminium is associated with the neuropathological changes in Alzheimer's disease (Perl, 1984; Perl, 1985; Perl et al., 1985a; Perl et al., 1985b) has been questioned as Jacobs and colleagues (1989) failed to confirm this finding. However, Candy and colleagues reported the presence of aluminosilicates at the centre of senile plaque cores suggesting that these salts is present at the onset of plaque formation (Candy et al., 1984; Candy et al., 1986). Other studies have shown a significantly higher serum aluminium concentration amongst patients with Alzheimer's disease as compared with age-matched controls, although the small differences were thought to be within the normal range: 21.9 *vs* 12.1 μg/l (Kellett et al., 1986); and increased concentrations of aluminium in the hair of patients with 'agitated senile dementia' as compared with age-matched controls: 19.5 *vs* 9.3 μg/g (Barlow et al., 1986); higher concentrations of aluminium in trabeculated bone (Mjoberg et al., 1997); and some increase in cerebrospinal fluid aluminium (Basun et al., 1991; Kapaki et al., 1993).

Although there has been some controversy, there is now little doubt both that aluminium can be absorbed through the intestinal tract and retained within the body (Priest, 1993; Sharp et al., 1993; Priest et al., 1995; Talbot et al., 1995; Exley et al., 1996; Priest et al., 1996), and that it is a neurotoxin, possibly playing a role in Alzheimer's disease (Doll, 1993; Flaten et al., 1996). A number of factors influence aluminium absorption and handling within the body (Lote & Saunders, 1991), including environmental fluoride (Still & Kelly, 1980; Alfrey, 1985; Shore et al., 1985; Savory et al., 1987) and silicon which may reduce the bioavailability and subsequent absorption of the

element by the body (Birchall & Chappell, 1988a; Birchall & Chappell, 1988b; Birchall et al., 1989; Birchall, 1990; Birchall, 1992; Birchall, 1993; Edwardson et al., 1993; Nieboer & Gibson, 1993; Parry et al., 1998). Birchall's elegant studies demonstrated how effectively silicon, at concentrations often present in water for human consumption, could abrogate the lethal intracellular toxicity of aluminium. Citrate on the other hand significantly enhances absorption (Slanina et al., 1984; Slanina et al., 1985; Slanina et al., 1986; Froment et al., 1989; Molitoris et al., 1989).

In addition, there are metabolic interactions between iron and aluminium, and disturbances in iron metabolism may be involved in the pathogenesis of Alzheimer's disease. Farrar and colleagues performed studies investigating the mechanisms by which aluminium is absorbed by the body and transported in the circulation by the protein transferrin. These studies, on the plasma of patients with Alzheimer's disease and Down's syndrome, demonstrated a reduced affinity of transferrin (<50%) for gallium (a chemical analogue of aluminium) possibly due to abnormally high transferrin-iron saturation (Farrar et al., 1988; Farrar et al., 1990). Patients with Down's syndrome have a very high risk of developing Alzheimer's disease. This difference in aluminium handling could, it was proposed, account for increased absorption of aluminium from the gut and also influence abnormal absorption of iron in Alzheimer's disease as others also suggested subsequently (Abreo & Glass, 1993; Abreo et al., 1999). Evidence for increased deposition of iron in the brains of affected individuals has been demonstrated (Connor et al., 1992; Dedman et al., 1992; Good et al., 1992; Bartzokis et al., 1994; Van Rensburg et al., 1995; Kennard et al., 1996; Savory et al., 1996). In addition there are now several studies reported suggesting increased gut absorption of aluminium in patients with Alzheimer's disease and Down's syndrome (Taylor et al., 1992; Zapatero et al., 1995; Mjoberg et al., 1997; Moore et al., 1997; Moore et al., 2000), despite earlier negative results (O'Mahony et al., 1995).

Following the Camelford incident in which a community's drinking water supply was heavily contaminated with aluminium sulphate, my group found a pattern of abnormalities that were similar to the findings previously described in our aluminium loaded but asymptomatic dialysis patients: normal pre-morbid IQ with discrepant and markedly impaired Symbol Digit Coding tests compared to other tests in the battery; and prolonged flash-pattern visual evoked potential differences. These results suggest that the Camelford subjects did suffer organic brain disease, and not merely the effects of anxiety. There were no other known causes for such effects in the Camelford subjects and so aluminium poisoning must be considered a possibility, although other contaminants may have contributed. As the effects of massive aluminium contamination of water for human consumption have not previously been described or investigated, it is possible that this neurotoxin may have been one of the causative factors, although conclusive evidence for this is unlikely ever to be forthcoming as far as the Camelford incident is concerned.

Our work on the Camelford group received some interesting criticism most of which had in fact been addressed in the paper. Several correspondents raised concerns about subject-motivated bias which we had controlled for as carefully as possible: the manner in which the computerised Symbol Digit Coding test assesses psychomotor function is subtle, to the subject being tested, compared to the superficially more demanding tests of memory such as the verbal recognition and visual spatial recognition memory tests.

Both in these and our previous studies, subjects commented, after testing, that the visual spatial ability, visual perceptual ability, verbal recognition memory and visual spatial recognition memory tests were far more daunting and difficult than the Symbol Digit Coding test. No mention regarding the sensitivity of the tests was ever made to the subjects and none had any prior knowledge of what type of testing was going to be done. The tests were run in a calm but most rigorous and uniform manner, and before each test the subject was given time to rest and it was explained that they should perform as fast and accurately as possible. Individuals who have early organic brain disease exhibit the pattern of abnormality exactly as described in the Camelford group we studied — a pattern that has been described repeatedly in other studies of psychomotor function in a variety of contexts including potentially psychotropic drugs. If litigation-motivated bias had been operating then one would expect the subject to under-perform on the tasks most overtly related to memory rather than in the Symbol Digit Coding test. As this was not the case there is no evidence that such bias affected these results.

With regard to the visual evoked potentials of the Camelford victims concern was raised about the possibility that in a given subject the flash-pattern difference may be large because of a quicker (i.e. shorter) pattern latency than 'normal'. However, as there is surprisingly little normative data for absolute flash and pattern visual evoked potential latencies, we chose to use the difference between the two types of visual evoked potential and not the absolute measures (as others have also done). In many studies looking at the relationship between severity of dementia and visual evoked potentials, it is noteworthy that it is the flash-pattern difference that correlates rather than absolute values. Indeed, the magnitude of the flash-pattern difference was significantly correlated with the magnitude of the Symbol Digit Coding abnormality (but not the other Bexley Maudsley tests), as in dialysis patients where it was also related to cumulative aluminium exposure. If there was a functional relationship between the two types of visual evoked responses one might expect the two measurements to correlate, whereas if they were independent of each other there should be little or no relationship. In our studies there was a very significant relationship between these two parameters in normals, which was nearly absent in the Camelford 'victims' and completely absent in our dialysis patients. How aluminium exposure might lead to this effect on visual evoked potential is not an area that we have addressed in our studies, but there is no reason to suppose that aluminium toxicity is manifested solely by causing neuronal death as one correspondent suggested.

Another correspondent raised the possibility that depression might have led to the changes in psychomotor function that we had observed, quoting results of one paper that he interpreted as demonstrating this. However, the authors of this work (Sloan & Fenton, 1992) had correctly concluded that there was no effect of depression on visual evoked potentials, as several other authors had also done (Jordan et al., 1989; Swanwick et al., 1996), and it was the correspondent that had misinterpreted the results in support of his own views.

If aluminium is important in Alzheimer's disease then would its chelation be of benefit? McLachlan and co-workers in Toronto, Canada, examined the effects of a therapeutic trial of the chelation agent desferrioxamine in patients with early Alzheimer's disease (McLachlan et al., 1991). They reported that the rate of progression of the disease was significantly reduced in the treatment group compared with the untreated group who

had been matched for age and severity of disease at onset of the study. There have not been any other studies to date investigating this possibility. Despite environmental factors, the dominant factors determining susceptibility are likely to be genetic, arising from an increased propensity to deposit beta-amyloid in the brain in these individuals (Mayeux & Schupf, 1995; Cacabelos, 1996).

There is little doubt about the neurotoxic effects of aluminium in dialysis patients, and even the occasional victim with normal renal function has been reported. Since the work done in the 19th century there have been thousands of studies performed to investigate the effects of aluminium on cerebral function and metabolism. Many metabolic processes have been investigated and shown to be adversely affected. Interest in this area of research remains great and in the last decade further studies have confirmed a wide range of toxic effects from neurobehavioral changes in animals fed aluminium to abnormalities of DNA transcription.

Similarities, both biochemical, morphological and functional, exist between circumstances where aluminium toxicity is known to cause animal or human disease (such as aluminium encephalopathy in renal failure) and Alzheimer's disease. These similarities provide what has become an increasingly large body of evidence supporting the role of aluminium in the genesis of Alzheimer's disease:

(i) Aluminium can inhibit tetrahydrobiopterin metabolism, which is important in the production of several neurotransmitters, and tetrahydrobiopterin metabolism is known to be depressed in Alzheimer's disease.

(ii) When transferrin, the protein responsible for the safe transport of iron in the blood stream and a powerful natural aluminium chelator, is added to brain homogenate of patients who had died with Alzheimer's disease some reversal of the tetrahydrobiopterin metabolic inhibition occurs. Clearly there may be other explanations for this effect other than the removal of aluminium, but it would support the view that aluminium may be involved and that its toxicity is not mediated simply through neuronal cell death.

(iii) Several studies from different groups have reported that aluminium is associated with the pathological lesions found at autopsy in the brains of patients with Alzheimer's disease. Aluminium appears to co-localise at the very centre of pathological plaque and in neurones bearing the characteristic neurofibrillary tangles. The fact that the aluminium is found at the core of such lesions might suggest a causative role.

(iv) A large number of studies have been reported in cats and other mammals using aluminium as a means of inducing a behavioural (and neuropathological) model for Alzheimer's disease, the first coming well before Alzheimer originally described the condition in 1907.

(v) In a relatively small number of brains of patients dying from aluminium encephalopathy, neuropathological changes have been identified which are similar although not identical to those in Alzheimer's disease. For example, single stranded tangles are found rather than the characteristic double stranded ones in Alzheimer's disease. The difference in the tangles may arise from the fact that the aluminium intoxication in haemodialysis patients is very acute compared to the supposed chronic or life-long intoxication of patients destined to develop Alzheimer's disease.

(vi) The syndrome of aluminium poisoning, both in the context of renal failure and others, causes clinical effects that are often similar to those seen in Alzheimer's disease.

(vii) Epidemiological evidence has linked drinking water aluminium concentrations with an increased risk of Alzheimer's disease, although genetic factors are now more strongly implicated.

(viii) Other very common environmental factors such as citrate, fluoride and silicon exposure may have a strong influence on the bioavailability and toxicity of aluminium.

(ix) Following heavy contamination of drinking water in Camelford by aluminium sulphate a number of exposed subjects developed short-term memory loss and subsequent studies suggest that this was due to organic brain disease.

(x) The fact that the Camelford victims studied were still significantly impaired several years later fits quite well with what is known about aluminium kinetics across the blood–brain barrier.

(xi) Transferrin regulated iron and aluminium metabolism is important for the absorption and subsequent delivery of aluminium to target organs, and a number of studies have confirmed increased absorption of aluminium in patients with Alzheimer's disease. Transferrin bound aluminium is relatively safe and can only enter cells via the transferrin receptor. If transferrin cannot bind aluminium either due to a defect in its configuration or lack of binding sites through iron saturation, then more 'free' aluminium, possibly in the form of aluminium citrate may circulate in the blood. This low molecular weight aluminium salt could then enter cells in a receptor independent and uncontrolled manner, thereby causing much more toxicity.

Some further questions should be considered if aluminium is to be accepted as anything more than a putative factor in the development of the Alzheimer brain lesion:

If aluminium is relevant, then why is it that dialysis patients do not appear to have a predisposition to developing Alzheimer's disease? This could be explained by reduced longevity of such patients due to other co-morbidity — also many eventually develop cerebrovascular disease. However it may be that through a serendipitous build up of silicon they are protected from the ill-effects of aluminium as so well demonstrated in animal and cell biological experiments. Even though our studies simply showed an inverse relationship between blood silicon and aluminium concentrations (Parry et al., 1998), the aluminium levels were still far higher than described in victims of Alzheimer's disease, and so one would have to postulate that silicon is detoxifying intracellular aluminium.

If the above is true then subjects chronically exposed to high silicon levels may also be protected from the ill-effects of environmental aluminium. As yet there is little epidemiological evidence to support this for silicon, although it is clear that increasing gut silicon reduces the absorption of aluminium. There is some epidemiological evidence for fluoride as a protective agent for Alzheimer's disease, and this element is known to reduce gut absorption (Still & Kelly, 1980). There may be a future role for raising drinking water silicon (not known to have any harmful effects on man) to provide such protection, if this were proved effective, or perhaps the administration of silicon salts to dialysis patients and others might be one method of combating the effects of aluminium.

Given the undoubted role of beta-amyloid deposition in Alzheimer's disease, is there a way in which aluminium could be involved in some way? Amyloid proteins are also acute phase proteins, in that their secretion and deposition may be stimulated by conditions of persistent and sometimes quite low-grade inflammation. Perhaps intracellular accumulation of even quite minute quantities of aluminium would be enough to set such a process off, or interact with the normal turnover of these proteins in the brain. A number of studies have now shown just such possibilities (Exley et al., 1993; Harrington et al., 1994; Exley et al., 1995; Exley, 1997), and if other factors such as a genetic predisposition to increased gut aluminium absorption are also present as discussed above, then it is possible that this type of 'double hit' pathological scenario could be an important step in the development of the disease.

Thus the reader should be persuaded that there is little doubt that aluminium is a neurotoxin both in the context of animal experiments and man. Because of its potential to cause insidious neurotoxicity, it remains a cause for environmental concern (Doll, 1993; Nieboer & Gibson, 1993) and the debate regarding its role in Alzheimer's disease continues (Savory et al., 1996; Forbes & Hill, 1998; Munoz, 1998). Although there is persuasive evidence that it may have a far wider role as a neurotoxin in humans, it is probably only relevant to the wider population if they are, in some way, genetically predisposed. The role of beta-amyloid deposition in the pathogenesis of Alzheimer's disease has become clearer over the last decade, and the gene defects leading to these changes are being unravelled. The ongoing debate about aluminium in this context is whether its known neurotoxic effects and interactions with beta-amyloid make it a likely factor in the pathogenesis of Alzheimer's disease — or is the observed presence of the element in the pathological lesions simply an epiphenomenon? The latter possibility seems a little unlikely, but the major challenge for further research in this field will be to carefully un-pick these genetic and environmental factors so that effective therapeutic strategies can be successfully developed.

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Aluminium and Alzheimer's Disease
The Science that Describes the Link
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CHAPTER 2

Aluminum-Induced Bone Disease: Implications for Alzheimer's Disease

Kenneth Abreo *

Section of Nephrology and Hypertension, Department of Medicine, Louisiana State University Health Sciences Center, Shreveport, LA 71130, USA

* Corresponding author, email: kabreo@lsuhsc.edu

Abbreviations: Al – aluminum; AD – Alzheimer's disease; OM – osteomalacia; ABD – adynamic bone disease; 1,25 D – 1,25 $(OH)_2$ vitamin D_3; PTH – parathyroid hormone; DFO – deferoxamine

Summary

Differences between the structure and function of bone and brain makes it difficult to extrapolate the insights gained from the study of bone aluminum toxicity to Alzheimer's disease. To prove that aluminum plays a critical role in Alzheimer's disease, substantial decreases in brain aluminum content and improvement in brain pathology would have to be demonstrated following chelation therapy. Observations in aluminum-related bone disease and Alzheimer's disease suggest either increased susceptibility to aluminum in some individuals or the presence of some other unknown factor(s). The absence of aluminum accumulation and toxicity in other organs in Alzheimer's disease patients argues against aluminum being the primary cause of this disorder. Brain aluminum deposits in Alzheimer's disease could be an epiphenomenon of prior brain disease similar to observations in aluminum-related bone disease. Low doses of aluminum stimulate bone cell growth and activity raising the intriguing possibility of a positive role for this metal in Alzheimer's disease. More evidence needs to be accrued to confidently state that aluminum is either an accessory or a culprit in the etiology of Alzheimer's disease.

Historical Perspective

Clinical Studies

Aluminum (Al)-related bone disease was initially suspected when patients with dialysis encephalopathy (Alfrey et al., 1976), were found to have severe bone disease and anemia (Parkinson et al., 1979, Pierides et al., 1980, Parkinson et al., 1981). This bone disease presented as a striking syndrome of bone pain, multiple fractures, and a tendency towards hypercalcemia particularly after treatment with vitamin D sterols (Parkinson et al., 1979,

Pierides et al., 1980). The histologic lesion seen on bone biopsy was osteomalacia, a lesion which is also seen in patients with vitamin D deficiency. Vitamin D deficiency can occur in dialysis patients because the kidney converts 25 (OH) vitamin D_3 to 1,25 $(OH)_2$ vitamin D_3 (1,25 D), the most potent form of the vitamin. This conversion is impaired with a reduction in renal mass. However, there was no improvement of this lesion with vitamin D therapy, pointing to Al as a probable etiologic factor (Hodsman et al., 1981). Further investigations focused on confirming whether Al was the etiologic agent in this unique form of renal osteodystrophy (Hodsman et al., 1982, Ott et al., 1982). With the development of specific histologic stains for Al and the ability to assay bone Al content with atomic absorption spectrometry, it was soon clear that these bone lesions were the consequence of Al toxicity (Hodsman et al., 1982, Ott et al., 1982). Following the initial reports from the United Kingdom and the United States, Al-associated bone disease was detected in Europe, Australia, Japan, and other countries suggesting that this problem was more widespread than initially believed (Dustan et al., 1986, Iwamoto et al., 1986, Poedenpant et al., 1986).

From the outset, contamination of water was suspected as the source of Al (Ward et al., 1978, Walker et al., 1982), and removal of Al form dialysate fluid with reverse osmosis and/or deionization was associated with improvement of bone disease (Smith et al., 1987, MacClure et al., 1984, Leather et al., 1981). But the prevalence of Al-related bone disease remained high, even in dialysis centers with low dialysate Al concentrations (Smith et al., 1986). This led to the suspicion that Al-containing phosphate binders, believed until then to be poorly absorbable, were a possible source of Al toxicity. Increased gastrointestinal absorption of Al was found in dialysis patients when compared to normal individuals. Also, a substantial amount of Al was detected in the bone of dialysis patients who had ingested Al-containing phosphate binders over an 8–10 year period (Smith et al., 1986, Kaehny et al., 1977, Bournerais et al., 1983). Other sources of Al were identified in non-dialyzed populations, such as patients receiving chronic parenteral nutrition and infants receiving parenteral therapy (Klein et al., 1982, Sedman et al., 1985) in whom osteomalacic bone disease was also seen (Klein et al., 1982, Ott et al., 1983). Following these reports a concerted effort was made to minimize oral and parenteral sources of Al. Calcium-containing and synthetic gastrointestinal phosphate binders were substituted for Al-containing binders. For patients with severe bone Al toxicity, deferoxamine (DFO), administered during dialysis sessions, proved to be an effective chelator and resulted in the cure of bone disease, as well as encephalopathy and anemia (Brown et al., 1982, Malluche et al., 1984, Abreo, 1988). Initial high-dose DFO regimens were replaced by low-dose regimens which were found to be equally effective but with decreased toxicity (Barata et al., 1996, D'Haese et al., 1996). With renal transplantation, the increased urinary excretion of Al resulted in healing of the osteomalacic bone (Malluche et a., 1984, Piraino et al., 1988, Nordal et al., 1992). This systematic effort to avoid exposure of dialysis patients to Al culminated in the near elimination of this disease in developed countries. The prevalence of Al-associated bone disease shrank from 30–50% two decades ago to 10% today (Cannata-Andia, 1998).

Normal Bone Histology

To understand the toxic effect of Al on bone a brief review of bone histology is necessary (Teitelbaum, 1984). Bone is made up of both mineral and cellular components since the skeleton has to provide structure, strength, and protection to the human body. Histologically bone can be divided into cortical (osteal) and cancellous (trabecular) bone. Bone cells are comprised of osteoblasts, osteoclasts, and osteocytes, and they are responsible for bone growth, modeling, repair, and remodeling. Osteoblasts, which are of bone marrow fibroblast-like lineage, line all bone surfaces. Fusiform in shape when inactive they become cuboidal or columnar when actively synthesizing and mineralizing osteoid. Osteoclasts, derived from the monocyte/macrophage lineage, become multinucleated when active and remove mineral from bone, forming pits called Howship's lacunae. Osteocytes are osteoblasts trapped in bone matrix to form Haversian canals. Their role is not well defined but they are supposed to sense deformation of bone, coordinate the formation and resorption of bone, and the flow of mineral ions between bone matrix and extravascular fluid spaces (Buckwalter et al., 1995).

The mineral component of bone exists in two phases, organic and inorganic. The majority of the organic phase or osteoid (95%) is a matrix of collagen. The inorganic phase of bone consists of hydroxyapatite, an insoluble microcrystalline mineral, which is initially deposited on the organic matrix as calcium phosphate salts, and is later transformed to apatite crystals. Various quantities of other ions, such as carbonate, magnesium, sodium, and fluoride, are also found in bone, and may play important structural and metabolic roles.

Bone growth and modeling occur early in life whereas remodeling occurs throughout life at discrete areas (bone remodeling units) of the endosteol, periosteal, Haversian, and trabecular surfaces of bone. The largest surface or envelope at which remodeling occurs is the trabecular surface. Remodeling consists of removal of mineral followed by its replacement, a process regulated by metabolic, hormonal, and other factors. The process is initiated at discreet sites in bone by osteoclasts that become multinucleated, and remove mineral and collagen from bone to form Howship's lacunae (resorption bays). After a brief resting phase osteoblasts become active, migrate to sites of bone removal, and begin laying down osteoid followed by its mineralization. The activities of osteoblasts and osteoclasts are coupled, in that the mineral removed is replaced, resulting in the absence of a net change in mineral balance.

Once osteoblasts lay down osteoid, mineralization does not occur immediately because osteoid has to mature before calcium can be incorporated into it. The unmineralized osteoid forms the osteoid seam and the time taken to mineralize osteoid is called the mineralization lag time. The autofluorescent antibiotic tetracycline chelates calcium and deposits along with it at the junction of osteoid and mineralized bone, the calcification front (Fig. 1). Administration of this antibiotic in doses given at specified intervals before a bone biopsy gives important dynamic parameters of mineralization. The length of the calcification front taking up the label and the distance between labels is an indication of the extent and rate of mineralization, respectively.

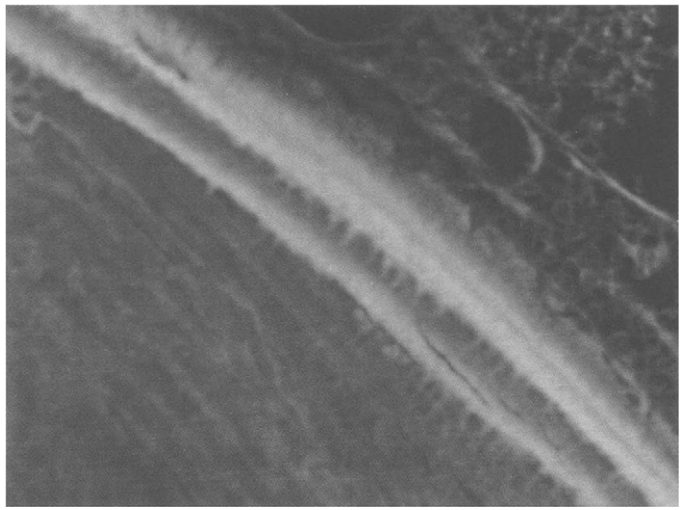

Fig. 1. Double tetracycline labeling: Two separate bands of tetracycline. Mineralized bone histology. Unstained section. Fluorescent light microscopy. Courtesy of MC Faugere, MC Langub, and HH Malluche.

Bone Histology in Aluminum Bone Disease

Al-related bone disease has two histological patterns: osteomalacia (OM) and adynamic bone disease (ABD). OM lesions (Fig. 2) comprise wide osteoid seams, few osteoblasts and osteoclasts, very little tetracycline uptake, and minimal separation of

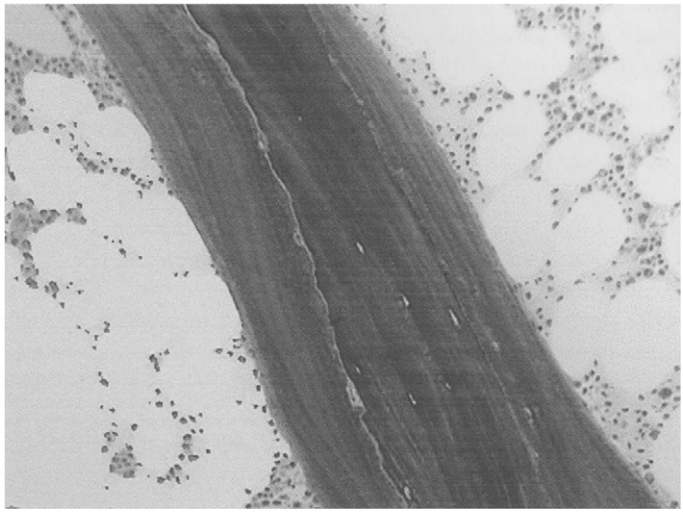

Fig. 2. Low turnover osteomalacia. High osteoid volume and surface. Thick osteoid seams. Lamellar osteoid. Paucity of bone cells. Modified Masson–Goldner stain. Bright light microscopy. Courtesy of MC Faugere, MC Langub, and HH Malluche.

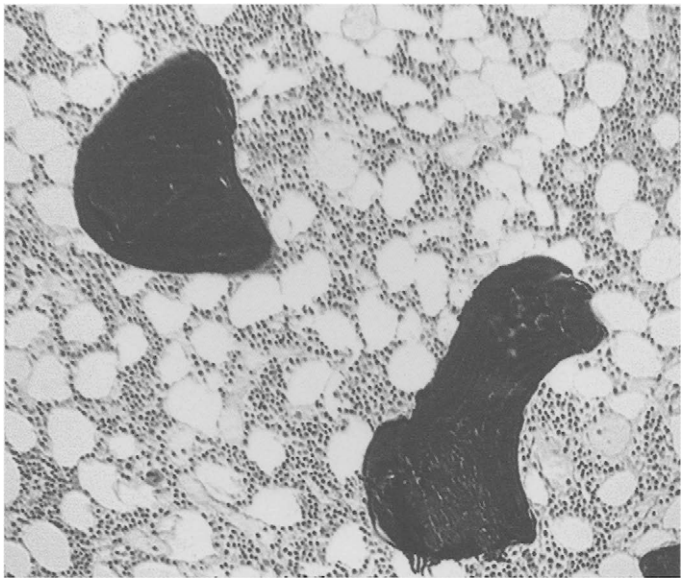

Fig. 3. Adynamic bone disease: Low bone volume. Few thin osteoid seams. Paucity of bone cells. Modified Masson–Goldner stain. Bright light microscopy. Courtesy of MC Faugere, MC Langub, and HH Malluche.

labels suggesting a primary defect in mineralization. In ABD (Fig. 3) the width of the osteoid seams are normal or decreased and the number of both osteoclasts and osteoblasts is greatly reduced. The decrease is characterized by a primary defect of bone formation accompanied by a secondary decrease of bone mineralization (Fournier et al., 1997). The width of the osteoid seam is dependent upon the rate of osteoid deposition and its subsequent mineralization. In osteomalacia, osteoblasts lay down more osteoid than they can calcify whereas in adynamic bone disease there seems to be a proportional defect in osteoid synthesis and calcification. In the original descriptions of Al-related bone disease almost all patients had the osteomalacic bone lesions but over the past two decades there has been a decrease in OM and an increase in ABD (Monier-Faugere & Malluche., 1996, Ballanti et al., 1996). This could be a consequence of a lower exposure to aluminum, the use of 1,25(OH) D and calcium-containing phosphate binders, correction of metabolic acidosis with bicarbonate baths, or perhaps a greater proportion of elderly and diabetic patients who are susceptible to this lesion (Pei et al., 1993, Lefevre et al., 1989, Fournier et al., 1991, Goodman et al., 1994, Couttenye et al., 1997).

Diagnosis and Treatment

Histochemical staining for Al and the measurement of bone Al content by atomic absorption spectrophotometry are the primary methods used to diagnose Al-related bone disease (Hodsman et al., 1982, Ott et al., 1982). Although other methods such as energy dispersive X-ray analysis and microprobe techniques have been used to assess the Al

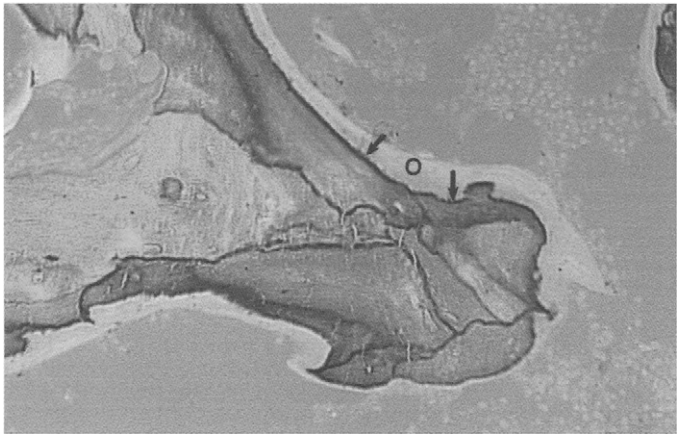

Fig. 4. Bone aluminum deposition: Aluminum deposits at the osteoid (O) bone interface (arrows) and with mineralized bone. Mineralized bone histology. Solochrome azurin stain. Combination or bright and fluorescent light microscopy. Courtesy of MC Faugere, MC Langub, and HH Malluche.

content of bone these methods are not readily available and therefore have not been used in large studies. Aluminum stains (solochrome azurin or aurin tricarboxylic acid) show linear deposits (Fig. 4) along the calcification front and within cement lines in calcified bone. The extent of staining and the location of deposits in areas of low remodeling suggests a causal role of the metal for these lesions. However, the fact that Al can be demonstrated within calcified bone also suggests that given the right conditions calcification can occur even in areas of Al deposition (Maloney et al., 1982). Therefore,

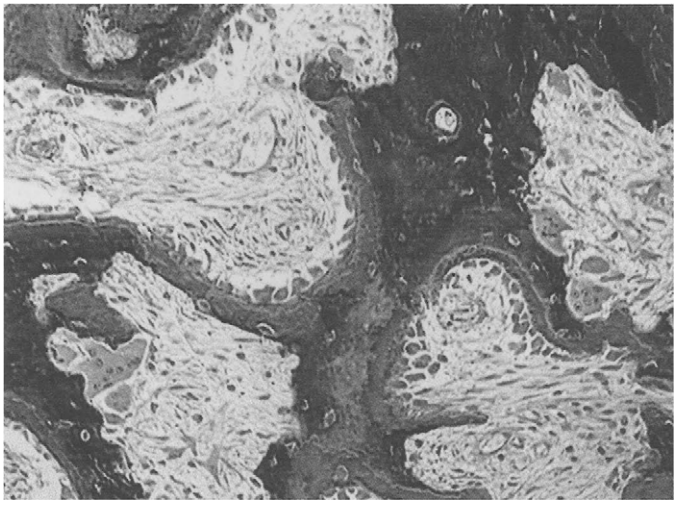

Fig. 5. Osteitis fibrosa: High osteoid volume and surface. Woven osteoid. Numerous osteoblasts and osteoclasts. Bone marrow fibrosis. Mineralized bone histology. Modified Masson–Goldner stain. Bright light microscopy. Courtesy of MC Faugere, MC Langub, and HH Malluche.

in the same bone sample one can sometimes find lesions of OM and osteitis fibrosa. Osteitis fibrosa (Fig. 5) is characterized by increased number and activity of osteoblasts and osteoclasts, peritrabecular bone marrow fibrosis, the presence of increased osteoid, and increase in the extent and separation of tetracycline labels. This lesion which has features of both Al-associated OM and parathyroid hormone (PTH)-driven osteitis fibrosa has been called a mixed lesion (Smith et al., 1986). Although Al staining and content are increased in patients with Al-associated OM and ABD, the pathologic changes seem to correlate better with histochemical staining than bone content, further suggesting that the location of Al deposits plays an important role in its toxicity (Faugere & Malluche., 1986). Chelation of Al with DFO administration to dialysis patients results in a decrease in stainable bone Al, increase in bone formation rate, and in some cases to the development of osteitis fibrosa (Brown et al., 1982, Malluche et al., 1984, Andress et al., 1987). Although bone biopsy is still considered the 'gold standard' for the diagnosis of Al related bone disease, the serum Al increment after DFO has been found to be a reliable non-invasive diagnostic alternative, and in combination with a PTH measurement, may even differentiate between Al accumulation, increased risk for toxicity, and Al-related bone disease (D'Haese et al., 1995).

Cellular and Subcellular Localization of Aluminum

Electron microscopy and microanalytic studies of bone sections from patients with Al-induced OM show Al located in isolated, hexagonal structures along the mineralization front (Plachot et al., 1984, Verbueken et al., 1984). It is not clear whether Al is part of the hydroxyapatite or separate from it. Its location, and the speculation that it may be in the form of Al citrate, which been shown *in vitro* to inhibit calcium phosphate crystallization, suggests that it may play a role in inhibiting the formation and aggregation of bone crystals (Thomas, 1982). Al deposits have also been located in mitochondria of osteoblasts (Cournot-Witmer et al., 1986).

Role of Parathyroid Hormone

Several studies indicate that PTH has a protective effect on Al-related bone disease, as it is associated with less Al accumulation at the calcification front (Cournot-Witmer et al., 1981). Parathyroidectomy has been shown to increase Al accumulation in bone and worsen osteomalacic lesions (Felsenfeld et al., 1987, Andress et al., 1985). Most patients with excessive bone Al accumulation have decreased PTH levels when compared to those without (Hodsman et al., 1982, Iwamoto et al., 1986). Al accumulation can itself suppress parathyroid gland function in dialysis patients. Parathyroid glands take up Al, perhaps by the Al-transferrin/transferrin receptor mediated mechanism, and suppression of PTH secretion by Al has been demonstrated (Smans et al., 2000, Morrissey et al., 1983, Bourdeau et al., 1987, Cann et al., 1979). In addition, Al-related OM can induce bone resistance to calcium entry, and the ensuing hypercalcemia can suppress PTH secretion (Cannata et al., 1983). Al-rich bone is resistant to the action of PTH, an important factor in bone for-

mation (Kawaguchi et al., 1986, Andress et al., 1983, Kraut et al., 1983). Healing of OM occurs in those patients who show rapid increases in PTH levels following elimination of dialysate contamination or after chelation with DFO, presumably from a simultaneous decrease in bone and parathyroid Al (Smith et al., 1987, Malluche et al., 1984).

Animal and Cell Culture Studies

A number of *in vivo* and *in vitro* studies have been conducted in an attempt to answer the following questions about Al-induced bone disease: 1) Does Al produce the bone lesions seen in man? 2) Does Al produce bone lesions in normal animals or is uremia a prerequisite for Al-bone disease? 3) Does uremia per se influence bone Al accumulation and its toxicity? 4) What are the mechanisms of Al toxicity to bone? 5) Can the bone alterations produced by Al be overcome with the administration of calcium regulating hormones? 6) Does bone disease heal by simply stopping Al administration or should a chelating agent be administered? 7) Do alterations of calcium regulating hormones influence the deposition of Al in bone and do they play a role in the types of bone lesions that develop? 8) How does Al enter bone cells? 9) How does Al alter the function of these cells?

Although a variety of model systems have been developed to answer the above questions, interpretation of the results should be tempered with the following caveats: (i) Very large doses of Al compounds have been administered by various routes, i.e. peritoneal, intravenous etc. often over a fairly short time interval, whereas Al accumulation in dialysis patients is a much slower process. (ii) Different animal species have been used such as dog, pig, rats, and chicks. Rodent bone undergoes modeling throughout life which is very different from human bone that is subject to cycles of remodeling. This makes the dog and pig data more relevant. (iii) Induction of uremia results in many changes in the level of PTH and 1,25 D. Whereas in primary hyperparathyroidism the synthesis of 1,25 D is stimulated, in secondary hyperparathyroidism seen in animals with chronic renal failure 1,25 D levels are low because the kidney plays a key role in the generation of 1,25 D. To further complicate matters, Al accumulation in the parathyroid glands has been shown to decrease PTH levels (Smans et al., 2000, Morrissey et al., 1983, Bourdeau et al., 1987) and Al accumulation in the kidney results in decreased 1,25 D levels (Goodman et al., 1984). In addition, in some animal models intravenous aluminum administration results in hypercalcemia which in turn is a potent suppressor of both PTH and 1,25 D (Goodman et al., 1984, Rodriguez et al., 1987). Given these alterations in calcium regulating hormones, their influence on each other, and their suppression by Al, it makes interpretation of results very difficult. In most of the uremic models that have been described the investigators have not measured or controlled all these variables.

Osteomalacia

There is absolutely no doubt that the administration of Al to the normal and uremic animal causes osteomalacic lesions similar to those seen in humans. Osteomalacia

was first shown to develop in Al-loaded rats by Ellis (1979), and subsequently was demonstrated in the dog and pig by others (Goodman et al., 1984, Sedman et al., 1987). This lesion would result if the rate of osteoid production outstripped the rate of mineralization, indicating that either mineralization was impaired or osteoid production enhanced.

When bone was evaluated shortly after Al was administered to rats, it was found deposited along the mineralization front but no alterations in bone histology or bone formation were noted (Ott et al., 1987). With continued administration of Al, defects in bone formation were seen, particularly impaired mineralization but no histologic alterations suggestive of OM developed (Ott et al., 1987, Goodman, 1984, Schrooten et al., 1998). Continued administration of Al resulted in full blown OM (Bourdeau et al., 1987, Cannata et al., 1983, Kawaguchi et al., 1986). The severity of OM correlated with both the bone Al content and the extent of Al deposition by histochemistry (Goodman et al., 1984, Ellis et al., 1979, Sedman et al., 1987)

In several models of Al toxicity, merely stopping the administration of Al resulted in improved mineralization over the next few weeks (Ellis et al., 1979, Ott et al., 1987, Finch et al., 1986). The bone Al content did not change but the percentage of surface showing stainable Al decreased significantly (Ott et al., 1987, Finch et al., 1986). A higher percentage of Al was seen in cement lines, suggesting that surface Al was buried under mineralized bone (Ott et al., 1987). These observations emphasize that it is the site of Al accumulation (the mineralization front), rather than the total bone Al content, which is important in the pathogenesis of OM.

Adynamic Bone Disease

The role of Al in the development of ABD in man is controversial because this bone disease has also been described in patients in the absence of Al. There are some animal models in which Al toxicity has been shown to produce this lesion. Goodman has described the occurrence of ABD in the cortical bone and OM in trabecular bone of rats administered Al (1984, 1985). Comparison of the amount of Al deposited in the two types of lesions showed that the adynamic lesions had less Al. Goodman has suggested that these differences are a consequence of the low bone turnover of cortical bone when compared to trabecular bone. Adynamic lesions with lower Al content have also been described in weanling rats administered Al in contrast to adult rats who developed OM at the same dose of administered Al (Ott et al., 1987). Contrary to the reasons given by Goodman, the higher bone formation rate of weanling rats was suggested as a possible explanation for the different age-related responses. In a recent study however, dietary administration of Al over a 12-week period to adult rats with chronic renal failure also resulted in the development of ABD in the presence of moderate bone Al levels (Schrooten et al., 1998). Finally, in another experiment Al-loaded parathyroidectomized rats developed ABD whereas those with intact parathyroid glands developed OM (Goodman, 1987). The adynamic lesions were seen in cortical and trabecular bone. There was no difference in the bone Al content between parthyroidectomized and intact animals. Since ABD is seen at lesser

Al concentrations, there must be other factors such as a low turnover state induced by parthyroidectomy to foster this lesion.

De Novo Bone Formation

In some circumstances Al does not impair bone formation and may in fact stimulate both matrix formation and mineralization. Quarles et al. have shown that low dose Al administration to dogs resulted in reduced bone resorption and decreased osteoblast number, findings indicative of low bone turnover (1988). At higher doses, however, Al stimulated the deposition of woven bone over the majority of the trabecular envelope. Another observation was that the new bone was deposited in unscalloped areas. Since new bone is only laid down in areas that have undergone osteoclastic reabsorption (Howship's lacunae), this suggests that Al induces uncoupling of bone resorption and formation (Quarles et al., 1988). Glaceran et al. have made similar observations in Al-loaded dogs and in addition they also found increased bone marrow fibrosis which was attributed to an Al stimulatory effect on osteoblasts and fibroblasts as they are of similar origin (1987). Increased rates of mineralization have also been noted in rats by Chan et al. (1983).

Mechanisms for the Development of Aluminum-Related Bone Disease

Defects in mineralization

To see whether Al directly affects mineralization independent of its effect on osteoblasts, cell free systems have been used by Thomas et al. (1982). Aluminum citrate complexes had a direct inhibitory effect on the growth of calcium phosphate crystals in aqueous solutions and also behaved as potent inhibitors of calcium uptake by a calcifiable matrix (collagen) (Thomas, 1982, Meyer et al., 1982). These observations were tested *in vivo* by Talwar by implanting bone matrix into Al-loaded rats (1986). Demineralized bone matrix when implanted subcutaneously in allogenic rats induces an invariant sequence of events resulting in de novo cartilage, bone, and bone marrow formation. Since the bone matrix cells were not exposed to Al until transplantation occurred, their function was intact. Impaired mineralization was seen in rats administered Al where as matrix synthesis was intact, if, somewhat delayed. When decalcified matrix was mixed with Al salts prior to transplantation, a toxic effect was seen on both mineralization and matrix synthesis. Similar observations have been made by others in long-term rat calvaria cell cultures (Bellows et al., 1995, Bellows et al., 1999) and embryonic chick bone (Miyahara et al., 1984). In embryonic chick bone both inhibition of mineralization and stimulation of demineralization was seen (Miyahara et al., 1984). Thus, it seems that Al may have a physicochemical inhibitory effect on bone mineralization prior to and independent of its toxic effect on osteoblasts. At high doses Al inhibits alkaline phosphatase activity in rat osteoblast-like cells in cultures (Lieberherr et al., 1987). Since alkaline phosphatase cleaves pyrophosphate, a known inhibitor of crystallization, altered function of osteoblasts would eventually affect mineralization.

Increased osteoid synthesis with or without mineralization

The stimulatory effect of Al on bone cells has been confirmed in cell culture studies. Contrary to the observation of Quarles et al. in aluminum loaded dogs (1988), low concentrations of Al in culture media stimulate osteoblasts and osteoclasts, whereas suppression is seen at higher concentrations (Lieberherr et al., 1987, Lau et al., 1991). Lieberherr et al. have shown that at low doses of Al osteoblast-like cells proliferate, take up calcium, and increase collagen synthesis while osteoclast-like cells release calcium, β glucoronidase, and tartarate resistant acid phosphatase (1987). Both proliferation and activity of both cell types decrease at higher doses of Al (Lieberherr et al., 1987). Stimulation by low dose Al of chicken and human osteoblast-like cells, and synthesis of collagen by human osteosarcoma cells has been described (Lau et al., 1991). Again biphasic activity is seen with suppression occurring at higher doses of Al. The studies of Kasai et al. suggest that transferrin may play a role in the entry of Al into bone cells, as the addition of transferrin to cell cultures resulted in increased suppression of osteoblast-like cells (1991)

Several potential mechanisms whereby Al stimulates bone cells have been explored. One possibility is an Al-activated receptor that is coupled to G proteins. Studies in osteoblast-like cells indicate that Al mimics the effects of a calcium sensing receptor to stimulate DNA synthesis, activate G proteins, and stimulate G-protein coupled signaling pathways (Quarles et al., 1994). Aluminum has been shown to stimulate bone cells to secrete powerful bone cell mitogens such as insulin-like growth factor (IGF) and inhibit its binding protein IGFBP-4, which would allow the unbridled activity of IGF (Lau et al., 1993)

Uremia

Since uremia is a syndrome in which there are multiple complex perturbations, its role in the genesis of Al-related bone disease is difficult to ascertain. In most studies animals with renal insufficiency retained more Al in bone when compared to their normal counterparts (Robertson et al., 1983). The simple explanation for this observation is that impaired renal function results in decreased clearance of the metal. However, it is possible that the uremic milieu may alter the organ distribution of Al, favoring its accumulation in bone as suggested by Chan and coworkers (1983). Normal rats given Al sequestered the metal preferentially in liver in contrast to uremic animals who sequestered it in bone. Unfortunately other animal studies do not duplicate these findings (Verbeelen et al., 1989). Further experiments by Alfrey et al. showed that parathyroidectomy in uremic animals given Al reversed the organ distribution of Al, suggesting that hyperparathyroidism of uremia may have also played a role (1985). Contrary to these findings the absence of PTH did not alter bone Al content in uremic dogs (Malluche et al., 1987).

Parathyroid Hormone and $1,25(OH)_2$ Vitamin D_3

As stated earlier, PTH and 1,25 D are so interdependent, that ideally the effects of both should be taken into account when assessing their influence on Al deposition and toxicity

in bone. This has not been the case and the majority of researchers have examined the effect of one or the other in their models of Al bone disease. It should be noted that Al accumulation and osteomalacic lesions have been described in animals without any change in PTH or 1,25 D levels suggesting that perturbations in these hormones are not a prerequisite to the development of Al associated bone disease (Goodman et al., 1984, Goodman, 1985, Ott et al., 1987).

Parathyroid hormone may play a role in the amount of Al taken up by bone and/or the type of bone disease that ensues. The literature on this subject is inconsistent and confusing. Seminal studies by Mayor et al. have suggested that PTH has a positive influence on the absorption and tissue distribution of Al (1977, 1980). In uremic rats with Al overload, parathyroidectomy resulted in decreased bone Al accumulation compared to non-parathyroidectomized animals (Alfrey et al., 1985). Despite the low bone Al concentration the parathyroidectomized uremic rats had severe OM suggesting that lack of PTH plays a role in this process. Since these differences in bone Al and histology were not found in normal animals who underwent parathyroidectomy, the uremic milieu may have also played a role. In similar studies by another group of investigators, uremic parathyroidectomized rats had lower bone Al content, but lack of parathyroid hormone did not induce OM (Vukicevic et al., 1989). Parathyroidectomy has also resulted in decreased bone Al uptake in normal dogs but has not resulted in osteomalacia (Quarles, 1990). In this study parathyroidectomy caused ABD and Al administration did not affect these lesions. Contrary to these findings other investigators found no differences in bone Al deposition and/or bone histology in uremic dogs and rats in the presence or absence of PTH (Malluche et al., 1987, Abreo et al., 1988). Finally, in one report, high doses of PTH seemed to protect Al-loaded rats from low turnover bone disease (Felsenfeld et al., 1991). Thus PTH can increase, decrease, or not alter bone Al content, and it can cause ABD or OM depending on the animal model evaluated.

The studies of Quarles et al. (1985) and Malluche et al. (1987) in dogs suggest that 1,25 D protects bone from Al deposition and toxicity. In the former study, 1,25 D deficient dogs with OM avidly deposited Al along the mineralization front and correction of this deficiency resulted in loss of bone Al and healing of bone lesions (Quarles et al., 1985). In the latter study, 1,25 D had significant protective effect on bone Al content and toxicity irrespective of PTH levels (Malluche et al., 1987). Similar observations have also been made in Al-loaded normal and uremic rats by others (Abreo et al., 1988, Hirschberg et al., 1985, Hodsman et al., 1984). However, other investigators have found no effect of 1,25 D on Al accumulation in the bone of uremic rats (Verbeelen et al., 1989, Vukicevic et al., 1987). The majority of investigations suggest that 1,25 D may have a protective role in bone Al accumulation and toxicity.

The effect of PTH and 1,25 D on Al uptake by bone cells in culture has not been evaluated. Parathyroid hormone was shown to potentiate the bone cell mitogenic activity of Al (Lau et al., 1991). On the other hand it has been shown that Al both inhibits and potentiates 1,25 D and PTH effects on bone cells. Mitogenic concentrations of Al potentiated 1,25 D stimulation of osteocalcin secretion but inhibited the hormone-mediated activity of cellular alkaline phosphatase (Lau et al., 1991). Aluminum suppressed the 1,25 D stimulated ornithine decarboxylase activity and PTH stimulated cAMP content in osteoblast-like cells (Lieberherr et al., 1987). It has also been shown in isolated perfused

dog bone that Al blunted the PTH stimulated release of cAMP, a marker of osteoblastic function (Glaceran et al., 1987).

Informed Opinion

A Possible Explanation of Aluminum-Induced Bone Disease

To ascribe a role for Al in the etiology of bone disease all four of Koch's (1882) postulates need to be fulfilled: (i) Is it found in high concentrations in the affected organ? (ii) Are the clinicopathological findings of the disease it produces constant? (iii) Can these findings be experimentally reproduced? (iv) Does removal of this agent result in amelioration of this disease? Aluminum does fulfill all four of Koch's postulates especially the first and last. Both clinical and experimental studies have consistently demonstrated a high bone Al content in patients with OM and ABD, and amelioration of these lesions with cessation of exposure to this metal. The second and third postulates are partially fulfilled, in that Al associated bone has a variable expression in man and animals. The remodeling of human bone is reminiscent of the whorls in the trunk of a tree. Just as the width and extent of the whorls provide clues to robustness or leanness of the previous years rainfall and nutrition, the effects of prevailing conditions are etched in bone with each remodeling cycle. Since uremia is a complex syndrome in which there are accumulated toxins, perturbations in calcium-regulating hormones, and poor nutritional status, all of which could individually or collectively affect bone remodeling. Bone metabolism is also influenced by the underlying disease process that induced the renal failure and medications that are taken by patients. Diabetes and corticosteroids for example can cause slowing of bone turnover. The internal milieu of the end-stage renal failure patient and the bone turnover status at the time of Al entry may have a profound effect on the toxicity of Al. This may explain why some patients are more susceptible to Al bone disease where as others seem to be protected.

Based on the clinical and experimental information four hypothetical stages of Al-related bone disease can be described. In the early stage of Al accumulation in bone there is no demonstrable toxicity (Table 1). At this stage (stage 1) mineralization is

Table 1. Stages of aluminum-associated bone disease

Stage	Al content	Mineralization	Histology	Mechanisms
1.	Mild	Decreased	Normal or osteitis fibrosa	Usually high PTH. Al in cement lines
2.	Moderate	Decreased	Mixed lesions: OM + OF	High PTH. Physicochemical effect of Al on mineralization.
3.	Severe	Decreased	Osteomalacia	Normal to low PTH. Osteoid synthesis > mineralization rate
4.	Variable	Decreased	Adynamic bone disease	Normal to low PTH. Osteoid synthesis = mineralization rate

OM = osteomalacia, OF = osteitis fibrosa.

intact and osteoblasts and osteoclasts are not affected. Bone mineralization continues unimpeded over the Al deposits, and Al is incorporated in cement lines in bone. The same events occur at some time point in the recovery process when OM and ABD disappear and mineralization normalizes. The bone in the dialysis patient will look either normal or have features of osteitis fibrosa. In stage 2 moderate amounts of Al will impair mineralization and also stimulate osteoblasts to increase synthesis of osteoid. Thus in trabeculae where Al is deposited there would be increased osteoid characteristic of OM and in areas where Al deposits are mild the lesions of osteitis fibrosa would develop, features characteristic of the mixed lesion. With severe bone Al accumulation (stage 3) osteoblast number and function decrease, there is a severe mineralization defect and wide bands of osteid are seen typical of OM. The lesion of ABD although categorized as stage 4 may have variable Al deposits. Severe Al toxicity could cause impairments in mineralization and osteoid synthesis giving rise to inactive bone with no increase in osteoid seams, the cardinal features of ABD. However, mild to moderate deposits of Al may be found in conjunction with ABD of other causes. In these instances the contribution of Al to ABD is not clear.

A unified hypothesis for the role of PTH and 1,25 D on bone Al accumulation and toxicity cannot be formulated based on the available data. In human studies PTH seems to prevent bone Al toxicity where as animal studies are inconsistent. On the other hand, 1,25 D plays a protective and therapeutic role in bone Al disease in animal models but has no effect in humans. It is possible that the relative concentrations of both hormones influence the uptake of Al in bone and the type of bone disease that ensues.

Relevance of Aluminum Bone Disease to Alzheimer's Disease

The sharp differences between the structure and function of bone and brain makes it difficult to extrapolate the insights gained from the study of bone Al toxicity to AD. The diagnosis of Al bone disease can be easily confirmed by bone biopsy and the effectiveness of therapy gauged by repeat biopsies at appropriate time intervals. In contrast the histopathologic findings of brain tissue in AD are based on autopsy studies. To prove that Al plays a critical role in this disorder, substantial decreases in brain Al content and improvement or stabilization of brain pathology would have to be demonstrated following chelation therapy in AD patients. This would require performance of brain biopsies in AD patients prior to and after chelation therapy, which is a daunting, if not, impossible task. Improvements in cognitive function following chelation therapy are not sufficient evidence to implicate Al as the culprit in this disorder as other metals, especially iron, are also chelated (McLachlan et al., 1991, McLachlan et al., 1993).

Epidemiological studies have shown an increased incidence of AD in areas with high Al content in drinking water (Flaten, 1990, Martyn et al., 1989, McLachlan et al., 1996, Neri & Hewitt, 1991). If these findings are accurate, studies need to be conducted in order to evaluate the effect of elimination of Al exposure on the incidence of AD. In this regard both clinical and experimental studies in dialysis patients and uremic animals have shown substantial improvement of Al bone disease following cessation of exposure to this metal. Despite similar exposure to Al, dialysis patients display a variety of responses in their susceptibility to bone disease, some are protected whereas other develop full blown OM.

Similar observations in AD patients suggest either increased susceptibility to Al in some individuals or the presence of some other unknown factor(s). Despite the generally high prevailing serum Al levels in dialysis patients when compared to those with normal renal function, it is surprising that AD has not been reported in these patients. This observation has led to the notion that Al therefore does not play a role in the etiopathogenesis of AD. Extrapolation of the findings of Al neurotoxicity in dialysis patients to those with normal renal function may not be appropriate as uremia is a complex state. In end-stage renal disease the accumulation of other compounds, such as silicone, may have a protective effect, by either affecting the bioavailability or reversing the Al-induced pathological changes of AD (Van Landeghem et al., 1997). Finally, one could argue that the longevity of patients on dialysis is considerably decreased, especially in the elderly, resulting in an inadequate length of Al exposure to develop AD.

The majority of dialysis patients with Al-related bone disease have high blood levels of Al. When the levels are equivocal, the increase in blood Al concentrations after the administration of DFO can be used as an indicator of Al overload (Millner et al., 1984) and in combination with a PTH measurement may allow to differentiate between overload, increased risk for toxicity, and Al-related bone disease (D'Haese et al., 1995). Similar non-invasive tests to assess the body burden of Al need to be developed in AD patients. Since AD patients do not have renal failure, urinary Al content would have to be measured after DFO administration. Is the increase in Al content in AD patients confined to the brain or is it found in other organs? Unlike AD, bone Al disease also afflicts other organs giving rise to encephalopathy and microcytic anemia. The absence of Al accumulation and toxicity in other organs in AD patients argues against Al being the primary cause of this disorder. On the other hand, it is also possible that acquisition of small amounts of Al over many decades may result in selective brain deposition of this metal.

Sufficient information is not available to delineate the pathways of Al entry into bone cells and matrix. Experimental observations suggest that Al may be harmless under certain circumstances. When the conditions are appropriate bone cells do not seem to be affected and mineralization can continue over Al deposits at the mineralization front. Thus it would seem probable that Al may exist as a dormant metal in the brain, not affecting function or pathology, but would exert its toxic effect if conditions changed. In dialysis patients, factors such as uremia, the state of bone turnover, and the activities of the calcium regulating hormones, PTH and 1,25 D may provoke Al toxicity. In AD there may be as yet unknown factors that play an important function in triggering Al toxicity. To my knowledge the relationship of calcium-regulating hormones on brain Al accumulation and toxicity has not been evaluated.

In some animal models it has been shown that the prior occurrence of bone disease facilitates Al deposition, suggesting that the altered bone pathology is not from Al toxicity (Hodsman et al., 1984, Quarles et al., 1985). A similar phenomenon could develop in the AD patient. Neurofibrillary tangles and senile plaques, the pathological hallmarks of AD, may result from another process but may allow the deposition of Al. Thus Al deposits in these lesions would be a mere epiphenomenon since they have nothing to do with the genesis of these lesions. In the animal models of bone Al disease it has been shown that healing of bone lesions resulted in elimination of Al from bone. Similarly it is possible

that once the major etiologic factor(s) for AD are identified and corrected brain Al content might automatically decrease.

Research in AD has focused on the presence of β-amyloid deposits in brain and suggest that this is a disorder of chronic inflammation. Some researchers have suggested a link between Al and the deposition of β-amyloid in brain (Exley et al., 1993, Exley et al., 1995). Although β-amyloid deposits can be found in bone, joints, and synovium of long-term hemodialysis patients, this amyloid is not the result of Al toxicity. The β-amyloid found in dialysis patients is a consequence of high blood β_2 microglobulin concentration, and the high β_2 microglobulin levels are ascribed to the chronic inflammatory state of uremia and poor clearance of β_2 microglobulin by hemodialysis (Koch, 1992).

At low concentrations Al has been shown to stimulate bone cells to proliferate and become more active. This suggests the intriguing possibility that this metal may even play a positive part in the healing and functioning of neuronal lesions affected by some other processes that ultimately lead to AD. Thus Al may act as a double edged sword depending on its concentration and the presence of associated factors in the brain.

In conclusion, the role of Al as a culprit in bone disease is based on the clinical and experimental information available. On the other hand, much more evidence needs to be accrued to confidently state that Al is either an accessory or a culprit in the etiology of AD.

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Aluminium and Alzheimer's Disease
The Science that Describes the Link
C. Exley (Editor)

CHAPTER 3

The Epidemiology of Aluminium and Alzheimer's Disease

Virginie Rondeau * and Daniel Commenges

INSERM U 330, Université Victor-Segalen, 146 rue Léo Saignat, 33076 Bordeaux Cedex, France

* Corresponding author, email: Virginie.Rondeau@isped.u-bordeaux2.fr

Abbreviations: AD – Alzheimer's disease

Summary

Although the hypothesis of a link between aluminium and Alzheimer's Disease (AD) has been supported by several epidemiological studies, there is much controversy regarding these findings and their interpretation. This chapter reviews the epidemiological results obtained regarding this hypothesis and the methodological difficulties in studying such an association. Further research leads are also discussed.

Historical Perspective

The epidemiological results are reviewed here under four headings according to the source of exposure; drinking water, occupational exposure, aluminium-containing products and other sources of exposure.

Drinking Water

Most of the epidemiological studies have focused on the association between aluminium in tap water and AD, despite the fact that this source of exposure represents a relatively minor proportion of intake compared with other dietary and non-dietary sources. Less than 5 percent of the daily intake of aluminium has been reported to come from aluminium in public water supplies (Priest et al., 1998). Nevertheless, data on aluminium in drinking water are relatively available and it is widely thought that aluminium in drinking water is more bioavailable, i.e. more readily taken up from the gut into the bloodstream than aluminium from food.

Table 1 summarizes 18 epidemiological studies which have been undertaken. They focused on cognitive impairment (a major predictor for dementia), dementia from all

Table 1. Main epidemiological studies for the last two decades on the link between aluminium in tap water and a neurological disease

Study	Design	Diagnosis	Association
Rondeau, 2000, France	Cohort (2698 subjects), 253 demented, 182 AD	Clinical diagnosis (65 years and over)	+
Moore, 2000, UK	Case-control, 13 AD, 13 controls	Clinical diagnosis (62–76 years)	+
Altmann, 1999	Retrospective study, 55 exposed, 15 non-exposed	Clinical and psychological tests (15–70 years)	+
Martyn, 1997, UK	Case-control, 106 AD, 441 controls	Clinical diagnosis, computerized tomographic records (42–75 years)	−
McLachlan, 1996, Canada	Case-control, 119 AD, 51 controls	Autopsy	+
Forbes, 1996, Canada	Population study (1041) AD	Death certificates (85 and over)	+
Forster, 1995, UK	Case-control, 109 PDAT, 109 controls	Clinical diagnosis (<65 years)	−
Forbes, 1994, Canada	Case-control, 199 cognitive impairment, 286 controls	Psychometric tests (75 and over)	+
Jacqmin-Gadda, 1994, France	Population study (3697), 906 cognitive impairment	Psychometric tests (65 and over)	+ −
Taylor, 1992, UK	Case-control, 20 AD, 20 controls	Clinical diagnosis, (65–89 years)	+
Neri, 1991, Canada	Case-control, 2232 AD or presenile dementia, 2232 controls	Death certificates (55 and over)	+
Wettstein, 1991, Switzerland	Population study (805)	Psychometric tests (81–85 years)	−
Michel, 1991, France	Population study (2792), 66 demented, 51 AD	Clinical diagnosis (65 and over)	+
Frecker, 1991, Canada	Population study (568345), 379 demented	Death certificates	+
Flaten, 1990, Norway	Population study (14727), 586 demented	Death certificates	+
Martyn, 1989, UK	Population study, 1185 demented, 666 AD	Computerised tomographic scanning (40–69 years)	+
Wood, 1988, UK	Population study (386)	Psychometric tests (55 and over)	−
Vogt, 1986, Norway	Population study	Death certificates	+

causes or more specifically dementia from AD. Three kinds of design have been used in these studies.

Ecological studies

Most of the studies were ecological in design in that they studied the relationship between rates of Alzheimer's disease in a geographical region and the concentration of aluminium in drinking water. The most widely publicized has been that of Martyn (1989). Cases of dementia between 40 and 69 years of age and ascertained from the records of seven CT scanning units were studied. Patients with dementia were classified into four

categories according to clinical information (probable AD, possible AD, cerebrovascular dementia and dementia from other causes). Rates of disease were age-standardized to the 40- to 69-year-old population of England and Wales. Mean aluminium concentrations in water supplying the 88 country districts of the study were estimated over the previous 10 years. The results showed a significant association for probable AD with a relative risk equal to 1.5 (1.1–2.2) for high (>0.11 mg/l) as compared to low aluminium concentrations (≤0.01 mg/l). However, there was however no dose–response effect of aluminium.

Furthermore a series of population studies has been done using death certificates (Vogt, 1986, Flaten, 1990, Frecker, 1991, Forbes & McLachlan, 1996). Age-adjusted death rates were calculated on the basis of the total number of death certificates from dementia or AD. These studies also reported a significant relationship between aluminium concentration in drinking water and the occurrence of AD. However, it must be noted that dementia is not always recognized as a distinct disease and recorded on death certificates. This can often lead to an underestimation of mortality rates, and probably to biases if the diagnosis of AD in certain regions is more easily recorded than it is in others.

Two studies from Britain and Switzerland, Wood et al. (1988) and Wettstein et al. (1991), did not find any relationship between impaired cognitive function and aluminium exposure in drinking water. Wettstein compared the degree of mental impairment in old residents (81–85 years) in two parts of a town with different levels of aluminium in their drinking water. The mean scores of the tests (using two simple subtests of the variant Zurick Mini Mental Status Tests) in the low and in the high district were almost identical. The failure to detect any relationship was perhaps due to the fact that only two drinking water sources were investigated and that the highest concentrations of aluminium that were measured in these waters were about 0.1 mg/l. It may be that other water components or other characteristics of the geographical area influenced the relationship between aluminium and AD. It is thus important to include diverse geographical areas in epidemiological studies.

Case-control studies

Several case-control studies have been done. The largest case control study was carried out in the province of Ontario by Neri & Hewitt (1991). They compared 2232 patients aged 55 years and over who had been discharged from an Ontario hospital with a diagnosis of Alzheimer's disease or presenile dementia with the same number of controls matched for age and sex and discharged with a non-psychiatric diagnosis. The results showed a relative risk of 1.46 for AD or presenile dementia for subjects living in areas where the drinking water aluminium concentration was greater than 0.200 mg/l (compared to <0.01 mg/l). More recently, Mclachlan (1996) compared 119 autopsy-verified cases of AD with 51 controls, but focused on the residential histories for estimating aluminium exposure. Using aluminium concentration of public drinking water at last residence before death as the measure of exposure, the odds ratio (OR) for AD to all controls was 1.7 (1.2–2.6). Estimating aluminium exposure from a 10-year weighted residential history resulted in estimates of OR of 2.5 (1.2–5.3) for a cutoff value of 100 μg/l of aluminium.

In contrast, two recent British studies failed to find any relationship between AD and aluminium in drinking water (Forster et al., 1995, Martyn et al., 1997). The study of Forster (1995) with a modest statistical power (109 cases vs 109 controls) did not find any association with aluminium in drinking water related either to a person's main place of residence 10 years before dementia onset or to birthplace. Martyn et al. (1997) also carried out a case-control study in which they assessed exposures from historical measurements of aluminium in water supplies. There was no tendency for an increased risk with aluminium concentration either when aluminium levels were averaged from 25 years to diagnosis, or from age 25 years to diagnosis, from age 25 years to 10 years before diagnosis, over 10 years before diagnosis. The association was in fact in inverse. The inconsistency of these results with those of other studies may be explained not only by methodological problems but also by the fact that the above mentioned studies examined younger subjects (aged 43–75 years), so they examined presenile rather than senile dementia of Alzheimer type.

More recently a retrospective study (Altmann et al., 1999) was undertaken in Camelford (South England) following an incident occurring in 1988 which contaminated the water supplies with 20 tons of aluminium sulphate used in the treatment of drinking water. Three years after the incident, an investigation was conducted to examine 55 self-selected adults (30 women and 25 men, aged 15–70 years, mean 41.8 (2.1) years), who complained of short-term memory loss and impaired concentration. They were compared to 15 siblings nearest in age to one of the group but who had not been exposed to the contaminated water. The results showed that the Camelford participants exposed to aluminium had lower psychomotor performances than their controls. However, several shortcomings in the 'design' of the study must be pointed out. The inquiry examined only a small number of exposed and non-exposed subjects. Moreover, the population was self-selected and was certainly not representative of the population of Camelford. Thus, the results of psychomotor testing were probably influenced and biased by the participants who felt victims of this incident. Nevertheless, the results found on the visual evoked potential test, which is an objective test, are probably resistant to bias caused by litigation.

Cohort studies

A series of papers (Forbes et al., 1991, Forbes & McAiney, 1992, Forbes et al., 1994) presented the results of the Ontario Longitudinal Study of Aging (LSA) whose participants were followed up for about 30 years. Forbes and McAiney (1992) reported an odds ratio of 1.86 ($p<0.01$) for symptoms of cognitive impairment where aluminium concentrations in the drinking water supply at the subject's place of residence were above the 50th centile.

The effect of aluminium in drinking water on the risk of AD was examined in a large prospective cohort (Paquid), including 3,777 subjects aged 65 years and over and living at home in 75 civil parishes in Southwestern France in 1988–1989. The first results (Michel et al., 1991) obtained on 40 prevalent cases of probable AD among a sub-sample of 2792 subjects showed a significant linear association (RR = 4.53 (3.36–6.10), for a difference of 0.1 mg/l between the two values compared). However this was based on retrospective measures of the aluminium concentrations that were not reliable; in particular some of

these measures were old, and sampling and dosage techniques have changed in recent decades. In a second analysis of the Paquid sample, a weighted mean measure of exposure to aluminium was computed using results of two surveys conducted in 1991. To evaluate the past exposure of the subjects, a history of the water distribution network over the previous 10 years (1981–1991) was established. The results (Jacqmin-Gadda et al., 1994) reported an association of baseline cognitive impairment (defined as a score of less than 24 on the Mini-Mental State Examination) with aluminium, pH and calcium (negatively associated). Some of these results were difficult to explain; aluminium was positively associated when the pH was less than 7.3 and negatively associated when the pH was higher. Results obtained on incident cases of AD after 8 years of follow-up were recently published (Rondeau et al., 2000). In this study, additional results of chemical analyses of drinking water carried out by the sanitary administration between 1991 and 1994 were used to evaluate exposure to aluminium. The sample studied included 2,698 non-demented subjects at baseline, for whom components of drinking water and covariates were available. A total of 253 incident cases of dementia (with 17 exposed to high levels of aluminium) including 182 AD (with 13 exposed to high aluminium levels) were identified. The relative risk of dementia adjusted for age, gender, educational level, place of residence and wine consumption was 1.99 (95% CI 1.20–3.28) for subjects exposed to an aluminium concentration greater than 0.1 mg/l. A similar result was found specifically for AD (adjusted relative risk = 2.14, 95% CI 1.21–3.80). However, no dose–response relationship was found.

Other water constituents

There are now more and more studies showing that the relationship between aluminium in drinking water and the risk of AD or cognitive impairment must be examined after adjustment for other water constituents, which could play a role of confounding factor by affecting the neurotoxicity of aluminium. In particular, the effect of silicon in tap water has been studied. Birchall et al., (1989) showed that silicon could reduce the toxicity of aluminium for fish, because hydroxyaluminosilicate compounds prevent the absorption of aluminium. In a study of five men, Edwardson et al. (1993) showed that gastrointestinal absorption of aluminium from orange juice is reduced if sodium silicate is added to the beverage. From these studies, two hypotheses may be proposed. The first was suggested by Birchall and Chappell (1989) who pointed out that the aluminium and silicon concentrations in drinking water generally are negatively correlated. Thus, silicon may be a confounding factor in the statistical association observed between aluminium in drinking water and AD. Taylor et al. (1995) showed a significant inverse correlation between soluble silicon and soluble aluminium ($r = -0.43$, $p < 0.001$). Moreover, silicon from drinking water could be an important part of daily silicon intake and in a form particularly available for association with aluminium. Thus, Birchall and Chappell (1989) suggested that the association between aluminium levels in drinking water and Alzheimer's disease might be a consequence of the protective effect of silicon in water. Silicon in water might protect against the effect of dietary aluminium intake, and not only from aluminium in water. More specifically, Exley and Birchall (1993) suggested that concentrations of silicic acid above 100 μmol/l may prevent the absorption of

aluminium from the gastrointestinal tract and facilitate its excretion by the kidney. Recent epidemiological results on the Paquid cohort (Rondeau et al., 2000) after 8-years of follow-up have shown that high silica levels ($\geq$11.25 mg/l) are associated with a lower risk of dementia and Alzheimer's disease (adjusted relative risk = 0.74, 95% CI 0.58, 0.96 and respectively, adjusted relative risk = 0.73, 95% CI 0.55, 0.99). These results are concordant with the hypothesis of Birchall and Chappell (1989). If this assumption is true, the exact risk attributable to aluminium is probably underestimated in the Paquid cohort, which does not consider total daily aluminium intake (which is difficult to measure). However, results obtained by Martyn et al. (1997) on a case-control study did not support a protective role of silicon.

In addition, several investigators have reported that the pH of drinking water could affect the solubility of aluminium components and the type of aluminium containing species that are formed. It is plausible that the biological availability of aluminium is higher for low than for high pH, which could lead to an interaction between pH and aluminium. Forbes et al. (1994) found that at neutral pH, relatively low aluminium concentration and relatively high fluoride concentration decreased the odds of exhibiting cognitive impairment by a factor of about five, compared with other types of drinking water. Iron concentrations are also of interest because compounds like transferrin are thought to be involved in the transport of aluminium to the brain (Farrar et al., 1990) and iron and aluminium compete for binding with these molecules. In a multivariate analysis Forbes et al. (1997) found a relationship between the level of iron in tap water and the risk of AD reported on death certificates for persons over the age of 85.

Occupational Exposure

Workers in refineries or smelters may be exposed to aluminium in the form of dusts, fumes and/or skin contact. Benke et al. (1998) reviewed the different specific chemical exposures and exposure assessment methods relating to epidemiological studies in the aluminium industry.

Since it has been shown that aluminium dust may reach the central nervous system via the nasal olfactory pathway (Perl & Good, 1987), several epidemiological studies have been undertaken to examine the potential relationship between aluminium exposure in the workplace and the risk of neurological disorders. The neurotoxic effect of aluminium with occupational exposure began with a fatal case of encephalopathy associated with inhalation of aluminium dust (McLaughlin et al., 1962). Then, chronic exposure to aluminium and its possible neurotoxicity were reported in a study of 607 Canadian gold miners exposed to McIntyre aluminium powder used as a prophylactic agent against silicosis (Rifat et al., 1990). The exposed miners were almost two times more cognitively impaired than the unexposed miners. The length of treatment ranged from 6 months to 36 years and the proportion with impaired cognitive function increased progressively with duration of treatment.

This topic is reviewed by several papers (Doll 1993, McLachlan 1995, Flaten 1996, Kilburn 1999) and no clear-cut trends have yet emerged. Most of the epidemiological studies have failed to establish clear exposure–response relationships between specific

workplace chemicals and neurological disorders. Akila et al. (1999) reported cognitive impairment in aluminium welders in Finland that seemed proportional to urine concentrations of aluminium. Nevertheless, Letzel et al. (2000) did not find any cognitive decline after a chronic exposure to aluminium dust in workers of a powder-producing plant. No evidence was obtained that a long-term aluminium exposure in the workplace led to dementia and AD. Three recent case-control studies did not find any association between workers in contact with aluminium dust and fumes, and AD (Salib & Hillier, 1996, Gun et al., 1997, Graves et al., 1998).

The inconsistency in these data may be due to differences in the ways exposure is measured. Namely, subjects are often exposed to several potential toxicants other than aluminium, making it difficult to identify the role of aluminium in the occupational environment. Furthermore, most of the studies are based on a small number of subjects.

Aluminium-Containing Products

The regular ingestion of aluminium-containing antacids represents a major source of exposure to aluminium. However, very few studies have related their use to the development of AD. Two case-control studies found a significant association (Graves et al., 1990, Forster et al., 1995). The latter study obtained an Odds Ratio equal to 1.6 (0.77–3.51) for prolonged (6 months) antacid users. The results of Graves et al. (1990) were surprising; in a case-control study of 130 matched pairs, the adjusted OR for AD was 3.1 (95% CI 1.3–7.5) when considering all antacids, and a dose–response gradient was found. However, when only aluminium-containing antacids were analyzed, the overall adjusted OR was only 0.7 (95% CI 0.3–2.0). The data of a long-term mortality follow-up study of 9928 patients did not suggest that these patients, who may be presumed to have ingested large amounts of antacids containing aluminium, are of greater risk of AD (Colin-Jones, 1989). More recently, Roberts et al., 1998, showed that serum and urine aluminium concentrations were significantly raised for the patients aged 39–70 years with peptic ulcer/dyspepsia on regular aluminium hydroxide therapy for at least 6 months compared with healthy volunteers aged 30–65 years. Similar results were obtained on demented patients. The data also showed that the silicon concentrations were no different in the sera of the control and treated groups, but were increased in the urine of the treated group. This suggests that an increased absorption of silicon may be associated with a considerably increased renal excretion of both silicon and aluminium.

Dialysis encephalopathy is one of the main observations in favour of the neurotoxicity of aluminium. Alfrey et al. (1976) measured aluminium levels in bone, muscle and brain in 10 control subjects, 9 uremic patients and 12 uremic patients with encephalopathy syndrome. All dialysed patients had received oral aluminium-hydroxide gels to control phosphate levels. High levels of aluminium were found in tissues and particularly in the grey matter of the brain of patients dying from this syndrome. In this study the aluminium content of the water used for the preparation of the dialysate was repeatedly measured and was negligible. However, other studies (McDermott & Smith, 1978, Flendrig et al., 1976) have strongly implicated the high concentration of aluminium in tap waters used to prepare the dialysate and which can pass into the bloodstream. Perazella & Brown (1993)

reported a case of a patient with renal failure following a bone marrow transplantation who developed an acute encephalopathy from apparent aluminium intoxication following intravesical aluminium. Dialysis encephalopathy has become relatively rare. However Nakamura et al. (2000) recently described a case with acute encephalopathy successfully treated with the combined use of deferoxamine and haemodialysis. The symptoms associated with acute aluminium toxicity appear to be reversible with deferoxamine, which is recognized as an effective chelator of aluminium (Day & Ackrill, 1993).

Aluminium salts are the major constituent of many widely used antiperspirant products. The possibility that antiperspirant aluminium might exert an effect on health should not be ignored (Exley, 1998). Experiments on mice have shown that aluminium salt could pass the skin barrier, and the transdermal uptake of aluminium resulted in the accumulation of aluminium in the hippocampus of the mouse brain (Anane et al., 1995). However, in the epidemiological field, little consideration has been given to the effect of use of antiperspirants with aluminium and the risk of AD. To our knowledge, only one epidemiological study has revealed an increased risk of AD for subjects using aluminium-containing antiperspirants (Graves, 1990). The effect of aluminium in antiperspirants is developed in another chapter of this book by Flarend.

Other Exposures

Epidemiological studies on aluminium exposure from food are uncommon, probably due to the difficulties of measuring such exposure. In a recent preliminary case-control study (23 AD cases versus 23 matched controls by age, gender and date of admission to the center), Rogers and Simon (1999) examined the association between consumption of food containing aluminium additives and the risk of Alzheimer's disease. Next-of-kin for patients with AD and controls completed an interview on the subject's medical history, lifestyle habits and dietary history (using the Health Habits and History Questionnaire), so the use of aluminium-containing food could be ascertained over a five-year period preceding the diagnosis for cases and the same period for controls. Consumption of food with a high aluminium content, at least once per day, yielded a crude OR of 2.0 and an adjusted OR of 8.6, but this association was not significant ($p = 0.19$). No clear association was found with the consumption of tea, although tea infusions are a potentially important source of dietary aluminium. In a case-control study Forster et al. (1995) study found an elevated odds ratio ($OR = 1.4$) with tea consumption (>4 cups/day), but this association was not significant.

Significant amounts of aluminium may also be supplied from aluminium cooking utensils, although this exposure has attracted less attention. The bioavailability of aluminium in cookware is unclear, even if it is known that such utensils may release aluminium when used to cook acidic food (Firmreite et al., 1997, Rajwanshi, 1997b, Lin et al., 1997). Aluminium might be more bioavailable when dissolved in water than in foodstuffs. In a prospective study (Rondeau et al., 2000), no influence was found with the use of aluminium cookware on the risk of dementia ($RR = 1.04$, $p = 0.86$).

A further link between aluminium and neurodegenerative disorders has been found in areas of the Western Pacific such as the island of Guam, which has unusual soils with

high amounts of aluminium and low amounts of magnesium and calcium. The inhabitants are particularly prone to amyotrophic lateral sclerosis or Parkinsonism with dementia and in either case the brains of affected subjects show neurofibrillary tangles like those of AD (Garruto & Yase 1986). This topic is reviewed in another chapter by Perl.

Informed Opinion

A Plausible Hypothesis

Based on what we know about Alzheimer's disease and on the numerous studies about a possible link between Alzheimer's disease and exposure to aluminium, a plausible hypothesis may be formulated. Alzheimer's disease is a complex disease involving a long causal pathway with multiple stages and probably multiple ramifications (Cummings 1998). It is known that several genetic mutations at several loci (mutations on presenilins on chromosomes 1 and 14, mutations of the APP gene on chromosme 21) are linked with the onset of Alzheimer's disease with a high probability, and that the presence of the E4 allele on the APOE gene (on chromosome 19) is linked with an increased incidence of the disease. Moreover, other susceptibility genes may remain to be discovered. Epidemiological studies have also revealed that several factors like age, gender, educational level, consumption of wine or tobacco modify the risk of development of the disease. It is quite likely that other factors, and in particular environmental factors (including nutritional, see Commenges et al., 2000), play a role at some point on this complex pathway, accelerating or slowing the evolution of the pathological process. It is also quite likely that a brain which has suffered stress of any nature will be more frail and less resistant to the development of the disease. Oxidative stress certainly plays a role in the ageing process and thus directly or indirectly in Alzheimer's disease (Ceballos-Picot 1997). Aluminium in the brain may increase the oxidative stress (Bondy et al., 1998, Savory et al., 1999, Christen, 2000) or may have a specific toxic effect, or both, which may pave the way to the disease. So, without playing a key role in Alzheimer's disease, aluminium when present in the brain may accelerate the pathological process, as suggested by McLachlan (1995). If a specific exposure of subjects to aluminium leads to increased levels of aluminium in the brain, this exposure will appear as a risk factor for AD. It is likely that a small amount of aluminium accumulates in the brain with age, so aluminium is likely to play a more important role in late-onset subjects than in early-onset ones. To penetrate the brain, it must first be present in some form in the blood and then cross the blood–brain barrier (BBB). It is likely that a very small part of Al in the blood enters the brain while most is excreted (some part will also be accumulated by other organs) (Yokel et al., 1999). Aluminium may normally enter the blood either through the gastro-intestinal tract (Berthon, 1996, Powell et al., 1999, Greger & Sutherland, 1997), the respiratory tract or across the skin (Exley et al., 1996). In dialysis it may enter the blood more directly through the membrane. The main problem here is that of absorption of aluminium from non-systemic to systemic sites such as the blood. How much of Al will really enter the blood from a given exposure? It is known for instance that bioavailability is increased in the presence of citrate; conversely silicon decreases

gastrointestinal absorption (Edwardson et al., 1993, Belles et al., 1998, Jugdaohsingh et al., 2000). Another even more difficult question is whether the form in which Al enters the blood is different from one exposure to another, because some forms may be more prone to be excreted and others are more likely to cross the blood–brain barrier (here transferrin may play a crucial role in transporting Al) (Harris et al., 1996, Rollin & Nogueria, 1997, Van Landeghem et al., 1998). Thus it is plausible that there are several possible types of exposure to Al which may be just one risk factor of AD, among many others.

What Do Epidemiological Studies Tell Us about this Hypothesis?

If it is admitted that Al in the brain accelerates the pathological process of Alzheimer's disease, it would then be useful to show that certain types of exposure increase the concentration in the blood of a form of Al able to cross the BBB. Unfortunately we know little about the metabolism of Al and this approach will take time to reach any conclusion. Another way is to directly study whether the risk of developing AD is increased for people having a given exposure. This is the classical epidemiological approach (in a certain number of studies AD has been replaced by a proxy, cognitive impairment). Although this precludes studying the very complex intermediary steps mediating the effect of the given exposure at least in the first stage, this approach also presents huge difficulties, especially when the magnitude of the effect is small. For instance, the expected RR in our context are of the order of 2, as opposed to 20 for the effect of tobacco consumption on lung cancer.

The most solid results are obtained with dialysis encephalopathy. Dialysis encephalopathy is not AD but is a 'model' in that when a large quantity of Al comes into the blood from water, then part of it may cross the BBB and cause lesions which have some similarities with AD lesions (Alfrey et al., 1976). In this case, the relatively large quantities of Al reaching the brain are responsible for a disease which is not exactly like AD. When exposed to drinking water with a high concentration of Al, a much lower quantity of Al is able to cross the BBB, the effect of which is simply to accelerate an Alzheimer pathological process which may have already started.

As for more conventional epidemiological studies trying to find a direct association between an exposure to Al and AD, the results are highly controversial, whatever the type of exposure. These inconsistencies may first be explained by the methodological problems which are common to all epidemiological studies. Of course one must not forget that chance may play a major role: the confidence intervals of the effects of Al are often large. Perhaps the most important source of bias comes from the selection of subjects, since most studies are ecological studies or case-control designs. For instance, subjects are selected because they have had a CT-scan while only a small fraction of AD have this examination (Martyn et al., 1989, 1997), or because they have donated their brain (McLachlan et al., 1996). The case-control study of Martyn et al. (1997) has the defect that cases and controls had a CT-scan in the same neurological center and thus were likely to have similar recent exposure to aluminium in drinking water. There is only one cohort study on this topic in which subjects were regularly followed (Rondeau et al.,

2000). Cohort studies are not completely free of selection bias which comes back (in an attenuated form) through missing data: during a follow-up many people die or quit the study. Another aspect linked to selection is the choice of the population, particularly with respect to age distribution. Most studies starting from clinical cases concern rather early onset AD: mean age 64 for Martyn et al. (1997), age below 65 for Forster et al. (1995) while the mean age of onset of AD in a population is about 80 (Rondeau et al., 2000). This may be important since genetic factors might be more important for early onset AD while toxicity of Al is a result of an accumulation which may be expressed at a later age.

Other biases may come from misclassification errors (subjects erroneously classified as AD or non-AD), measurement errors of exposure, and confounding factors. The analysis may also be complicated by correlation of the data. There is no room here to detail all these sources of bias. We will rather discuss the important issue of exposure measurement. The simplest model of measurement error is as follows. What is measured is the value of the true exposure plus an independent error. This type of error is not very severe in principle and simply leads to a loss of power and an attenuation of the effect. However, even in this case, the power of the test will decrease as the correlation between the available measurement and the effective exposure decreases, and will become quite low if this correlation is low. For instance, in our case the value of the effective exposure is the mean concentration (during a period of time) of a certain form (prone to cross the BBB) of Al in the blood. The contribution to the mean concentration from one oral intake, for instance can be obtained as a product of consumption c, bioavailability b, and a coefficient f which represents the fraction of Al in the blood which is prone to cross the BBB. The effective mean concentration is the sum of such products for all types of exposure; b and f are mainly unknown and may vary from one subject to another while c is measured with error, i.e. we measure $c' = c + e$, where e is a measurement error. The risk of AD may depend rather strongly on the effective mean concentration but very weakly on one particular c' (a measurement of a particular consumption). There are so many unknowns in such a model that it is useless in practice; however, it does help to explain why we cannot expect strong effects of particular exposures to Al on AD. However, the reverse of this argument is that if we do find some effect, the true effect is probably larger than what we observed. For instance, Rondeau et al. (2000) estimated the relative risk equal to 2 for subjects exposed to water containing more than 0.1 mg/l versus non-exposed subjects. The first thing is to establish that the true relative risk is really different from 1. If the time relative risk with these data is around 2, then if we could measure the individual exposure to bioavailable species more reliably, the relative risk would be higher.

Another problem is the shape of the effect as a function of the intensity of exposure. For instance, for drinking water several studies (McLachlan et al., 1996, Rondeau et al., 2000) suggest that there is no effect below 0.1 mg/l. In some countries only a small fraction of the population is exposed to this concentration, so misspecifying the shape of the effect (e.g. by taking a threshold at 0.03 mg/l) would completely dilute the effect. In conclusion, the results of previous epidemiological studies are not unexpectedly controversial.

Need for Future Studies

There is a need of further studies in order to reach reliable conclusions concerning the role of Al exposure in AD. These studies should be cohort studies. Such studies must ensure that a sufficiently large fraction of the sample has a high level of exposure (e.g. drinking a water with more than 0.1 mg/l). The only problem is that such studies are long and costly. Such studies should focus on those most at risk in the population, e.g. older than 70 or 75. There are two reasons for this; there would be more cases in a given period of time and the effect of Al would probably be larger in old people. The authors of this paper have already begun such a study (called ALMA+) as a prolongation of their first work (Rondeau et al., 2000). There is also the tantalizing prospect of studying the metabolism of Al and its possible genetic variability. This variability could concern transferrin for instance, which plays a key role in the transport of Al (Farrar et al., 1990, Van Landeghem et al., 1998). Gene-environment interactions should also be studied. It may be that only a fraction of the population is susceptible to Al toxicity. This is part beyond the scope of epidemiology. However in such a complex context, epidemiologists and biologists should work together and multidisciplinary teams are likely to delve more deeply into the possible mechanisms leading from exposure to Al to development of AD.

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Aluminium and Alzheimer's Disease
The Science that Describes the Link
C. Exley (Editor)

CHAPTER 4

Absorption of Aluminum from Antiperspirants and Vaccine Adjuvants

Richard Flarend *

Division of Mathematics and Natural Science,
The Pennsylvania State University–Altoona College, 3000 Ivyside Park,
Altoona, PA 16601, USA

* Corresponding author, email: ref7@psu.edu

Abbreviations: ACH – aluminum chlorohydrate; AD – Alzheimer's disease; Al – aluminum; AMS – accelerator mass spectrometry; AZH – aluminum–zirconium salt; BBB – blood brain barrier; DTP – diphtheria, tetanus, pertusis; FDA – Food and Drug Administration; GI – gastrointestinal; Hep B – hepatitis B; ICRP – International Commission on Radiation Protection; IP – intraperitoneally; IV – intravenously; MMF – macrophagic myofasciitis; Td – tetanus

Summary

This review discusses the exposure to aluminum from antiperspirants and vaccine adjuvants and whether that exposure is likely to be a significant factor in the development of Alzheimer's disease. Information is given about the bioavailability, metabolism, and retention of aluminum from these sources and from other common sources of aluminum. Emphasis has been placed on research utilizing the radioisotope aluminum-26. From this information, general estimates are presented which compare the body's exposure to aluminum and the aluminum load in the brain from antiperspirants and vaccine adjuvants to that originating from dietary sources of aluminum.

Historical Perspective

The availability and metabolism of aluminum is not understood as well as other metals, especially at physiological concentrations in the body. This is partially because there was no suitable tracer isotope to study the absorption and metabolism of aluminum until the development of accelerator mass spectrometry (AMS) allowed the use of ^{26}Al (Flarend & Elmore, 1997). Since this new technique has been developed, gallium-67 has now been shown to be an insufficient analog for aluminum (Priest et al., 1995). Research utilizing stable aluminum ^{27}Al is of limited use for quantitative research because of the unrealistically high doses or exposures to aluminum required or due to the limited analytical ability to detect aluminum following a physiological exposure.

Since 1990, a variety of studies have been published using ^{26}Al and AMS. Two reviews, published in 1994 and 1997, summarize the ^{26}Al research up to those times (Day et al., 1994; Flarend & Elmore, 1997). There is no natural background of ^{26}Al. Aluminum-26 can be detected using AMS at extremely low concentrations (about 10 attomole/g or 1 part in 10^{16}). Since the detection limit for ^{26}Al is so low, such small amounts can be used so that there are virtually no radiation risks. And its long life-time does not limit the duration of its use or detection. All of these factors combine to make ^{26}Al a rather ideal radiotracer when used with AMS. It is now possible to measure the metabolism of physiological concentrations of aluminum throughout the body, pharmacokinetics of aluminum-containing drugs, and even transport of aluminum at the cellular level.

But even with this powerful tool for studying the biology of aluminum, there is little support for aluminum research today. It has even been suggested that this lack of sufficient support for research on aluminum in biology may be due to the controversy of aluminum and Alzheimer's disease (Flaten et al., 1996). Hence, the most significant problem with the research on the biology of aluminum using ^{26}Al, is that most studies have not been conducted with large subject pools or reproduced by independent research groups. This is not to say that the data is questionable, just that most studies have not been attempted by other research groups because there are only a handful of scientists who use this isotope and they do so with little support.

Sources of Aluminum

In order to understand the potential impact from the exposure to aluminum from antiperspirants and vaccine adjuvants, one must also consider the daily exposure to aluminum from other common sources.

The intake of aluminum from the diet has been studied by the FDA in an ongoing total diet study (TDS). The results of the TDS have been published in 1989 (Pennington & Jones, 1989) and more recently in 1995 (Pennington & Schoen, 1995). Both of these publications give the aluminum content of many varieties of food and also a breakdown of aluminum intake by age and gender. The typical amount of aluminum ingested by adult males is about 10 mg Al/day, 7 mg Al/day for adult females and children, and 0.7 mg Al/day for 6–11 month infants (Pennington & Schoen, 1995). A study of newborn and premature infants found that the intake from milk and formulas for preterm infants is about 0.03 mg Al/day and about 0.04 mg Al/day respectively (Bougle et al., 1992). Table 1 contains typical values of aluminum intake for various age groups.

The aluminum content of foods vary greatly, but only 7% of the foods studied by Pennington & Schoen contained greater than 1 mg Al/serving. Of these 7%, most were over 1 mg Al/serving due to aluminum-containing food additives such as baking powder, emulsifying agents, and anti-caking agents (Pennington & Schoen, 1995).

Drinking water can contain a large range of aluminum, from less than 0.014 mg Al/L to 2.7 mg Al/L, although the median amount is about 0.02–0.03 mg Al/L based on the locale (Miller et al., 1984). Thus, even in the rare locality with the highest aluminum concentration, drinking water would account for just 1/3 of the aluminum absorbed from

Table 1. Aluminum intake by age group

Age group	Mean aluminum intake
Premature infants	0.03 mg Al/day
0–3 months	0.04 mg
6–11 months	0.7 mg
Children (2 years old)	5 mg
Children (6+ years)	7 mg
Adult females	7 mg
Adult males	10 mg

food and water. In localities with a median concentration of aluminum in water, the water adds a negligible amount of aluminum to the diet. To date, the bioavailability or metabolism of aluminum from food has not been compared to that from water.

Ingestion of aluminum by infants from human breast milk is very small as it contains 0.02 mg Al/L (about the same as typical drinking water). Formulas based on cow's milk contain around 0.2 mg Al/L, and soya-based formulas for lactose intolerance are fairly high in aluminum at 0.5 to 1 mg Al/L (Coni et al., 1993; Fernandez-Lorenzo et al., 1999).

Some common oral pharmaceutical products contain aluminum such as some calcium supplements (12 mg Al/day), buffered aspirin (10–20 mg Al/tablet), and aluminum-containing antacids (50 mg Al/tablet) (Greger & Sutherland, 1997; Pennington & Schoen, 1995). Much effort is being taken to reduce the aluminum content of the above products or to switch to alternative products which contain less aluminum.

Metabolism of Aluminum

Most ingested aluminum is eliminated in the feces within several days (Priest et al., 1996). The remaining aluminum which then enters the blood via the intestinal system is then eliminated in urine.

From a study using an intravenous (IV) injection of aluminum and gallium citrates, it was found that 85% of the aluminum was removed in two weeks (Priest et al., 1995). The amount of aluminum which is not eliminated in urine is then retained in the body by deposition in tissue. This deposited aluminum is then removed very slowly by the body over several years as seen in Fig. 1. This study also showed that the metabolism of aluminum and gallium are quite different and that the retention of aluminum is very different from the exponential function proposed by the ICRP (ICRP, 1981).

The intestinal uptake of aluminum, F (see Eq. 1), seems to vary with the conditions and form of aluminum which is ingested. A range from 0.01% to 1% has been observed in adults (Edwardson et al., 1993; Hohl et al., 1994; Day et al., 1991). However, many of the techniques used with ^{26}Al to establish this range have underestimated the absorption rate due to the calculation methods used (Priest et al., 1996; Flarend & Elmore, 1997). In addition to the range noted above, it is also known that different chemical species of aluminum can differ in their bioavailability by more than an order of magnitude (Priest et al., 1996). Although there is not direct evidence specifically for aluminum, it is suspected

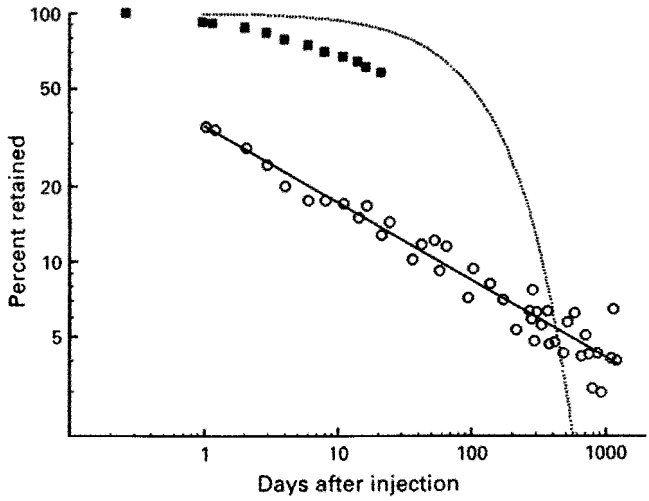

Fig. 1. Long-term whole-body retention of aluminum (○) and gallium (■) injected intravenously as a citrate. Broken line represents the retention of aluminum proposed by the ICRP. Reprinted with permission of *Human & Experimental Toxicology*.

that, like other metals, the absorption of aluminum in infants is greater than the typical 0.1% observed in adults (Barton, 1987).

$$F = \frac{\text{Al absorbed}}{\text{Al ingested}} \tag{1}$$

Several studies have found that between 2×10^{-4} % and 5×10^{-5} % of the ^{26}Al ingested by rats is accumulated in the brain after 2 to 30 days (Fink et al., 1994; Jouhanneau et al., 1997; Drueke et al., 1997). When this is compared to the typical gastrointestinal uptake for aluminum of 0.1%, it can be approximated that the uptake fraction across the blood–brain barrier from an internal dose of aluminum is about 0.1%. This 0.1% is also consistent with the amounts found in rat brains several weeks after exposure to an intraperitoneally (IP) or IV injection of aluminum (Walker et al., 1994; Kobayashi et al., 1990).

Use of Vaccine Adjuvants

A vaccine adjuvant is any material which is incorporated with a vaccine for the purpose of enhancing the immune response of the vaccine. An adjuvanted vaccine will tend to have a higher, earlier, and longer-lasting immune response than a non-adjuvanted vaccine. But the actual mechanism by which adjuvants enhance the immune response of a vaccine is unknown. Vaccines containing whole cell toxins as antigens tend not to require a separate adjuvant because the whole cell toxin acts as its own adjuvant. However, newer vaccines containing partial cell toxins or synthetic toxins are greatly enhanced with the use of a separate adjuvant (Gupta & Siber, 1995).

Until recently, the only adjuvants approved for human use were aluminum salts generically referred to as alum. Although these aluminum adjuvants are given a variety of generic and/or trade-names, they are either aluminum oxyhydroxide (commonly called aluminum hydroxide adjuvant) or aluminum hydroxyphosphate (commonly called aluminum phosphate adjuvant) (Shirodkar et al., 1990). Aluminum hydroxide and aluminum phosphate adjuvants have different physical and chemical properties which makes each useful for different vaccine formulations (Gupta & Siber, 1995).

Recently, a non-aluminum containing vaccine adjuvant, MF59 was approved for human use in Europe for one particular vaccine. It is likely that other vaccine adjuvants not containing aluminum will also be approved in the coming years (Singh & O'Hagan, 1999). However, it is important to note that adjuvants are not approved for human use independently, but rather each vaccine formulation (antigen *and* adjuvant) is approved together. Thus, as new aluminum-free vaccine formulations are tested and approved, currently approved aluminum-containing vaccine formulations are unaffected. For a currently used aluminum-containing vaccine formulation to be switched to a new non-aluminum adjuvant, the new vaccine formulation would have to undergo regular clinical and approval procedures as an entirely new vaccine. This is so costly and time consuming that current adjuvanted vaccines are unlikely to be reformulated with other adjuvants just to remove aluminum from the formula.

Commonly used aluminum-adjuvanted vaccines include: diphtheria, tetanus, hepatitis, Hib (Haemophilus influenzae type B), lyme, rabies, and anthrax (Baylor, 2000). The FDA legal limit for aluminum in a single vaccination is 0.85 mg, however most vaccines contain less than this upper limit. Also vaccine combinations such as DTP (diphtheria, tetanus, pertusis) can have 0.85 mg Al for each vaccine component. Table 2 shows the common vaccines administered, age of administration, and range of aluminum content of available vaccines (Baylor, 2000).

Known side-effects from aluminum adjuvants include short-term effects such as swelling, inflammation, and the formation of granulomas (Vogel, 1998). Recently, there has been a controversial finding that macrophagic myofasciitis (MMF) (Gherardi et al., 1998), a rare muscle disorder, is associated with aluminum-containing adjuvants. However the causal relationship between adjuvants and MMF is a matter of spirited discussion which is just now beginning (Workshop on Aluminum in Vaccines, 2000). It must be

Table 2. Age, vaccine, range of aluminum content

Age	Vaccine	Range of aluminum content	
		min. (mg)	max. (mg)
Birth	Hep B	0.68	0.75
1 month	Hep B	0.68	0.75
2 months	DTP, Hib	0.51	3.00
4 months	DTP, Hib	0.51	3.00
6 months	Hep B, DTP, Hib	1.19	3.75
18 months	DTP	0.51	2.55
5 yrs	DTP	1.53	4.56
Every 10 years	Td	0.28	0.85

stressed that this finding is preliminary and, as yet, has not been corroborated by other independent researchers. In general, all adjuvants must be viewed from a risk–benefit perspective since it is believed that many of the side-effects caused by aluminum adjuvants are directly related to the immunogenicity of the adjuvant itself due to some irritation mechanism related to the irritating side-effects (Workshop on Aluminum in Vaccines, 2000). Thus an adjuvant with no side-effects may also not be effective. Furthermore, adjuvanted vaccines save countless lives every year from fatal infectious diseases and this life-saving benefit clearly outweighs the risk of rare non-life threatening side affects caused by the adjuvanted vaccine.

Biological Fate of Injected Vaccine Adjuvants

There are only two known studies which have been published to date concerning the fate of aluminum injected as vaccine adjuvants. This dearth of information will probably be filled very soon because the preliminary findings relating MMF and aluminum adjuvants is spawning new research in this area. One of the published studies looked at aluminum in blood and brain tissue of control and study mice following an intraperitoneal (IP) injection of aluminum hydroxide-adjuvanted vaccines (Redhead et al., 1992). This study used young mice which were divided into groups and then part of each group was sacrificed each day for 5 days. The study found that there was no measurable increase in blood aluminum concentrations after the IP injection, but that there was a slight increase in brain aluminum content (Fig. 2) which returned to normal after 3 days.

Another study, which was more quantitative, utilized aluminum hydroxide and aluminum phosphate adjuvants labeled with ^{26}Al to study the metabolism of these vaccine adjuvants (Flarend et al., 1997). Since ^{26}Al does not exist in nature, its use eliminates many concerns about levels of significance in measured results (e.g. any ^{26}Al measured in blood or brain must have originated from the adjuvant). In this study, rabbits (2 per group)

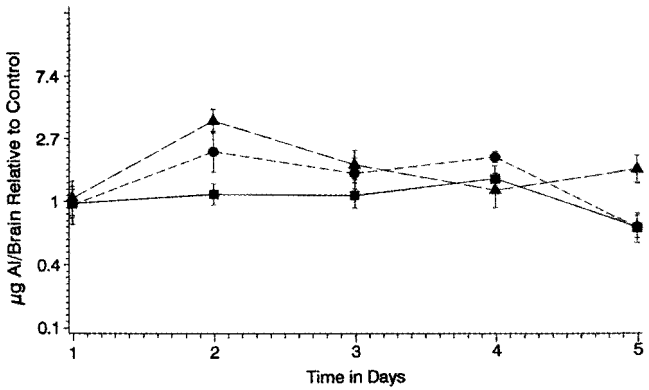

Fig. 2. Transient rise in aluminum concentration within the brain after IP injection of aluminum-adjuvanted vaccine. ■, saline control; •, aluminum adjuvanted DT vaccine; ▲, aluminum adjuvanted DTP vaccine. Reprinted with permission of *Pharmacology & Toxicology*.

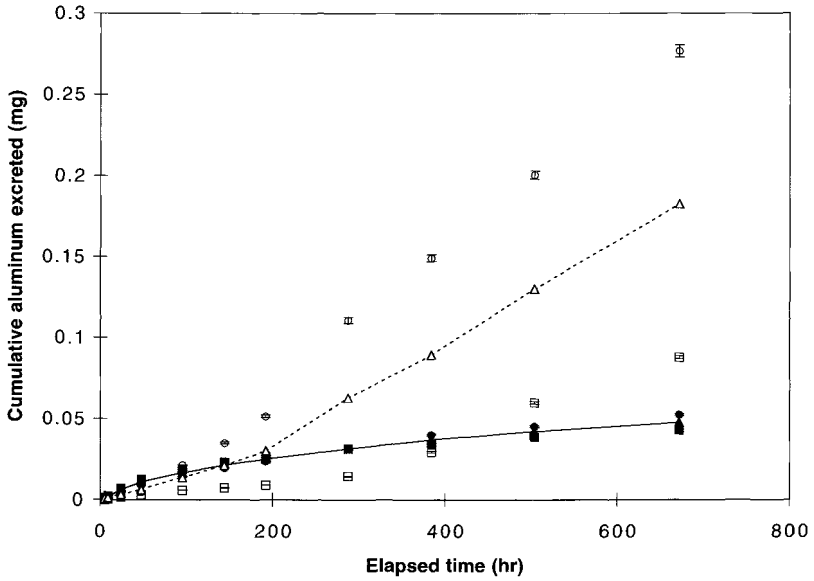

Fig. 3. Cumulative amount of aluminum eliminated in urine following injection of ^{26}Al-labeled vaccine adjuvants. Dashed line (hollow symbols) are aluminum phosphate adjuvant, solid line (solid symbols) are aluminum hydroxide adjuvant.

were given intramuscular (IM) injections of adjuvant only (no toxoid was incorporated with the adjuvant) containing 0.85 mg aluminum. Blood and urine samples were then analyzed for 28 days while tissue samples were collected at 28 days. This study found that the aluminum adjuvants began to be mobilized by the body and transported within the bloodstream within just one hour after injection. However the study also showed that the dissolution rate of both adjuvants was very slow and that it would take greater than 28 days for the adjuvants to be dissolved. This is clear in Fig. 3 which shows the amount of aluminum (^{26}Al) recovered in urine from the rabbits. Fig. 3 shows that there is a relatively steady elimination of, and therefore dissolution of, the aluminum adjuvants over 28 days, and that the dissolution is still continuing at 28 days. Since most aluminum in the body is quickly eliminated by the kidneys, the urinary excretion of aluminum is very similar to and provides the best measure of the rate at which the adjuvants are dissolved.

After 28 days, urinary data indicates that 6% of the aluminum hydroxide adjuvant was dissolved and 22% of the aluminum phosphate adjuvant was dissolved. If the dissolution of the adjuvants is assumed to continue at a constant rate, this would predict that the aluminum hydroxide adjuvant will be dissolved over 18 months and the aluminum phosphate adjuvant will be dissolved over 4 months. Tissue measurements indicate that very little aluminum (<1 ng) from the adjuvants was incorporated into the brains of the rabbits at 28 days.

Use of Antiperspirants

Antiperspirants and deodorants are widely used by over 90% of the US and 85% of the UK populations. This discussion will focus solely on the aluminum-containing antiperspirants, since deodorants do not contain aluminum compounds. The first antiperspirant, based on aluminum chloride, was introduced in 1903 (Laden, 1999). Since this first product, all successful antiperspirants have contained aluminum salts. The aluminum salt in antiperspirants is applied in a soluble form and the aluminum then precipitates inside the sweat duct to temporarily block the sweat duct. Depending on the level of physical activity, this sweat-blocking effect can last several weeks after the application of the antiperspirant.

Aerosol varieties contain aluminum chlorohydrate (ACH), whereas most non-aerosol varieties contain an aluminum–zirconium salt (AZH). AZH formulations are banned from aerosols due to the formation of zirconium particulates in the lungs (Food and Drug Administration, 1977). The amount of aluminum which is used for a single application varies widely but a typical amount is about 10 mg Al (based on author's unpublished data). The type of application and vehicle used also varies greatly. In 1970, 80% of users preferred aerosols. But in the U.S. today, only 16% of users prefer aerosols and 70% prefer a stick or roll-on type application. In other countries, the popularity of aerosols is much greater (e.g. over 80% in the UK) (Laden, 1999).

Epidemiological Evidence Relating Alzheimer's Disease and Antiperspirants

A study on the relationship between Alzheimer's disease (AD) and antiperspirant use was conducted by Graves et al. (1990). In this study a slight correlation between AD and any use of antiperspirants was found with an odds ratio of 1.6. This study defined 'any use' as at least one use per month for the year prior to AD onset. In further analysis, the case and control subjects were split into subgroups of varying frequency (none, low, moderate, high) of antiperspirant use. There was a significant trend between these odds ratios and the frequency of use, and the odds ratios for each subgroup were all >1. However, the odds ratios for these subgroups were not significant.

The data used in this study were collected, via telephone, from case and control surrogates which were usually the spouses of the case and control subjects. However, in order to create the antiperspirant groupings, data on frequency of use, duration of use, and brand name of most commonly used product were collected from the surrogates. Since many surrogates could not provide all this information, half of the case-control subjects could not be grouped for the antiperspirant analysis. As a result, the large number of rejected case-control subjects may have had a negative impact on the significance of the results. In addition, some of the control subjects were questioned directly about the frequency, duration, and brand name of underarm products in order to correlate their responses to the responses from the control subject surrogates. Since there was a non-significant association between dose and observance of AD, the subgroup antiperspirant analyses are in question (Graves et al., 1990)

Graves attempted a more sophisticated follow-up study in which the actual products

used and type of application were determined using pictures of the various products, but the statistical power was low for that study and the results have not yet been published. (Graves, 2000).

A more recent epidemiological study performed by The Canadian Study of Health and Aging found an insignificant risk factor for AD from the use of any antiperspirant or deodorant or from the use of antiperspirants only (Canadian Study of Health and Aging, 1994). Both of these odds ratios were 1.33 which was not significant. However, it is interesting to note that when only institutionalized cases and controls were analyzed, the odds ratio jumped to 3.03 for any antiperspirant or deodorant use and 4.81 for antiperspirant use only, a much more significant odds ratio. This marked increase for the institutionalized group may just be an anomaly in the data, or it could be related to a better understanding of exactly which products were used by the case and control subjects who were institutionalized. The latter hypothesis would further reinforce the notion from the study by Graves et al. that epidemiological studies attempting to relate AD and antiperspirant use must clearly identify the products being used and the methods of application.

Absorption of Aluminum from Antiperspirants

Despite the popularity and long history of safe antiperspirant use, very little is known about the absorption of aluminum from antiperspirants. There are four routes of absorption which need to be considered (Exley, 1998). All antiperspirant formulations could be absorbed dermally. However this route of absorption is likely to be quite small since the aluminum salts used in antiperspirants are water soluble and should not be able to penetrate the epidermis. The other three routes of absorption, inhalation, ingestion, and nasal-olfactory should only be considered for aerosol formulations. For the inhalation or ingestion route, aerosol particles could be deposited in the lung or along the airways and then either absorbed directly in the blood or indirectly via ingestion. For the nasal-olfactory route, aerosol particles could be deposited within the nose and then absorbed directly into the olfactory bulb of the brain via the olfactory neurons and mucosa (Tjalve & Henriksson, 1999).

Two studies have measured the dermal absorption of aluminum from antiperspirant ingredients. The first study, by Anane et al., used aluminum chloride applied to the shaven skin of mice (Anane et al., 1995). At one time aluminum chloride was a common active ingredient in antiperspirants, but it is less common today because of its acidity and tendency to cause irritation (Laden, 1999). In this study, mice were shaven and aluminum chloride was applied to the shaven skin for 130 days at the rate of 0.1 μg Al/day for one group, and 0.4 μg Al/day for another. One day after the last application, urine, blood, hippocampus, and remaining brain samples were collected. Aluminum concentrations were significantly increased in both study groups compared to controls. There are three major problems with this study: it is not clear if any measures were taken to prevent the mice from ingesting the aluminum due to grooming, if aluminum shed from the skin's surface contaminated the cage and food supply, and that mouse skin does not contain sweat glands as does human skin.

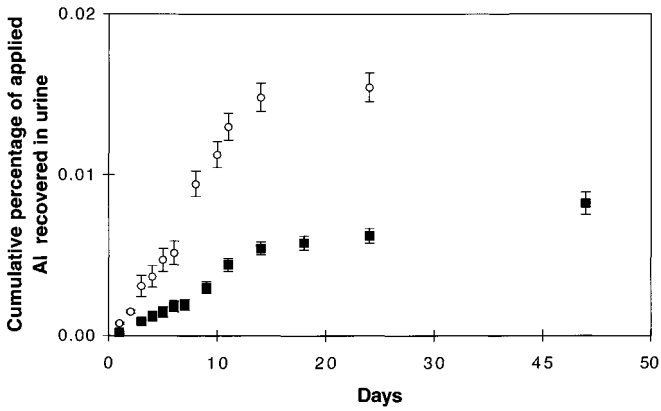

Fig. 4. Cumulative percentage of aluminum eliminated in urine which had been applied dermally as aluminum chlorohydrate. ○, subject A; ■, subject B.

In a different pilot study, aluminum chlorohydrate was labeled with ^{26}Al (Flarend et al., 1999) and then applied to the underarm of two human subjects (Flarend et al., 2001). ACH is the active ingredient in modern aerosol antiperspirant formulations and a few non-aerosol formulations. Since this study utilized human subjects instead of laboratory animals, it did not have the same difficulties as the previous study. This pilot study also used a non-invasive method of determining aluminum absorption by measuring the clearance of aluminum in urine and then correcting for the amount absorbed and not yet removed from the body. Results showed that only 0.012% of the applied ACH was absorbed through the skin. This 0.012% amounted to only 4 μg of aluminum from a single application of ACH (to both arms).

Fig. 4 shows that the aluminum was cleared (and thus absorbed) at a relatively steady rate for the first two weeks following the application of the ACH. The slight rise in cumulative urine after two weeks is probably due to wash out from the body and not further dermal absorption. This is interesting since the underarm was washed lightly for the first week after the application (per study protocol), and presumably the subjects washed this area quite well at the start of and during the second week. This two week period of absorption, along with other evidence that the sweat reducing effect of antiperspirants lasts for several weeks after discontinued use, suggest that some percentage of the applied antiperspirant remains on the underarm despite washing and continues to be absorbed. This would also indicate a strong likelihood that continuous use of an antiperspirant saturates the underarm area with aluminum so that the total amount of aluminum absorbed through the skin from continuous use would not be too different from the amount absorbed from just one application repeated weekly.

This pilot study clearly demonstrates that aluminum is absorbed, albeit only slightly, through the skin from the use of ACH. This study did not consider the effects of long-term usage, specific formulation, vehicle for delivery, or physical activity of the subject. All of these factors will probably have some effect on the dermal absorption of aluminum.

Inhalation of Aluminum

No study to date has measured the absorption of aluminum from antiperspirants via inhalation or the nasal olfactory route. One recent study measured aluminum in the rat olfactory bulb after it had a nasal exposure to aluminum chlorohydrate (Divine et al., 1999). This study was not designed for the purpose of measuring aluminum absorption and did not use a control group. Thus the source of the aluminum cannot be determined and thus this study does not provide conclusive evidence for the olfactory uptake of aluminum. However, these routes are potentially the most vital to the aluminum–Alzheimer's disease hypothesis. Data from welders exposed to aluminum fumes shows that aluminum can be absorbed by inhalation (Alfrey, 1997). This rate of absorption is estimated at about 0.2% of the inhaled aluminum.

ACH is a water soluble form of aluminum which tends to convert to and precipitate as aluminum hydroxide in the sweat duct (Teagarden et al., 1983). As a water soluble chemical, any amount which is inhaled and deposited in the lungs or other airways may have a significant absorption by the body before its conversion to aluminum hydroxide. If not absorbed directly in the airways, it could also be swallowed and absorbed in the GI tract. An important issue here is how much aluminum is inhaled daily by a typical aerosol user. This question has been addressed in a variety of studies within the pharmaceutical industry which are described by Wulf (Wulf, 1999). From these studies it can be approximated that from a single use, 1 μg of aluminum reaches the nasal area of the face where it can be inhaled. No study has been reported which has measured the aluminum absorbed from the inhalation of antiperspirants, but drawing upon the example of aluminum inhalation by welders, this route must also exist for aerosol antiperspirants. But even if this 1 μg is completely absorbed (by either ingestion or inhalation), it would only add an extra 10% to the aluminum which is absorbed each day from the diet. Thus, even in an exaggerated worst case, inhaling 1 μg of aluminum leads to less aluminum loading than eating a muffin (a food item particularly high in aluminum due to baking powder). Or in a more conservative estimate, assuming that the absorption of aluminum from antiperspirants is similar to welding fumes (0.2%), only 2 ng of aluminum would be absorbed daily via inhalation of aerosol antiperspirants. This more conservative estimate represents a trivial increase in the body burden of aluminum when compared to the diet.

The only remaining route of absorption for aluminum from antiperspirants, specifically from ACH, is the nasal-olfactory route. This is a very significant route since it would bring the aluminum directly into contact with the brain, bypassing the efficient elimination of the kidneys and the blood–brain barrier. Once in the olfactory region of the brain, these metals can then be transported to other areas of the brain. Therefore, this route of absorption requires careful scrutiny.

From the use of isotopic tracers, metals other than aluminum such as manganese and cadmium are known to be absorbed directly into the brain via the olfactory route (Tjalve & Henriksson, 1999). Unfortunately, very few studies have been conducted to measure the absorption of aluminum through the nasal-olfactory route, and no study has been conducted specifically for antiperspirants. This is even more surprising considering that some researchers believe that AD may begin in the olfactory region of the brain (Perl & Good, 1991). But this hypothesis is still a matter of debate (Kovacs et al., 1998).

The first study to demonstrate that aluminum could be absorbed via the olfactory pathway was by Perl and Good (Perl & Good, 1987). In this study, aluminum lactate and aluminum chloride (different study groups) were applied directly to the nasal recess using foam strips inserted into a hole drilled in the frontal bone of rabbits. After four weeks of exposure, selected brain tissue was stained for aluminum and analyzed using laser microprobe mass analysis (LAMMA). Results indicate that aluminum was absorbed into the olfactory bulb, cerebral cortex and other areas of the brain, whereas the control group showed no evidence of aluminum adsorption into the brain.

A more recent study has also shown deposition of aluminum into the brain of rats following a nasal exposure to aluminum acetylacetonate (Zatta et al., 1993). Both of these studies cannot totally rule out the possibility that aluminum detected in the brain did not arise from the aluminum being absorbed systematically and then transported to the brain via another route. But more importantly, neither of these studies specifically utilized an antiperspirant exposure and thus they only loosely support the hypothesis that aluminum can be absorbed directly into the brain from aerosol antiperspirants.

Informed Opinion

Without focusing on whether aluminum is linked with Alzheimer's disease, this opinion will attempt to determine whether antiperspirants or vaccine adjuvants should be considered a significant source of aluminum contributing to the overall body-burden of aluminum, or to aluminum deposition in the brain. If antiperspirants and vaccine adjuvants are not a significant source of bioavailable aluminum in either the whole body or the brain, then neither source could be a significant factor in the development of AD. This assumption does preclude the possibility that one of these two sources of aluminum plays an active role in the development of AD because of their method of delivery or specific interactions within the body, but at this time there is no evidence to the contrary.

For comparison purposes, the daily exposure to aluminum from the diet can be estimated using the typical dietary exposure rates given in Table 1 and a GI uptake fraction of 0.1%. Using a blood–brain barrier (BBB) uptake fraction from the diet ranging from 2×10^{-4} % to 5×10^{-5} % the cumulative aluminum exposure to the brain is also estimated. These estimates are given in Fig. 5. Also dividing the BBB uptake fraction by the GI uptake fraction, a range of 0.2% to 0.05% is obtained for the brain uptake of aluminum from internal exposures of aluminum.

It should be noted that the uptake fraction for the GI-tract has been measured in adult human and animal models many times. The aluminum content of the diet is also fairly well known. Thus Fig. 5a should be reasonably close to the actual internal exposures of a typical person. The cumulative aluminum exposure to the brain, Fig. 5b, is a rough estimate. This is because the brain uptake for aluminum has been quantitatively measured in just a few animal models and the intersubject variability is bound to be quite significant. However, Fig. 5b can provide a useful comparative tool provided that one acknowledges its limitations.

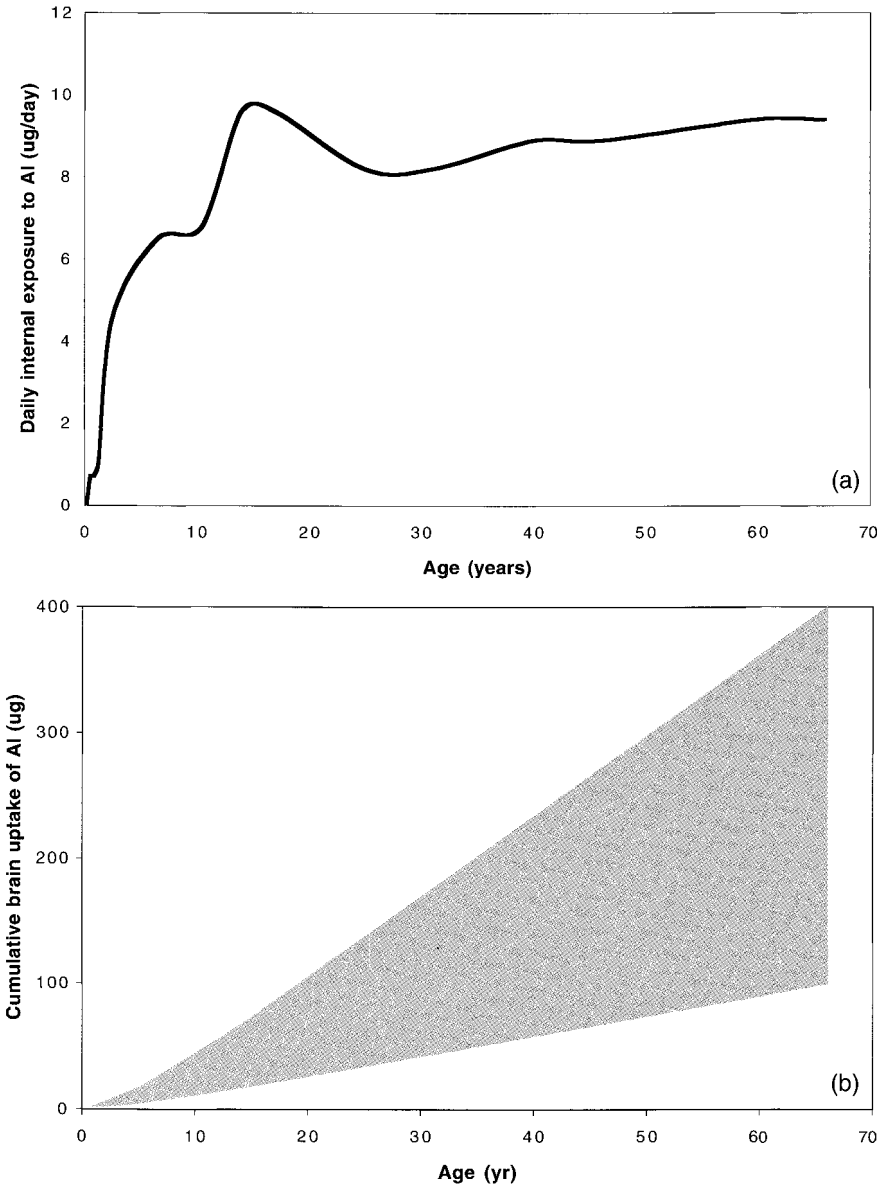

Fig. 5. (a) Estimated daily internal exposure to aluminum. (b) Estimated cumulative aluminum exposure to the brain (the shaded region indicates the estimated range of possible cumulative brain exposure to aluminum).

Vaccine Adjuvants

Using the data from Table 2, which gives the aluminum content of recommended childhood and adult vaccinations, the range in the daily contribution to the body's internal

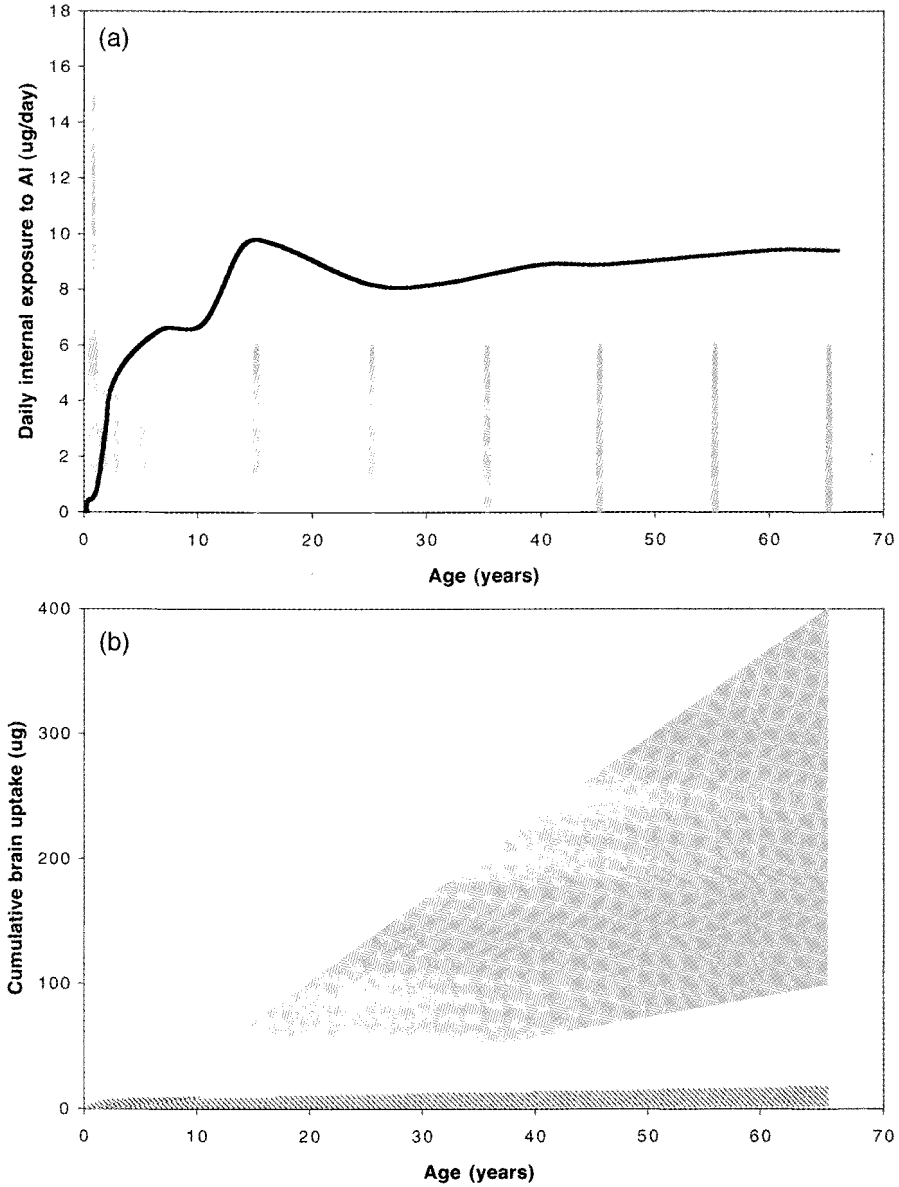

Fig. 6. (a) Estimated range of daily internal exposure to aluminum from the recommended regime of vaccines (shaded area) compared to the dietary intake estimate from Fig. 5 (bold line). (b) The estimated range of cumulative aluminum exposure from vaccine adjuvants (hashed region) compared to the estimated range of cumulative aluminum exposure from the diet (shaded region).

exposure to aluminum from vaccine adjuvants was determined and is shown in Fig. 6a. For this estimation, it was assumed that aluminum hydroxide adjuvants are completely dissolved by interstitial fluid at a steady rate within 15 months of intramuscular (IM)

injection and are equilibrated with other aluminum in the body. Aluminum phosphate adjuvants are assumed to be dissolved within 5 months. This calculation is based upon an extrapolation of the study by Flarend et al. (1997) which indicated that only 6% of aluminum hydroxide and 22% of aluminum phosphate adjuvants are dissolved within a time period of 1 month.

After an age of about 2 years old, the estimated internal exposure to aluminum from vaccine adjuvants, Fig. 6a, is less than (and usually much less than) the exposure to aluminum which is absorbed from the diet. Only for short intervals following the occasional booster vaccination does the adjuvant exposure even approach the dietary exposure. During the first 18 months of age for a term-delivered infant, the adjuvant exposure is much greater than the dietary exposure to aluminum for two reasons. First infants receive multiple vaccinations over just a few months, and second, infants, especially breast-fed infants, receive very little exposure to aluminum in their normal diet. Premature infants may receive much more aluminum due to their special diet, but that situation is not discussed here because they represent a small population group and there are special considerations for their diet and metabolism of aluminum (Bishop et al., 1997).

The estimated range of cumulative brain exposure to aluminum, Fig. 6b, is likewise greater from vaccine adjuvants over the first several years of life than from dietary aluminum. After several years, the estimated cumulative exposure from the diet continues to climb, whereas the exposure from adjuvants levels off at about 1–10 μg Al.

Considering the exposure to aluminum during the majority of a person's life, vaccine adjuvants account for much less of an exposure to aluminum than from the diet. The 'blip' which occurs early in life from childhood vaccinations should be considered very carefully. The risk–benefit ratio of receiving the full regiment of childhood vaccinations is heavily tilted to the benefit side. If children, in large numbers, were not to receive these needed vaccinations, the mortality rate from preventable diseases would certainly dwarf any observable effect which aluminum may cause in vaccinated children. Because the metabolism of aluminum in adults has been extrapolated to infants and children, the estimations given in Figs. 5 and 6 (also Figs. 7 and 8 below) are weak during the early years because very little is known about aluminum metabolism in young age groups.

Nonetheless, because the cumulative exposure to aluminum from vaccine adjuvants is much less than from the diet for older ages (past 5 years old), it can be safely assumed that aluminum containing vaccine adjuvants do not contribute significantly to the brain's accumulation of aluminum.

Dermal Antiperspirants

Based upon the preliminary data from the absorption of aluminum from ACH (Flarend et al., 2001), the exposures to aluminum have been estimated for the dermal absorption of aluminum from antiperspirants being applied on a daily basis beginning at age 13. The upper limit of the range given in Fig. 7 represents a dermal absorption of 4 μg Al per daily application of antiperspirant. This upper limit is probably an unrealistically high assumption based on the preliminary study by Flarend et al. because it assumes a large daily application of 30 mg aluminum for both underarms, an absorption rate equal to that found

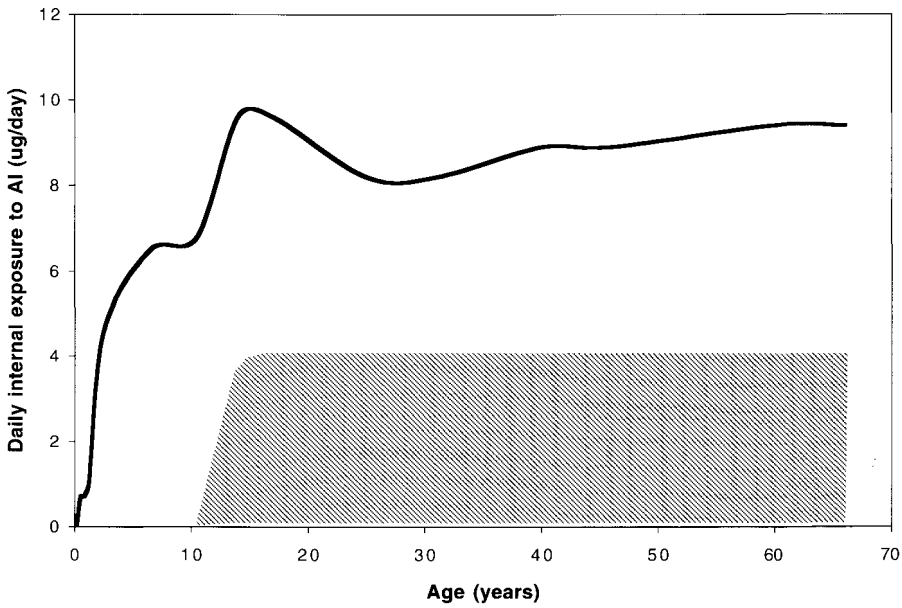

Fig. 7. Estimated range of internal exposure to aluminum from the dermal use of antiperspirants (hashed region) compared to the estimated dietary intake of aluminum (bold line).

in the preliminary study (0.012%), and that the steady-state absorption rate is equal to the single application absorption rate. The lower limit of this range, 0.03 μg Al, is estimated on the assumption that daily usage of antiperspirants will saturate the dermal absorption route resulting in an absorption rate of about 0.001% and a smaller daily application rate of about 3 mg for both underarms. This range (0.03–4 μg Al) should account for the fact that the study by Flarend et al. is a preliminary study and that several factors concerning delivery, formula, frequency of use, and personal hygiene need to be considered for the absorption of aluminum from dermal antiperspirants to be accurately estimated.

Using the estimated range above, dermal application of antiperspirants would increase the body's internal exposure to aluminum by 0.3% to 40% compared to the typical 10 μg of Al which is absorbed each day from the GI tract. Although the 40% upper limit may appear to be significant, it is probably an unrealistically high estimation. To be completely fair, this extreme limit should be compared to an equally high level of dietary intake for aluminum of over 95 mg Al/day which occurs for about 5% of adults (Greger & Sutherland, 1997). By comparing upper limits, 4 μg Al/day would be about 10% of the aluminum from a high dietary intake.

In the lower limit estimate, dermal usage of an antiperspirant does not result in a significant amount of aluminum being absorbed by the body compared to that absorbed from the diet. In either comparison, it must be warned that there is not enough data to make a reliable statement on the relative amounts of aluminum absorbed from the diet and from dermal use of antiperspirants. It can only be said, with some degree of reliability, that dermal use of antiperspirants is a secondary source of aluminum when compared to the diet.

Aerosol Antiperspirants

In the case of aerosol antiperspirant use, it will be assumed that about 1 μg Al is inhaled per day (Wulf, 1999). If this amount is completely absorbed systemically via the lungs and air-ways (an absolute maximum), the body-burden of aluminum would increase by only 10% compared to the typical 10 μg Al absorbed daily from the diet. A lower estimate would be that the aluminum from antiperspirant aerosols would be absorbed at the same 0.2% rate as is estimated for welding fumes. This lower estimate would amount to just 0.002 μg Al/day which is trivial compared to the diet. Thus in neither extreme is the inhalation (followed by systemic absorption) a primary source of aluminum.

There is not yet enough information available to determine if the nasal-olfactory route of absorption is a significant route of aluminum accumulation in the brain. To understand the potential for this route of absorption, consider the approximate 1 μg of aluminum which is inhaled per day from aerosol antiperspirant use. If 0.1–1% of this amount is absorbed directly into the brain, this would amount to 1–10 ng Al accumulating in the brain per day. Several studies using rats have found that between 2×10^{-4}% and 5×10^{-5}% of the aluminum ingested from food is accumulated in the brain (Fink et al., 1994; Jouhanneau et al., 1997; Drueke et al., 1997). Thus about 0.2 to 5 ng Al/day would accumulate in the brain from a diet containing 10 mg Al/day. In this comparison, the nasal-olfactory route could represent the single most important route of aluminum into the brain, possibly by more than an order of magnitude depending on how future research pins down these absorption rates. Fig. 8 shows an estimation of the cumulative uptake

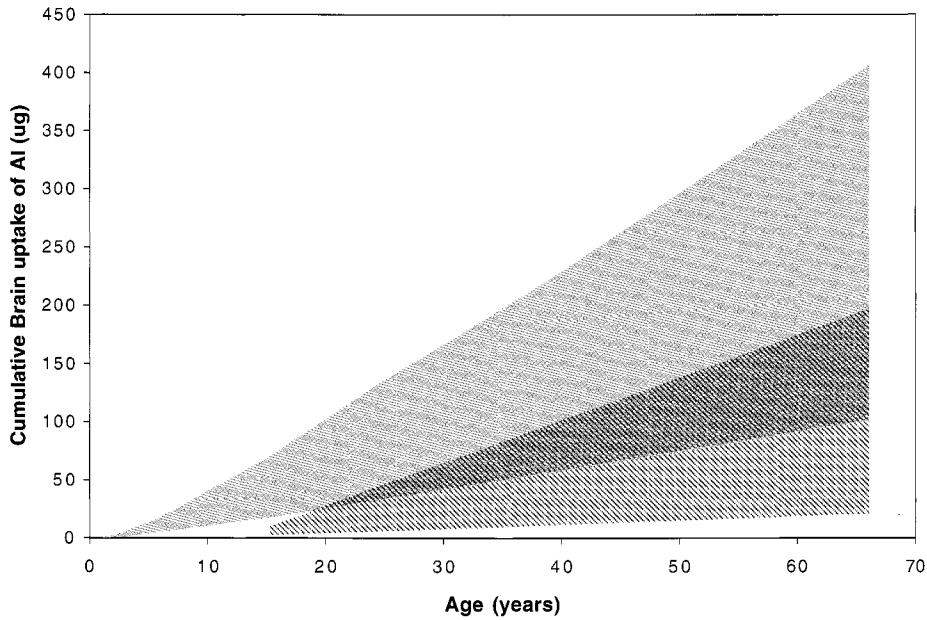

Fig. 8. Hypothetical range of cumulative brain exposure to aluminum from the aerosol use of antiperspirants (hashed region) compared to the estimated brain exposure to aluminum from dietary sources (shaded region).

of aluminum in the brain via the nasal-olfactory route compared to the accumulation of aluminum from the diet.

Fig. 8 is very hypothetical. This is because the transport of aluminum into the brain is poorly understood and the 0.1–1% range for the rate of absorption via the nasal-olfactory route is merely an informed guess. Nevertheless, Fig. 8 does illustrate the need for this route of absorption to be studied carefully and for all airborne sources of aluminum to be scrutinized.

Needed Research

In general, more information on the metabolism and bioavailability of aluminum from a variety of sources is needed. This bioavailability information should include a variety of different dietary sources of aluminum, food additives and colorings, inhalation of aluminum, use of aluminum-containing drugs (aspirin, anti-ulcer and anti-diarrhea drugs), and the dermal absorption of aluminum from cosmetics. This research should be conducted using the appropriate aluminum species since there can be a large range in the bioavailability of aluminum depending on the chemical form of the aluminum. These questions are best solved using the radiotracer ^{26}Al since this is the only method which can utilize realistically small exposures and still quantify the absorbed aluminum from the intended exposure.

Information is also needed about metabolism and bioavailability of aluminum in infants and children. Most studies using ^{26}Al involve very little exposure to radiation. In fact, many questions can be addressed while keeping the radiation exposure much less than background levels. Thus if the socio-political difficulties can be overcome, there is no scientific reason why the radiotracer ^{26}Al cannot be used with infants and children in certain applications. In other applications, appropriate models should be substituted, but this information must be collected.

An area of critical importance is to determine the absorption of aluminum from inhalation and from the nasal-olfactory route. Very little data has been gathered on the inhalation of aluminum and even less for the nasal absorption of aluminum other than the concept that aluminum can be absorbed directly into the brain from the nose. These routes are very important to the safe use of aerosol antiperspirants. Since there are a variety of other delivery methods for antiperspirants other than aerosols, the aerosol delivery formulation may have a very high risk to benefit ratio (compared to other antiperspirant delivery formulations) and is likely popular only for its convenience. It is quite possible that when the nasal absorption of aluminum has been studied in detail, that aerosol antiperspirants could be responsible for a substantial portion of the brain's burden of aluminum. In such a situation, the convenience of the aerosol antiperspirant would clearly not outweigh its risk.

If it is determined that aluminum accumulation in the brain is mildly significant to AD, the lack of supporting epidemiological data from the use of antiperspirants is not surprising. This is because the epidemiological studies may have failed to detect a connection due to a methodological flaw. It is likely to be only the aerosol antiperspirants (NOT deodorants and NOT stick or roll-on antiperspirants) which would present a risk

factor. Since the studies performed thus far use proxies (spouses for example) for the control and AD subjects, it may be unrealistic to expect the proxy to correctly identify the actual antiperspirant product used. This methodological flaw could be the reason why the two published studies to date (Graves et al., 1990; Canadian Study of Health and Aging, 1994) have observed slight risk-factors for AD from antiperspirant use, and yet have problems determining its significance and showing a correlation between risk and degree of antiperspirant use. If it is possible to separate aerosol antiperspirant usage from all other antiperspirant or deodorant usage, these studies may become more clear. It is interesting, that in the event that it is technically difficult for a single study to identify whether case and control subjects used aerosol or non-aerosol antiperspirants, it may be possible to repeat the two epidemiological studies discussed above in either the UK, France, or Japan where about 80% of the population still uses aerosol antiperspirants and/or deodorants today, compared to only 16% in the U.S. (Laden, 1999). If such a study were performed, the results may be more clear due to the higher popularity of aerosol antiperspirants which probably yield a higher amount of brain-available aluminum.

It can be concluded that neither aluminum-containing vaccine adjuvants nor *dermal* usage of antiperspirants are risk factors for Alzheimer's disease. Even if aluminum is found to be a risk factor for AD, the risk from vaccine adjuvants and dermal usage of antiperspirants would pale in comparison to the normal diet because the diet provides a much larger amount of bioavailable aluminum than vaccine adjuvants or dermal antiperspirants.

At present, there is not enough data available to conclude whether aerosol antiperspirants are a significant source of aluminum in the brain or not. Further research must be focused on this particular question and the nasal-olfactory route of absorption for aluminum.

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Aluminium and Alzheimer's Disease
The Science that Describes the Link
C. Exley (Editor)

CHAPTER 5

The Aetiology of Alzheimer's Disease: Diverse Routes into a Common Tau Pathway

Charles R. Harrington *

Department of Mental Health, University Medical Buildings, University of Aberdeen, Foresterhill, Aberdeen, AB25 2ZD, UK

* Corresponding author, email: c.harrington@abdn.ac.uk

Abbreviations: AD – Alzheimer's disease; Aβ – amyloid β-protein; APP – amyloid β-protein precursor; AGE – advanced glycation endproduct; ALS, amyotrophic lateral sclerosis; ApoE – apolipoprotein E; BACE – β-site *A*PP *c*leaving *e*nzyme; CBD – corticobasal degeneration; CNS, central nervous system; ER – endoplasmic reticulum; FTDP-17 – frontotemporal dementia with parkinsonism linked to chromosome 17; GSK-3 – glycogen synthase kinase-3; MAP – microtubule-associated protein; NFT, neurofibrillary tangle; PHF – paired helical filament; PrP – prion protein; PS – presenilin; PSP – progressive supranuclear palsy; RNA – ribonucleic acid

Summary

Alzheimer's disease is a complex heterogeneous disorder. Mutations in three genes (the amyloid β-protein precursor and presenilins 1 and 2) are associated with the rarer early-onset forms of the disease. Possession of an apolipoprotein E ε4 allele predisposes individuals to a greater risk of developing sporadic AD, which is most frequent in the elderly. Despite this heterogeneity, dementia is consistently associated with the accumulation of tau aggregates and filaments. The accumulation of such deposits implicates a common pathway leading to dementia, in which protein conformational change and tau capture play critical roles in the rapid progression of the disease.

Historical Perspective

Heterogeneity of Alzheimer's Disease

Alzheimer's disease is the major cause of dementia in the elderly, although the frequency of other types of dementia increases as improvements in their diagnosis become available. These include a whole spectrum of disorders including dementia with Lewy bodies, frontotemporal dementias and other less common disorders. From epidemiological studies, the established risk factors, which predispose individuals to an increased risk of acquiring AD, include the following: increased age, family history of dementia,

head trauma and Down's syndrome. Mutations in three genes have been identified which co-segregate with disease in families with early-onset AD: the amyloid β-protein (Aβ) precursor (*APP*), and two presenilin genes, *PSEN1* and *PSEN2* (Table 1). An updated list of these mutations can be found at http://www.alzforum.org. Nevertheless, subtle differences in age of onset and duration of disease have been observed and these may be sufficient to account for the genetic heterogeneity seen in familial AD. Nonetheless, a genetic factor in perhaps as many as half of the early, autosomal dominant AD cases has yet to be found (Tanzi et al., 1996). While various mutations are associated with some differences in clinical presentation, the pathological changes at autopsy are relatively constant. Mutations in *APP*, *PSEN1* and *PSEN2*, when combined, account for only a small percentage of the incidence of AD. A polymorphism in the *APOE* gene, however, can account for as much as 60% of the susceptibility to AD and is the first gene identified in which 'normal' alleles predispose to dementia. Unlike mutations in the familial forms of AD, inheritance of an ε4 allele does not reliably predict whether or when a carrier will develop AD.

It seems likely that independent genetic defects that lead to a similar clinical and neuropathological profile would share some part of a common neurodegenerative pathway. Thus, although the successful search for genes that accelerate the progression of AD provides important starting blocks for further investigation, the identification of common pathway(s) can provide critical points at which therapeutic strategies might be targeted to prevent dementia.

Amyloid β-protein precursor

Mutations in the *APP* gene, near to or within the Aβ domain, are linked with AD in a few families throughout the world and in different ethnic populations. The AD mutations are either within or flanking the Aβ domain of APP that gives rise to the deposits of Aβ plaques throughout the cortex and, to a lesser extent, in the cerebellum (Selkoe, 1999). APP exists in at least 5 isoforms, but its physiological role in the brain is not understood. In the numbering of the largest APP isoform (770 residues), the 42-residue Aβ domain corresponds to residues 672 to 713, which are encoded for by exons 16 and 17 (Fig. 1). Eleven mutations in APP are associated with disease, ten with AD and one with hereditary cerebral haemorrhage with amyloidosis — Dutch type (HCHWA-D). In one family, a Swedish mutation results in the replacement of 2 amino acids (K670N and M671L). Other point mutations associated with AD are: A692G, E693G, V715M, I716L, V717I, V717G, V717F, V717L and L723P (Fig. 1). HCHWA-D, which exhibits severe congophilic angiopathy in the absence of plaques and tangles, is associated with the mutation E693Q.

Although APP mutations co-segregate with disease in rare families with early-onset AD, the mechanism by which such mutations cause disease is not clear. Some mutations result in over-production of longer $A\beta_{42}$ forms that aggregate more readily than $A\beta_{40}$. Recent studies have suggested that the ratio between Aβ peptides terminating at 40 and those with 42 or 43 residues is important, and this may have an effect on the acceleration of plaque formation (Jarrett et al., 1993). The predominant form of Aβ in diffuse and neuritic plaques tends to be that which terminates after 42 residues (Iwatsubo et al., 1995) in contrast to the vascular deposits of Aβ that tend to be of 40 residues in length

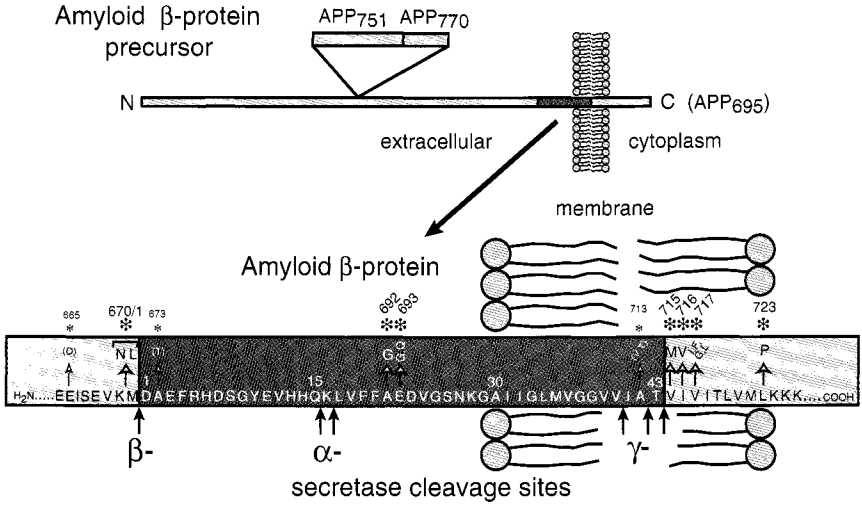

Fig. 1. Amyloid β-protein is derived from a transmembrane precursor. The positions of the major cleavage sites for α-, β- and γ-secretase are shown. Asterisks indicate mutations associated with disease, with the numbering corresponding to the amino acid position in the largest isoform (APP_{770}). Mutations that are not associated with disease are indicated in lower case and by smaller asterisks, whereas E693G is associated with HCHWA-D.

(Suzuki et al., 1994). $A\beta_{42}$ is also more resistant to proteolysis; diffuse Aβ deposits can be removed from rat brain by a neutral protease, similar to neprilysin, that is sensitive to specific inhibition by thiorphin (Iwata et al., 2000). Evidence that extracellular Aβ deposits can be cleared is supported by the finding that deposits of amyloid in transgenic APP mice are removed by immunisation with $A\beta_{42}$ (Schenk et al., 1999).

Degradation-resistant Aβ is found within neurons and it may be that this intracellular product is more important in AD than the extracellular deposits of Aβ. Cells can respond abnormally to aggregated proteins or, alternatively, resistant Aβ could act as a focus for seeding the aggregation of other cellular proteins such as tau protein. Evidence that Aβ is toxic to neurons *in vivo* is unconvincing, and very elderly patients with abundant Aβ deposition can be found to be clinically normal (Delaère et al., 1993). Furthermore, the distribution of Aβ deposition does not follow the same pattern as that of neurofibrillary degeneration, which is more closely correlated with cognitive decline (Arriagada et al., 1992; Wilcock & Esiri, 1982). Nevertheless, recent data suggests that soluble Aβ is correlated with cognitive decline (Näslund et al., 2000). Finally, models of amyloidosis in mice (Games et al., 1995) do not induce neurofibrillary tau pathology.

Three pathways for the processing of APP have been identified so far (Citron, 2000) and the sites of cleavage by three secretases are shown in Fig. 1. There are two secretory pathways: one in which the Aβ domain is cleaved within the middle (α-secretase) and the second, a potentially amyloidogenic pathway which leaves the Aβ peptide intact (β-secretase). APP can also be internalised and degraded via the endosomal/lysosomal pathway (γ-secretase) to yield C-terminal fragments of APP that also contain an intact Aβ domain. The β-secretase (Hussain et al., 1999; Vassar et al., 1999) and γ-secretase

Table 1. Genetic heterogeneity of Alzheimer's disease and fronto-temporal dementias

Chromosomal location	Frequency (%)		Age at onset (year)	Gene	Protein	Number of mutations or polymorphism	Reference
	Autosomal dominant	All cases					
(a) Autosomal-dominant loci linked with AD							
14	50	7	30–68	*PSEN1 (AD3)*	Presenilin 1	>75	Sherrington et al. (2000)
21	1	<1	41–67	*APP (AD1)*	Amyloid protein precursor	11	Goate et al. (1991)
1	20	2	50–70	*PSEN2 (AD4)*	Presenilin 2	6	Levy-Lahad et al. (1995); Rogaev et al. (1995)
12	3	<1	40–70	–	Unidentified locus	–	Pericak-Vance et al. (1997)
(b) Established genetic risk/susceptibility factors associated with AD							
19		45–60	60–90	*APOE (AD2)*	Apolipoprotein E	polymorphism	Strittmatter et al. (1993)
21		*	35–60	*APP*	Amyloid protein precursor	trisomy 21	Oliver and Holland (1986)
(c) Autosomal dominant FTDP-17							
17	40	20	45–65	*MAPT*	Tau protein	17	See Fig. 3.
(d) Risk factor for progressive supranuclear palsy and corticobasal degeneration							
17				*MAPT*	Tau protein	polymorphism	Conrad et al. (1998)
(e) Genetic risk factors associated with AD (not established)							
14				*AACT*	α_1-Antichymotrypsin	polymorphism	Kamboh et al. (1995)
9				*VLDLR*	Very low density lipoprotein receptor	polymorphism	Okuizumi et al. (1995)
14				*PSEN1*	Presenilin 1 intron	polymorphism	Wragg et al. (1996)
4				*SNCA*	α-Synuclein (NACP)	polymorphism	Xia et al. (1996)
8				*CLU*	Clusterin (apolipoprotein J)	polymorphism	Tykco et al. (1996)
17				*SLC6A4*	Serotonin transporter (5-HTT) promoter	polymorphism	Li et al. (1997)
6				*HLA-DRA1*	Major histocompatibility complex, class II, DRα	polymorphism	Curran et al. (1997)

14	*DLST*	Dihydrolipoyl succinyltransferase	polymorphism	Nakano et al. (1997)
6	*HLA-HA2*	Minor histocompatibility antigen, HA2	polymorphism	Payami et al. (1997)
3	*BCHE*	Butyrylcholinesterase	polymorphism	Lehmann et al. (1997)
12	*A2M-2*	α2-Macroglobulin	polymorphism	Blacker et al. (1998)
11	*ESRRA*	Estrogen receptor α	polymorphism	Isoe et al. (1998)
12	*NTF3*	Neurotrophin 3	polymorphism	Kunugi et al. (1998)
17	*BLMH*	Bleomycin hydrolase	polymorphism	Montoya et al. (1998)
11	*APBB1*	Fe65 (APP binding, family B, member 1)	polymorphism	Hu et al. (1998)
19	*APOE*	Apolipoprotein E (regulatory regions)	polymorphism	Roks et al. (1998)
19	*APOE*	Apolipoprotein E (APOE4 Pittsburgh)	polymorphism	Kamboh et al. (1999)
1	*HTR-6*	Serotonin 5-HT-6 receptor	polymorphism	Tsai et al. (1999)
17	*DCP1*	Dipeptidyl carboxypeptidase 1 (ACE)	polymorphism	Hu et al. (1999)
11	*CTSD*	Cathepsin D	polymorphism	Papassotiropoulos et al. (1999)
2	*IL1A*	Interleukin-1α	polymorphism	Grimaldi et al. (2000)
12	*LBP-1c*	Transcription factor CP2	polymorphism	Lambert et al. (2000)
7	*NOS3*	Nitric oxide synthase (endothelial NOS)	polymorphism	Dahiyat et al. (2000)
20	*CST3*	Cystatin C	polymorphism	Crawford et al. (2000)
6	*HFE*	Haemochromatosis (class I-like MHC)	polymorphism	Moalem et al. (2000)
mtDNA	*MTND1*	Complex I, subunit ND1	1	Hutchin and Cortopasi (1995)

* Approximately 50% of Down's syndrome patients who survive into their 4th decade are found to have AD pathology.

(Wolfe et al., 1999b) cleave APP to create Aβ peptides. β-Secretase, which is also referred to as BACE (β-site *A*PP *c*leaving *e*nzyme) is an aspartic protease located in the endoplasmic reticulum (ER) and Golgi compartment. The processing of APP is modulated by presenilin 1, or by a complex containing this protein. It is not clear whether presenilin acts either as the β-secretase itself or by increasing γ-secretase activity or whether other associated proteins, such as nicastrin, are involved (Yu et al., 2000). γ-Secretase cleavage generates Aβ of different lengths — $A\beta_{42}$ in pre-Golgi compartments and Aβ40 in later compartments and in the endocytic pathway. Aβ itself, when aggregated, possesses proteolytic activity and thus may be responsible for inactivation of other proteins (Elbaum et al., 2000). Growth of amyloid fibrils occurs by the assembly of seeds into protofibrils which, in turn, self-associate to form mature fibres. Many factors have been identified which either promote or inhibit amyloid aggregation or fibril formation, but the relevant importance of such modulators has not been established yet (McLaurin et al., 2000).

Presenilin proteins

The human presenilin proteins, PS1 and PS2, were identified in 1995 (Levy-Lahad et al., 1995; Rogaev et al., 1995; Sherrington et al., 1995). The *PSEN1* gene was identified by classical positional cloning techniques, while *PSEN2* was isolated on the basis of its homology to *PSEN1*. More than 75 mutations in these two proteins have now been identified as being associated with AD, the majority of these being mutations in *PSEN1* (Fig. 2). In certain cases, onset can occur as early as the age of 30 years. The biology and function of the presenilin proteins is, at present, relatively poorly understood, but is reviewed in greater detail elsewhere (Annaert & De Strooper, 1999; Wolfe et al., 1999a). Both are membrane-spanning proteins, with at least 6 trans-membrane domains and a large hydrophilic loop. The two proteins show extensive homology with each other and they undergo proteolytic processing, with a cleavage site situated within the large hydrophilic loop (Fig. 2). The full-length *PSEN1* and *PSEN2* genes encode predicted proteins of 463 and 448 amino acids, respectively. They are both cleaved into N- and C-terminal fragments of approximately 35 and 20 kDa. They are normally localised in the nuclear envelope, the endoplasmic reticulum and the Golgi, with PS2 being more predominant in the Golgi compartment than the PS1 protein.

Clues about their function in mammalian cells are derived from the observation that both presenilin proteins share significant amino acid homology with two *Caenorhabditis elegans* proteins: *sel-12* and *spe-4* (Levitan & Greenwald, 1995). Thus the presenilin proteins are thought to play a role in the intracellular trafficking or localisation/recycling of proteins in the brain. Just as mutations in APP lead to increases in the ratio of $A\beta_{42}$: $A\beta_{40}$, mutations in PS1 increase the proportion of $A\beta_{42}$ in transfected cell lines (Citron et al., 1996), transgenic mice (Borchelt et al., 1996; Duff et al., 1996) and brain tissue from AD patients carrying PS mutations (Lemere et al., 1996). Recent studies suggest that γ-secretases involved in APP processing are aspartyl proteases that catalyse novel intramembranous proteolysis. This proteolysis requires a protein complex containing presenilin 1 and other proteins (Wolfe et al., 1999a; Yu et al., 2000).

PS1 mutations facilitate apoptotic neuronal death *in vitro* (Chui et al., 1999; Vito et al., 1996) and, by analogy, their presence may account for some of the neurodegenerative

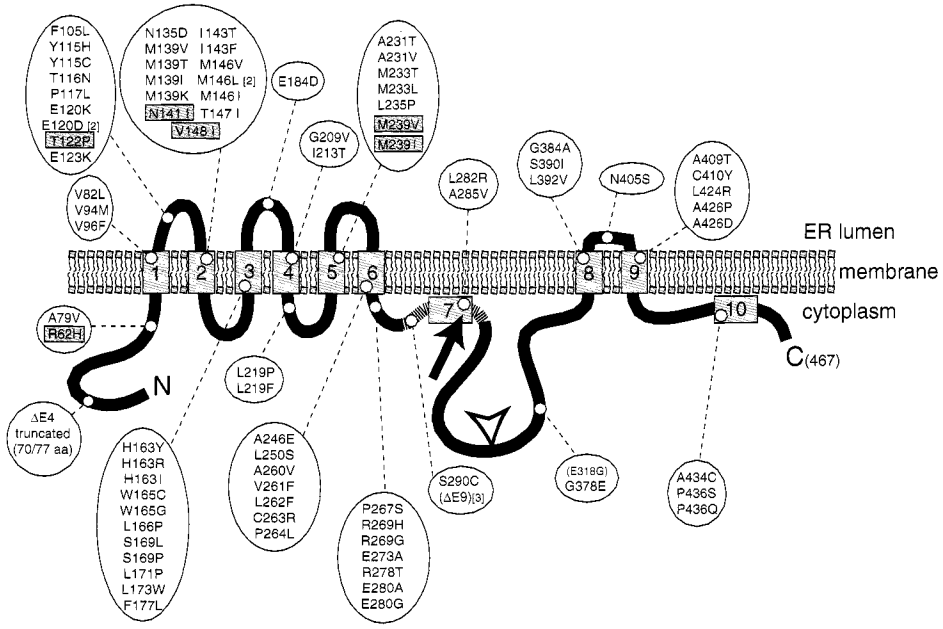

Fig. 2. Presenilin proteins are multiple transmembrane-spanning proteins showing considerably homology with each other. A model of PS1 indicates the putative topology of the protein, where transmembrane or membrane-associated domains are indicated by boxes. The location of over 70 different mutations associated with early-onset AD is indicated. The location of mutations is shown, with those for PS2 being boxed. Positions for constitutive proteolytic processing by the putative presenilinase occurs at residue Met-298 (arrow) and an alternative site for caspase cleavage at residue Asp-345 (open arrowhead) are shown.

process that occurs in the AD brain. The accumulation of misfolded proteins in the lumen of the endoplasmic reticulum (ER) results in an 'unfolded-protein response' in which increased production of chaperones alleviates the increased demand on the existing protein folding machinery. In neuroblastoma cells, mutations in PS1 downregulate the endoplasmic reticulum to nucleus signalling pathway that adjusts the levels of chaperones (Katayama et al., 1999). In addition to affecting APP processing, altered chaperone levels may influence the aggregation of other proteins. To this extent, further research in this area could provide clues to a link between tau processing, and either APP or the presenilin proteins. Finally, there is evidence that presenilin proteins may be involved in mitosis and that the mutations in the presenilin genes may predispose to chromosome mis-segregation (Geller & Potter, 1999).

Apolipoprotein E and other genetic susceptibility factors

Apolipoprotein E (ApoE) is a plasma protein involved in cholesterol transport. In the CNS, it is secreted by astrocytes as a constituent of high-density lipoprotein complexes but is also found in neurons (Mahley & Huang, 1999). Three major alleles exist: ε2, ε3 and ε4, with ε3 being the most common. An association between the *APOE* ε4 allele

and AD was first demonstrated at Duke University (Strittmatter et al., 1993). The ε4 allele frequency in AD and control Caucasian populations is approximately 0.38 and 0.13, respectively (Harrington et al., 1994a), and an earlier age of onset is often associated with possession of an ε4 allele (Farrer et al., 1997). In the very elderly (>85 years), however, the *APOE* gene does not influence cognitive decline or dementia (Farrer et al., 1997). *APOE4* is also associated with increased risk for dementia with Lewy bodies which, in distinction from AD is a condition associated with minimal accumulation of PHF-tau (Harrington et al., 1994a). Although many studies have now confirmed the association of *APOE* with AD, testing populations for *APOE* genotype cannot be used to predict development of AD; presence of the ε4 allele is neither necessary nor sufficient to cause disease. Furthermore, the distribution of the ε4 allele varies considerably between different populations (Uterman, 1994).

Possible biological explanations for the association of *APOE* genotype with AD have included isoform-specific neurotoxic and/or neuroprotective effects of apoE and the binding of apoE to Aβ or tau proteins in an isoform-dependent fashion (Strittmatter et al., 1994). Recent studies with transgenic mice indicate that apoE promotes the deposition and fibrillisation of Aβ and that this is greatest in mice expressing the apoE4 isoform (Bales et al., 1999). It has been speculated that this results from decreased clearance of Aβ. Alternatively, apoE influences neurite outgrowth: whereas apoE3 stimulates outgrowth in cell culture, apoE4 has the converse effect (Nathan et al., 1994). At present, however, these explanations remain hypotheses whose relevance *in vivo* has yet to be established. The disease specificity of the association is uncertain and the extent to which apoE interacts with other gene products is not known. Furthermore, other potential confounding factors such as cerebrovascular disease may impinge on the development of AD, affecting its association with *APOE4* genotype (Harrington & Roth, 1997).

The ε4 allele is over-represented in AD patients with late-onset symptoms and *APOE* genotype represents an important biological marker for the disease, accounting for 45%–60% of the genetic component of AD (Nalbantoglu et al., 1994). A recent study, however, suggests that *APOE* represents less than 10% of the total variance in age at onset of familial AD and that it is but one of several important genes (Daw et al., 2000). At least 4 additional loci were identified that have an effect equal to or greater than that of *APOE*. Associations between AD and genetic polymorphisms other than *APOE* have been investigated extensively in recent years (Table 1). In some studies, polymorphisms have been found to modify the risk attributed to *APOE* (Brandi et al., 1999; Crawford et al., 2000a; Farrer et al., 1997; Kamboh et al., 1995). None of these association studies, however, have been established in subsequent investigations and so the role of those factors listed above remains equivocal. Similarly, evidence for autosomal recessive AD in an Arab population is unsubstantiated (Bowirrat et al., 2000).

Mitochondrial DNA mutations

Evidence that oxidative stress is involved in AD is less compelling than for Parkinson's disease. Nonetheless, AD brains show evidence of iron dysregulation (that could promote free radical generation), changes in antioxidant levels and oxidative damage (*e.g.* lipid peroxidation) (Beal, 1995). The unconfirmed finding that mitochondrial DNA mutations

were associated with a subset of AD (Hutchin & Cortopassi, 1995) suggested that the bioenergetic capacity of neurons might be affected in these patients (Wallace, 1994). Since many of the mitochondrial disorders occur sporadically, it is conceivable that the common and sporadic, late-onset AD might be due, in part, to mitochondrial dysfunction and further genetic studies seem warranted.

Extrinsic factors in AD pathogenesis

Genetic factors, so far identified, do not account for all cases of AD, leaving the possibility that one of a number of non-genetic, environmental factors could be involved in AD pathogenesis. Just as the late-onset nature of AD has hindered genetic analysis, so have the epidemiological studies designed to identify environmental factors been complicated.

Epidemiological evidence for a role of aluminium as a risk factor in AD remains inconclusive and is reviewed in this book by Rondeau and Commenges. Aluminium has been found in both plaques (Candy et al., 1986) and tangles (Perl & Brody, 1980) in the brain and it can alter the metabolism of both Aβ (Exley et al., 1993; Kawahara et al., 1994; Mantyh et al., 1993) and tau protein (Abdel-Ghany et al., 1993; Guy et al., 1991; Kawahara et al., 1992; Mesco et al., 1991; Scott et al., 1993; Yamamoto et al., 1990). Aluminium interacts with PHF-tau through phosphorylated residues and, in so doing, masks their recognition by phosphorylation-dependent antibodies (Murayama et al., 1999). Furthermore, aluminium-induced aggregation of phosphorylated tau *in vitro* can be inhibited by desferrioxamine (Murayama et al., 1999), an agent tested in clinical trials for the treatment of AD (Crapper-McLachlan et al., 1991).

The neurofibrillary degeneration observed in rabbits injected with aluminium is morphologically distinct from neurofibrillary tangle-mediated degeneration found in AD and has been reviewed in this book by Savory. Recent findings (Rao et al., 2000), however, suggest that the differences may not be as great as originally reported. PHF preparations injected with $AlCl_3$ into rat brains show that aluminium increases the resistance of PHF-tau to *in vivo* proteolysis (Shin et al., 1994), suggesting that aluminium might serve as a cofactor in the formation of neurofibrillary lesions (reviewed by Shin in this book). Likewise, there is evidence that aluminium might effect Alzheimer-like tau pathology in the human brain (Harrington et al., 1994b). AD-like changes to tau protein were observed in the absence of overt neurofibrillary pathology in the brains of patients with renal failure subjected to chronic aluminium exposure. These changes include depletion of normal tau, the appearance of hyperphosphorylated tau and a soluble form of truncated tau; and, in occasional cases, the presence of PHFs in frontal cortex. These changes were correlated with aluminium levels in the brain and may represent early events similar to those involved in the formation of PHFs in AD. It remains unclear, however, why overt neurofibrillary pathology was not observed in these patients, where excessive accumulation of aluminium in the brain occurs.

Dementia pugilistica in ex-boxers is associated with the presence of large numbers of neurofibrillary tangles (Corsellis et al., 1973) and the existence of both diffuse and neuritic plaques (Roberts et al., 1990). Furthermore, Aβ deposition may commence rapidly following head trauma (Roberts et al., 1991). Those possessing an apolipoprotein

E type ε4 allele are more susceptible to Aβ accumulation than those without (Nicoll et al., 1995).

Other extrinsic factors, for which roles in the pathogenesis of AD have been proposed, include the following: trace metals (*e.g.* iron, zinc, cobalt and mercury); neuroleptic treatment; infectious agents such as viruses and spirochaetes; pesticides; electromagnetic fields; organic solvents; and hypertension. Similarly, protective factors such as smoking, estrogen replacement, and exposure to non-steroidal anti-inflammatory drugs and H2-blocking drugs have been proposed.

The strongest evidence in support of an acquired environmental form of AD comes from monozygous twin studies; twin pairs show clear discordance for AD (Breitner & Welsh, 1995; Kumar et al., 1991). The critical factor(s) responsible for this observation, however, have not been identified. An environmentally-based etiology for the production of NFTs in Guam-ALS is suggested by two findings: the decreased frequency of the disease and an altered pattern of neuropathology that have occurred over a 30-year period since its discovery. The issue has been reviewed in this book by Perl in Chapter 6.

Tau Protein and Dementia in Alzheimer's Disease

Tau proteins belong to the family of microtubule-associated proteins. They are mainly expressed in neurons where they play an important role in the assembly and stabilisation of tubulin monomers into microtubules that constitute the neuronal cytoskeletal network. Microtubules are essential in morphogenesis, cell division and intracellular trafficking of organelles. At physiological concentrations tau stabilises microtubules as tracks for intracellular transport but, in excess, it interferes with transport down the axon. The full extent of its biological function *in vivo* remains to be seen. Tau plays a role in signal transduction, through its interaction with phospholipase C-γ; interacts with actin and the plasma membrane, is involved in anchoring protein kinases and phosphatases and is important in neurite outgrowth (Buée et al., 2000).

Human tau protein in the CNS exists in 6 isoforms, ranging from 352 to 441 amino acids in length, that are derived by alternative mRNA splicing from a single gene (*MAPT*) located on chromosome 17. Each isoform contains either 3- or 4- tandem repeats of 31- or 32- amino acids located in the C-terminal half of the molecule. These repeats are both rich in basic amino acids and capable of binding to an acidic domain of tubulin. Similar microtubule-binding repeats are found in the carboxy-terminal domain of high molecular weight microtubule-associated proteins, such as MAP2.

Tau proteins are the major constituents of intraneuronal and glial fibrillar lesions described in Alzheimer's disease and numerous neurodegenerative disorders referred to as 'tauopathies'. It is now well established that the density of neurofibrillary tangles is closely linked to intellectual status ante-mortem (Arriagada et al., 1992; Delaère et al., 1989; Delaère et al., 1991; Delaère et al., 1990; Duyckaerts et al., 1987; Giannakopoulos et al., 1997; McKee et al., 1991; Nagy et al., 1995; Wilcock & Esiri, 1982). The link is stronger than that found with the density of Aβ deposits, although the latter correlation can be improved somewhat by measuring the 'amyloid load' or the area occupied by Aβ deposits in the neocortex (Cummings & Cotman, 1995) or by the levels of soluble

Aβ (Näslund et al., 2000). On the other hand, the deposition of diffuse amyloid can be extensive in intellectually normal elderly cases (Giannakopoulos et al., 1997). In contrast, there are no reports of preservation of normal cognitive functioning in the presence of numerous neocortical tangles. Neuritic plaque densities tend to correlate better both with the biochemical measure of PHF-burden (see below), and also with cognitive dysfunction. Other studies also confirm that Aβ load does not predict neuronal loss (Gómez-Isla et al., 1997), synapse loss (Terry et al., 1991) or dementia (Gómez-Isla et al., 1997). Furthermore, there is considerable overlap in the levels of Aβ deposition found in AD and non-demented patients (Harrington et al., 1994a) that is not the case with PHF accumulation (Mukaetova-Ladinska et al., 1993). The PHF content in AD tissue is 19-fold greater than that found in control tissue, and the difference is even greater in brain regions such as temporal cortex. The accumulation of PHFs is accompanied by a corresponding loss of normal soluble tau, in excess of the loss due to normal aging (Mukaetova-Ladinska et al., 1996). In contrast, the levels of Aβ in nearly half of the cases of AD are found to overlap the levels found in controls, regardless of *APOE* genotype (Harrington et al., 1994a). These findings argue against the hypothesis that Aβ deposition induces a direct toxic effect on neurons in the human brain.

Tau pathology is closely linked with loss of synapses. It has been reported that loss of synapses provides a better correlate of cognitive deficit than neurofibrillary tangles (Terry et al., 1991). When comparing tangles and Aβ plaques, Terry and colleagues found that only tau pathology was associated with synapse loss. Others have confirmed these findings (DeKosky & Scheff, 1990; Price et al., 1991; White et al., 1995). More recently, the extent of synapse loss has been found to be as great in 'Lewy body variant' cases as in AD but, in the former, synapse loss is not linked to tau pathology (Brown et al., 1998). This may imply that in dementia with Lewy bodies, synapse and neuronal loss are mediated by quite different mechanisms (Harrington et al., 1994a). In AD, the failure of axonal transport is associated with early aggregation of tau protein, whereas the loss of synaptic proteins appears to be a relatively later phenomenon, occurring well after the onset of clinical symptoms (Mukaetova-Ladinska et al., 2000).

Mutations in the *MAPT* Gene in Neurodegenerative Dementias

The absence of any tau mutations in AD has been used as evidence that tau pathology was merely a secondary epiphenomenon in the disease. Recently, it has been found that mutations in the *MAPT* gene co-segregate with a number of familial dementias associated with a variety of phenotypes that may be broadly categorised as frontotemporal dementias. The locations of the mutations identified to date are shown in Fig. 3. Although mutations have not been found in AD, the discovery that tau mutations are associated with dementia in the absence of Aβ pathology further asserts the view that tau pathology need not be a secondary consequence of altered APP metabolism.

There are two main classes of mutation: those that affect alternative splicing of the *MAPT* gene and those that directly affect the function of the protein. Mutations in the 5′-splice site of exon 10 destabilise the intron 10 stem-loop structure, giving rise to an increased splicing in of the exon, which increases production of the 4-repeat isoforms

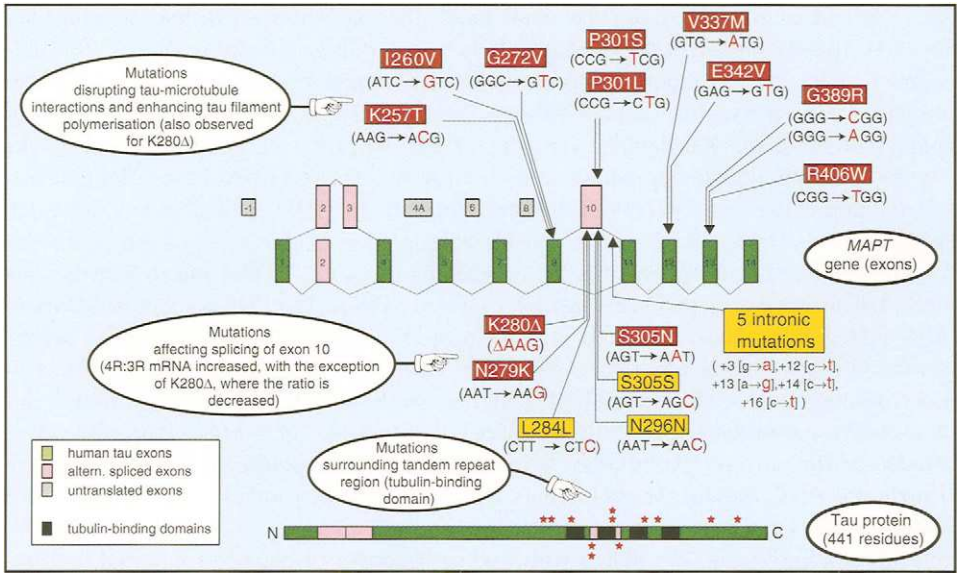

Fig. 3. Tau proteins are derived by alternative splicing of exons 2, 3 and 10. The largest isoform, with all three inserts, is 441 amino acids in length. Mutations associated with FTDP-17 can be separated into two major classes of mutation, as indicated. The location of the 12 mis-sense mutations (red) are indicated by asterisks. The other mutations (yellow) do not affect the coding sequence.

(Grover et al., 1999; Hutton et al., 1998; Spillantini et al., 1998; Varani et al., 1999). At the ultrastructural level, the tau filaments in these patients consist mainly of twisted-ribbon filaments, rather than the PHFs typical of AD (Spillantini et al., 1998).

Mutations that affect the coding sequence have been found in exons 9, 10, 12 and 13 of the *MAPT* gene. These mutations are clustered around the tandem repeated microtubule-binding region of the tau protein, with the exception of G389R and R406W in the C-terminal segment. Mis-sense mutations in exon 10 will only affect those tau isoforms containing 4 repeats. In contrast, all tau isoforms will be affected by those mutations on other exons. Intronic mutations and those close to the exon–intron boundary (S305N, S305S, L284L and N279K) have destabilising effects on the stem loop in the pre-mRNA at the exon 10 5′-intron boundary. Recombinant tau proteins carrying mis-sense mutations or the deletion mutation (K280Δ) have a decreased ability to promote microtubule assembly, which is more marked for 3- repeat than for 4- repeat isoforms (Bugiani et al., 1999; Hasegawa et al., 1998; Rizzu et al., 1999). A decrease in the binding of tau to microtubules could destabilise them and disrupt axonal transport. The tau mutations could also result in a toxic gain of function because decreased binding of mutant tau leads to increases in free, cytosolic tau with an increased propensity to form insoluble aggregates (Hong et al., 1998). Of the mutations tested, the P301L and K280Δ mutations in exon 10 have the greatest effect on microtubule assembly. The P301L mutation has the greatest potential for fibril formation, and spherical structures are obtained by incubation of the mutant protein in the absence of heparin (Nacharaju et

al., 1999). Furthermore, filaments are produced in the brains of mice that are transgenic for this mutant form of tau protein (Lewis et al., 2000). The mutations N279K and S305N, on the other hand, do not show any decreased ability to promote microtubule assembly. The latter mutations increase the splicing-in of exon 10. It is possible that subtle changes in the ratio between 4- and 3-repeat tau isoforms may be sufficient to cause neurodegeneration over a prolonged period. Greater distances between the microtubules and fewer microtubules per process were observed with the V337M mutant isoform (Frappier et al., 1999).

The varied ways in which mutations in tau affect the processing and function of this protein is probably responsible for the phenotypic heterogeneity observed in patients with frontotemporal dementia with parkinsonism linked with chromosome 17 (FTDP-17) (Bird et al., 1999; D'Souza et al., 1999; Goedert, 1999). Despite variations, the characteristic hallmark for all of these tauopathies is the accumulation of aggregated, insoluble tau protein. What other factors are involved in the phenotypic variability is not known at present. In contrast to AD, *APOE* genotype does not influence age of onset in FTDP-17 (Bird et al., 1999; Houlden et al., 1999).

The regional distribution, ultrastructural and biochemical characteristics of the tau deposits in FTDP-17 differentiate them from those present in AD, corticobasal degeneration (CBD), progressive supranuclear palsy patients (PSP) and Pick's disease. In many families no Aβ deposits are present, indicating that FTDP-17 is a disorder distinct from AD. This implies that the tau aggregation in the brain is not dependent upon prior deposition of Aβ. Conversely, it implies that intraneuronal tauopathy does not necessarily lead to the deposition of Aβ. If tau and amyloid pathologies are linked in AD, then some other factor(s) are required to account for the association between these two deposits in the brain in AD.

Animal Models of Alzheimer Pathology

Animal models of AD are essential to understand the relationship between the biochemical and pathological changes in the brain and impairment of memory and behaviour. Furthermore, they enable the pathogenesis of the disease process to be examined *in vivo* and provide a model in which therapeutic strategies can be tested. Having said this, all existing models fail to display the combination of tangles, plaques and cognitive impairment that is characteristic of AD. Aged dogs and non-human primates develop β-amyloidosis, but tau pathology is not a feature in these animals (Walker, 1997). Similarly, transgenic mouse models of β-amyloidosis have been created, but these fail to exhibit abnormal deposition of tau (Janus et al., 2000).

Conversely animal models of tau pathology fail to demonstrate amyloid pathology. The tangles that accumulate in the brains of rodents treated with aluminium differ in their ultrastructure from those found in AD (see above). Filamentous cytoskeletal changes associated with abnormally phosphorylated tau have been observed in aged baboons, bears, sheep and goats (Braak et al., 1994; Cork et al., 1988; Nelson et al., 1994; Nelson & Saper, 1995; Roertgen et al., 1996; Schultz et al., 2000).

Some experiments have implicated links between tau and amyloid in animal models.

Hyperphosphorylated tau accumulates in the somatodendritic compartment of neurons in rat brains following chronic intraventricular infusion of okadaic acid, an inhibitor of protein phosphatase 2A (Arendt et al., 1995). Furthermore, the okadaic acid treatment also led to the formation of extracellular deposits of Aβ and memory impairment. Two other studies have implicated Aβ in the accumulation of tau in animal models. Microinjections of fibrillar Aβ in the cortex of aged rhesus monkeys resulted in focal accumulations of intracellular phosphorylated tau (Geula et al., 1998). This was dependent upon both age and species; the same result was not observed for rats or young rhesus monkeys. Secondly, focal deposits of tau have been observed in mice that were transgenic for APP carrying AD-associated mutations (Sturchler-Pierrat et al., 1997).

The brains of tau-deficient mice appeared immunohistochemically normal and axonal elongation was not affected in cultured neurons (Harada et al., 1994). Microtubule stability, however, was decreased and its organization altered in some small-calibre axons. Furthermore, an increase in the levels of microtubule-associated protein 1A (MAP 1A), which might compensate for a functional loss of tau in large-calibre axons, was found. Thus, tau seems to be crucial in the stabilization and organization of axonal microtubules in certain axons. Subsequent studies have demonstrated that tau-deficient mice exhibit signs similar to certain symptoms characteristic of frontotemporal dementia patients, *i.e.* personality changes (disinhibition/aggression) and deterioration of memory and executive function. The mice showed muscle weakness and impaired balance control, hyperactivity in a novel environment, and impairment in contextual fear conditioning (Ikegami et al., 2000). Spatial learning tasks, however, were unaffected in the mice as is the memory function in FTDP-17 patients.

Although neurofibrillary tangles and phenotypic alterations were not reported in animals transgenic for 3- or 4-repeat tau isoforms (Brion et al., 1999; Götz et al., 1995), these findings may reflect low levels of protein expression. Impaired motor function, in the presence or absence of tauopathy, has been observed in mice expressing higher levels of human tau protein (Ishihara et al., 1999; Spittaels et al., 1999). In these animals the filamentous inclusions did not exhibit the ultrastructural features of AD PHFs. Over-expression of tau in sea lamprey neurons *in situ*, however, did result in PHF-like formation and provides evidence of neurodegeneration in a simple vertebrate model (Hall et al., 2000).

A recent transgenic mouse model expressing human tau with the P301L mutation develops neurofibrillary tangles, neuronal loss and motor dysfunction (Lewis et al., 2000). The tau inclusions in these mice show both straight and twisted ribbon filaments similar to those found in human patients. Attempts to combine this model with amyloidosis are underway by crossing these mice with those transgenic for APP.

Aluminium maltolate-induced neurofibrillary degeneration in rabbits offers a further model in which shared mechanisms with the process of neurofibrillary tangle formation in AD appear to be indicated. The degenerative process in the rabbit model is associated with normal and hyperphosphorylated tau and the presence of APP, Aβ, α_1-antichymotrypsin and ubiquitin (Huang et al., 1997). Furthermore, evidence for oxidative stress and apoptosis in hippocampal neurons have been reported in a similar rabbit model (Rao et al., 2000). This experimental system is reviewed by Savory in this book.

Modifications of Tau Protein in Disease

In AD, there is a substantial redistribution of tau protein from its normal axonal location into somatodendritic PHFs (Harrington et al., 1991; Mukaetova-Ladinska et al., 1993). The mechanism whereby this occurs, however, is not understood. Tau protein is subject to a number of post-translational modifications that could potentially affect this distribution (Fig. 4). These include ubiquitination, phosphorylation, *N*- and *O*-glycosylation, non-enzymatic glycation and oxidation. Several of these modifications are probably relevant to the insoluble, protease-resistant state of PHFs found in Alzheimer's disease. Whether they occur as early events in the formation of the filaments that constitute tangles or whether they are secondary phenomena is less easy to demonstrate. Post-translational modifications are discussed in the following paragraphs, whereas the mechanism of tau aggregation and proteolytic truncation of tau are described later.

Non-enzymatic glycation

Protein glycation occurs in diabetes and is a frequent feature of ageing. In AD the neuropathological lesions in the brain are glycated. Whether or not such glycation is a primary or secondary event in AD is uncertain (Harrington & Colaco, 1997). Tangles, plaques, Hirano and Lewy bodies, granulovacuolar degeneration and lipofuscin are structures in the brain that are all modified by advanced glycation end products (AGEs) (Kimura et al., 1998; Münch et al., 1998; Sasaki et al., 1998; Smith et al., 1994; Takeda et al., 1998; Vitek et al., 1994; Yan et al., 1994). The addition of AGEs can affect protein structure and function, induce oxidative stress and lead to inflammation and tissue damage. Tau protein has abundant lysine residues suitable for glycation (Ledesma et al., 1995; Ledesma et al., 1994; Nacharaju et al., 1997).

Glycation of tau protein, rather than being involved in the assembly of PHFs, seems

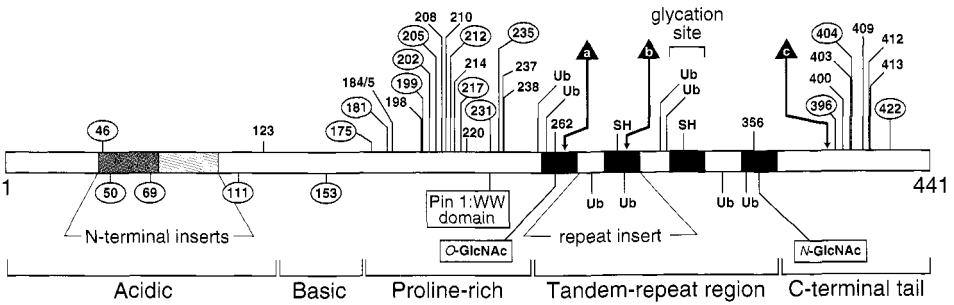

Fig. 4. Post-translational modifications of tau protein. Phosphorylation sites of tau are numbered; above are those which have been identified in PHF-tau. The fragment of tau found in the PHF core indicates proteolytic N-terminal truncation at either Leu-266 (a) or Ile-297 (b) and C-terminal truncation at Glu-391 (c). SH denotes the two Cys residues in the repeat region that can form either intra- or inter-chain disulphide bonds. Four lysine residues in PHF-tau serve as ubiquitin acceptors (Ub); other potential sites are indicated below. Sites of glycation in PHF-tau and enzymatic glycosylation sites that may modify tau within the repeat region are shown. The WW domain of the prolyl isomerase Pin 1 binds to phosphorylated Thr-231, placing the isomerase site of the enzyme in the vicinity of its Ser-/Thr-Pro substrates.

to be involved in the cross-linking of filaments which, in turn, leads to the increased insolubility and protease resistance associated with advanced neurofibrillary pathology (Harrington & Colaco, 1997; Münch et al., 1997; Smith et al., 1995).

Enzymatic glycosylation

O-linked *N*-acetyl glucosamination is a dynamic and abundant post-translational modification that can be reciprocal with Ser-/Thr- phosphorylation (Hayes & Hart, 1994). In an initial study bovine tau was modified by *O*-linked glycosylation at multiple sites, and it is likely that this modification may play a role in modulating tau function (Arnold et al., 1996). More than 12 different sites were glycosylated to the extent of at least 4 mols *O*-GlcNAc per mol of tau. One of these attachment sites resides within the first microtubule-binding domain. The phosphorylation of Ser-/Thr- residues in PHF-tau would decrease the chance of finding *O*-GlcNAc in PHFs. Modification of PHF-tau, but not normal tau, by *N*-linked glycosylation has been reported (Wang et al., 1996). In contrast to *O*-linked glycosylation, *N*-linked glycosylation usually occurs co-translationally and is a considerably less dynamic modification. Deglycosylation of PHFs, however, converts them into straight filaments, a property that suggests N-linked glycosylation may contribute to the maintenance of the PHF structure (Wang et al., 1996). *N*-glycosylation affected the helicity of PHFs as well as stabilising tau. Immunohistochemical lectin-binding studies indicated that both tangles and neuritic plaques are sites of glycan addition. Intracellular but not extracellular neurofibrillary pathology is positive for *Galanthus nivalis* agglutinin (Takahashi et al., 1999). Similar changes in tangle-free neurones suggest that glycosylation may be involved early in the pathogenesis of AD (Guevara et al., 1998). Studies with synthetic tau peptides indicate that N-glycosylation of Asn-359 within the fourth repeat not only favours the isomerisation of the adjacent Asp residue (Hoffmann et al., 1999) but also inhibits the normal tau–tubulin interaction (Otvos et al., 1998).

Ubiquitination

Ubiquitin is a conserved, 76-amino acid polypeptide that binds to target proteins and is involved in the removal of abnormal or damaged proteins, which become degraded in proteasome complexes. The amino-terminal portions of tau are cleaved to a greater extent in those PHFs that have been ubiquitinated (Morishima-Kawashima et al., 1993). The long-lived ghost tangles in the brain appear to be relatively resistant to degradation and removal. It may be that the proteasome complex fails to get access to the ubiquitinated sites located inside tightly packaged PHFs. However, it seems likely that ubiquitination occurs as a late event in the formation of neurofibrillary tangles as suggested by the results from immunohistochemical studies (Bancher et al., 1991).

Phosphorylation

Expression of the different tau isoforms is developmentally regulated. A combination of kinase and phosphatase activity contributes to the extent of phosphorylation of

tau both in normal situations and in Alzheimer's disease (Buée et al., 2000). Thus tau from the neonate is phosphorylated at more sites than tau from adult brain and tau phosphorylation in developing brain is more dynamic than in adult brain. Tau is phosphorylated to an even greater extent in PHFs, and at least 20 sites have been identified to date (Hanger et al., 1998; Morishima-Kawashima et al., 1995). Attempts to identify AD-specific phosphorylation sites, however, have not yielded conclusive results. When phosphorylated, tau loses its ability to bind microtubules (Drewes et al., 1995; Trinczek et al., 1995). Phosphorylation of tau also decreases the self-association of tau protein (Guttmann et al., 1995) and decreases the rate at which it can be degraded by the protease, calpain (Litersky & Johnson, 1992).

Phosphorylation on Ser-/Thr- Pro motifs decreases the rate of isomerisation of the Ser-/Thr- Pro bonds, and proteins with these motifs are substrates for the prolyl isomerase Pin 1. Pin 1 has been found to restore the microtubule-binding capacity of phosphorylated tau and is sequestered in NFTs in AD (Lu et al., 1999). Pin 1 also binds to several mitotic phosphoproteins that may be affected in AD. The capture of Pin 1 within tangles depletes the soluble pool of this enzyme in AD, a situation that mimics the triggering of mitotic arrest leading to apoptosis. Binding of Pin 1 to phosphorylated tau and subsequent isomerisation of the peptide bonds may change the conformation of domains in tau protein. Pin 1 specifically binds to the phosphorylated Thr-231 site in tau, via a WW domain that is separate from the domain involved with isomerase activity. Phosphorylation of Thr-231 is both necessary and sufficient for mediating the interaction between Pin 1 and tau. Thus Pin 1 might be needed to prevent abnormal activation of mitotic events in differentiated neurons. Inappropriate expression of mitotic proteins in AD brain (Vincent et al., 1997; Vincent et al., 1998) may contribute to hyperphosphorylation of tau and the neurodegenerative process in AD. Phosphorylated tau would also be capable of sequestering Pin 1, which itself can trigger apoptosis.

It is possible that tau may be linked with metabolism of APP through a signal transduction pathway (Lovestone & Reynolds, 1997). Protein kinase C can decrease both Aβ production and the phosphorylation of tau and tau interacts with a key enzyme in the phosphatidyl inositol signalling pathway, phospholipase C-γ (Hwang et al., 1996). A link between the cholinergic signal transduction pathway and tau phosphorylation has also been indicated (Sadot et al., 1996).

Other modifications

Because of the insoluble nature of the PHF-tau aggregates in AD brain tissue, it is difficult to be certain whether modifications of tau are involved in PHF assembly or merely responsible for increasing their insoluble status. Aside from those modifications described above, transglutamination and racemisation of amino acids can affect tau protein (Dudek & Johnson, 1993; Kenessey et al., 1995). Proteolysis and aggregation of tau protein are discussed in the following sections.

Aggregation of Tau Protein and Conformational Diseases

It has never been demonstrated crystallographically that PHFs have any underlying cross β-pleated structure. However, in view of the shared ability of PHFs to bind planar dyes, it seems likely that they share at least some of the generic ultrastructural features of amyloids. The behaviour of tau protein in solution is remarkable for its lack of ordered secondary structure, whether normal human tau, recombinant tau or hyperphosphorylated tau has been analysed (Lang et al., 1992a; Lang et al., 1992b; Schweers et al., 1994). It seems unlikely, therefore, that the aggregation of tau in PHFs is based on interactions between strands of β-sheets. There is only one report of the expected β-pleated aggregation *in vitro*, and this was obtained using fragments of tau restricted to the C-terminal tail, which are not the major component of the proteolytically stable core of the PHF (Yanagawa et al., 1998). Minor local regions might be important in conferring conformational change and it was reported recently that the peptide VQIVYK, corresponding to residues 306–311 in tau, forms a β-structure capable of inducing PHF-like formation (von Bergen et al., 2000).

Several neurodegenerative diseases, in which there is aberrant protein aggregation, are characterised by protein misfolding. These disorders have been given the terms 'conformational diseases' (Carrell & Gooptu, 1998) or 'prionoses' (Wisniewski et al., 1998). These include diseases that predominantly affect the cerebral cortex, such as AD, frontotemporal dementia and prion disorders, and those affecting basal ganglia, such as Huntington's and Parkinson's disease. Despite differences, these disorders share several major features (Carrell & Gooptu, 1998; Soto, 1999; Wischik et al., 1997). They generally occur as disorders of mid- to late-life and are associated with the abnormal processing of normal precursors, namely APP, PS, tau, prion protein, huntingtin and α-synuclein. In AD, FTDP-17, Parkinson's disease and prion diseases, mutations in these proteins are associated with inherited forms of the disorder. These disorders also occur in a sporadic form.

Altered processing of the different proteins leads to the accumulation of aggregates either as inclusions or deposits throughout selective areas of the brain. Plaques, tangles, Lewy bodies and intranuclear inclusions are all signs of advanced pathology that probably reflect earlier, more damaging changes within the brain. In Parkinson's disease and related disorders, intraneuronal α-synuclein fibrils are present in Lewy bodies and in Lewy neurites (El-Agnaf & Irvine, 2000; Wanker, 2000). Both tau protein and α-synuclein show a propensity to self-assemble into fibrils and form neuronal inclusions. Mutations in either of these proteins are associated with accelerated fibril formation. Both proteins are subject to post-translational modifications (*e.g.* phosphorylation, glycation, ubiquitination and proteolysis). Recently, a correlation between the formation of insoluble protein aggregates and disease progression has been identified in transgenic models. An important difference between the two is that tau is a cytoskeletal protein that can associate with the plasma membrane, whereas α-synuclein is a cytoplasmic protein enriched at synaptic terminals. Nonetheless, in both cases, protein aggregation occurs in neurons of defined vulnerable brain regions.

Determinants of tau aggregation

Filament assembly in solution has been used widely to study the aggregation properties of tau protein (Crowther et al., 1992; Crowther et al., 1994; Garcia de Ancos et al., 1993; Montejo de Garcini et al., 1986; Ruben et al., 1993; Troncoso et al., 1993; Wille et al., 1992). However, such studies have yielded inconsistent results (Schweers et al., 1995; Wille et al., 1992; Wilson & Binder, 1995), probably because it has been difficult to induce tau aggregation experimentally. Full-length recombinant tau does not aggregate in physiological conditions (Yanagawa et al., 1998), although full-length tau and fragments restricted to the repeat domain assemble in non-physiological buffers and at high protein concentrations, irrespective of phosphorylation status (Crowther et al., 1994; Wille et al., 1992). The conditions in which tau aggregation has been observed are artificial, including dialysis of urea-treated brain extracts, spraying of glycerol solutions of tau and hanging-drop crystallization conditions. Conversely, several non-tau proteins have also been shown to form PHF-like assemblies *in vitro*, including the C-terminal tail of APP (Caputo et al., 1992), α-1 antitrypsin (Janciauskiene et al., 1995; Lomas et al., 1992) and Aβ (Exley et al., 1995). As none of these proteins are found in PHF preparations, the morphology of filaments produced *in vitro*, even when this closely resembles the PHF, is an insufficient criterion to establish disease relevance.

Procedures have been developed whereby tau aggregation could be facilitated by co-incubation of tau protein with sulphated glycosaminoglycans (Goedert et al., 1996; Pérez et al., 1996). It appears that the N-terminal half of the protein inhibits aggregation, and this inhibition can be overcome by a range of polyanionic cofactors, including heparin, heparan sulphate, RNA and polyglutamine (Friedhoff et al., 1998a; Goedert et al., 1993; Hasegawa et al., 1997; Kampers et al., 1996). Although tau assembly can be induced in more physiological buffers, using this manoeuvre, the concentrations of tau required remain very high. Typically, concentrations between 40 μM to 100 μM are required, whereas direct measurements in the human brain indicate that tau protein concentrations are unlikely to exceed 1μM within pyramidal cells (Lai et al., 1995).

A property of protein aggregation systems is that the initial nucleation step is rate-determining, whereas the elongation step is rapid, indeed autocatalytic. Mandelkow and co-workers have shown that tau aggregation can be seeded, greatly increasing the efficiency of the process in a concentration-dependent manner (Friedhoff et al., 1998b). This was achieved using crosslinked dimers or pre-formed tau aggregates extracted from the brain. Furthermore, in this system, N- and C-terminal truncation increased the rate of *in vitro* filament formation. Similarly, PHF-tau extracts from the brains of AD and CBD patients facilitate tau aggregation in the presence of high levels of Ca^{2+} and Mg^{2+} (Yang & Ksiezak-Reding, 1999). With amyloids, aggregation begins with dimer formation. There is no evidence that the dimer has an independent existence, as argued by Mandelkow in the case of the disulphide cross-linked form of truncated tau (Friedhoff et al., 1998b). In other studies, however, neither oxidation nor phosphorylation of truncated tau fragments enhanced tau aggregation (Hoffmann et al., 1997).

The ability to form aggregates is not the peculiarity of a relatively small group of proteins known to be implicated in human disease; a large number of proteins have the capacity to form amyloid fibrils which aggregate (Dobson, 1999). At sufficient

concentrations aggregation can stabilise an aberrant conformational structure, which then acts as a seed for further polymerisation, recruiting more normal molecules into the aggregate (Carrell & Gooptu, 1998; Cohen & Prusiner, 1998; Kelly, 1998; Lansbury, 1999; Wisniewski et al., 1998). This represents the 'conformational change' hypothesis. Once the β-fibril configuration has been achieved, the protein, or those parts forming the core of the fibril structure, becomes indestructible under physiological conditions, because of the large number of hydrogen bonds that must be disrupted in order to rescue the peptide chain from the aggregated state (Dobson, 1999). Prion diseases exemplify the mechanics of transition proteins to the proteolytically stable aggregate (Cohen & Prusiner, 1998). In this instance, the normal transmembrane protein (PrP^{C}) is converted into a highly proteolytically stable shortened form (PrP^{Sc}). PrP^{C} is composed of about 40% α-helix and little β-sheet, whereas PrP^{Sc} is composed of about 30% α-helix and 45% β-sheet. The similarity between several neurodegenerative diseases characterised by protein aggregation suggests that common principles may underlie these disorders and that generic therapeutic approaches may be feasible.

Informed Opinion

AD and related neurodegenerative disorders are complex diseases. Different phenotypes can arise from a single mutation (*e.g.* P301S is associated with either CBD or FTD in a single family (Bugiani et al., 1999)). Conversely, mutations at different loci are associated with the 'same' disease (*e.g.* AD that is indistinguishable between patients with either APP or PS mutations). Furthermore, multiple pathologies can be found in a single individual (*e.g.* tauopathy and synucleinopathy may co-exist in AD and Parkinson's disease). In AD, neurofibrillary tau pathology is a close correlate of dementia at a stage where axonal transport is disrupted (Mukaetova-Ladinska et al., 2000). What drives tau aggregation and how the process is initiated is central to our understanding of the aetiology of AD (Fig. 5).

Initiation of Tau Aggregation

Normal tau protein has little in the way of physical structure, and PHF-tau shows no significant structural changes (Schweers et al., 1994). Nevertheless, tau aggregation through the repeat domain confers proteolytic stability on a short segment of the molecule that corresponds closely to that isolated form the PHF-core. This fragment has the intrinsic capacity to propagate tau capture (Wischik et al., 1996). Although extracellular matrix proteins (heparin) and acidic proteins (RNA) can promote tau aggregation *in vitro*, facilitation of tau aggregation may be due to a general property of macromolecular substrates that bind tau non-specifically (Wischik et al., 1997). The substrates that initiate tau aggregation *in vivo* remain to be identified (Fig. 5).

It is possible that stochastic conformational changes to tau molecules occur with aging (sporadic AD). Alternatively, conformational changes might be induced through interaction with other proteins or macromolecular complexes. This could include inter-

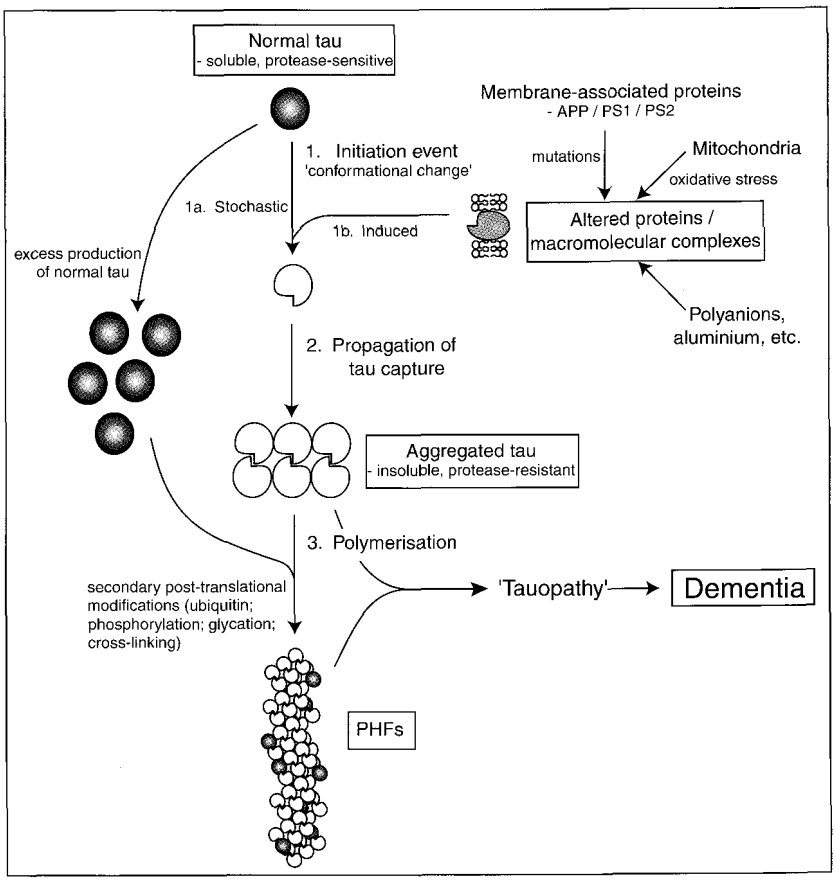

Fig. 5. Aggregation of tau protein in the aetiology of AD.

action with transmembrane proteins, such as mutant forms of APP, PS1 or PS2. Protein interaction could take place in intracellular compartments such as the ER or Golgi or within vesicles of the endocytic pathway. Alternatively, membrane proteins from damaged mitochondria might serve as a substrate for the capture of tau (Wischik et al., 1997). Two mitochondrial proteins (core protein 2 of the cytochrome *bc1* enzyme complex and porin) tightly complexed with insoluble and truncated PHF-tau have been isolated from AD brain tissue (Wischik et al., 1997). Likewise truncated tau aggregates are associated with abnormal mitochondria in AD using immunoelectronmicroscopy (Mena et al., 1996). Binding of endogenously truncated tau to mitochondria within pyramidal cells in AD raises the possibility that age-related defects in endosomal–lysosomal turnover of other membrane-spanning proteins, such as APP or the presenilin proteins, might provide substrates to initiate tau capture.

It has been reported that the transmembrane region of APP just C-terminal to the Aβ domain is immunochemically associated with PHFs (Giaccone et al., 1996). A peptide, corresponding to this region, forms fibrils *in vitro* and dense fibrillary assemblies are

generated in the presence of tau. These findings extend their observation that tau interacts with a conformation-dependent domain of APP encompassing residues 714–723. This forms part of the transmembrane domain of APP and one in which several residues are known to be mutated (Fig. 1). PHF-like structures are also assembled from the cytoplasmic APP peptide, consisting of residues 751–770 (Caputo et al., 1992) or from amyloid β-peptide in the presence of aluminium (Exley et al., 1995). Thus either of these peptides might conceivably act as substrates for the initiation of tau assembly into PHFs.

Despite the presumption that all neurodegenerative diseases might have a genetic aetiology (Hardy & Gwinn-Hardy, 1999), it still behoves us to examine whether non-genetic factors are involved. Aluminum, for example, could be implicated in a conformational change of normal tau protein, or in the acceleration of aggregation and polymerisation of tau into fibrils. Any one of the post-translational modifications of tau protein has the potential to alter the conformation of tau. It might only need a single modified molecule to initiate the seeding and capture of non-modified tau. There is no evidence to date to indicate that mutations in tau are involved in AD. In FTDP-17, however, mutations affect the microtubule assembly properties of tau protein (Hong et al., 1998). It is not clear how such mutations might accelerate fibril formation. Although tau bearing the mutations G272V, P301L, V337M and R406W had greater α-helical content than wild-type tau (Jicha et al., 1999), these results were not confirmed by others (Goedert et al., 1999). The P301L mutation increases the propensity for isolated repeats to aggregate (Arrasate et al., 1999) indicating that tau possessing mutations may be more susceptible to aggregation following proteolysis.

Propagation of Tau Capture

A role for proteolytic processing in the aggregation of tau protein has been studied *in vitro* as a result of the findings of biochemical studies using AD brain tissue (Wischik et al., 1996; Wischik et al., 1997). Tau–tau binding of high affinity through the repeat region confers proteolytic stability on this domain of the protein. Proteolytic truncation of the N- and C-termini of tau aggregates increases the capacity for more tau to be bound. This process is inhibited by phosphorylation of tau, suggesting that phosphorylation, rather than accelerating filament assembly, actually protects the protein from aberrant aggregation. The capture and propagation of tau capture *in vitro* is autocatalytic (Wischik et al., 1996). In other words the sequential capture and proteolytic processing of tau generates a substrate that has a greater affinity for the further capture of tau molecules. One explanation is that protein conformation is altered following the tau–tau interaction. This could also be the scenario were a non-tau protein to serve as the substrate for the initiation of tau aggregation.

The exponential increase in tau capture may represent a biological correlate of the rapid changes that occur around the stage when symptoms start to develop. In early stage AD, the redistribution of tau into PHFs is exponential with respect to the level of PHF-tau (Lai et al., 1995), *i.e.* the greater the PHF burden, the greater the rate of incorporation of normal tau into PHFs. The medial temporal lobe is the region most severely affected by tau pathology and its shrinkage, as measured by computerised tomographic scans,

progresses exponentially during AD (Jobst et al., 1992). Thus tau aggregation in early stages of AD is an essential feature of the disease. If tau aggregation is critical for the rapid progression of AD, then its inhibition should prevent the symptoms of dementia. In this respect, agents which inhibit tau aggregation and which disrupt isolated PHFs have been reported (Wischik et al., 1996).

In conclusion, various mechanisms to initiate tau aggregation in AD and other tauopathies have been proposed. These include a number of the post-translational modifications of tau protein, stochastic conformational changes in tau that increase in frequency with advancing age, mutations in the membrane-bound APP or the presenilin proteins, damage resulting from neuronal oxidative stress and the involvement of aluminium. Once started, aggregation of tau proceeds at an exponential rate that is consistent with the rapid development of clinical symptoms. Neurodegeneration might arise as a result of cytoskeletal destruction or be due to a gain of toxic function for the aggregated tau. The molecular mechanisms underlying neuronal dysfunction are still not known. With a better understanding of tau aggregation, it will be possible to combine roles for several factors in the aetiology of this genetically heterogeneous disorder. Finally, knowledge gained from tau aggregation may be of relevance to other neurodegenerative disorders involving other proteins. A demonstration that protein aggregation causes neurodegeneration will be established if agents that inhibit such aggregation are able to prevent the progression of the disease.

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Aluminium and Alzheimer's Disease
The Science that Describes the Link
C. Exley (Editor)

CHAPTER 6

The Association of Aluminum and Neurofibrillary Degeneration in Alzheimer's Disease, a Personal Perspective

Daniel P. Perl *

Professor of Pathology, Psychiatry and Neurobiology, Mount Sinai School of Medicine, New York, NY 10029, USA

* Corresponding author, e-mail: daniel.perl@mssm.edu

Abbreviations: ALS – amyotrophic lateral sclerosis; PDC – parkinsonism/dementia complex (of Guam); LAMMA – laser microprobe mass analyzer; Nd : YAG – neodymium : yttrium aluminum garnet; He : Ne – helium : neon; NINCDS–ADRDA – National Institute of Neurological and Communicative Disorders and Stroke–Alzheimer's Disease and Related Disorders Association; PIXE – proton induced X-ray emission; NFT – neurofibrillary tangle; ANOVA – analysis of variance

Summary

The association of aluminum and Alzheimer's disease has a long and controversial history. Using scanning electron microscopy with X-ray spectrometry we showed evidence of aluminum accumulation in the neurofibrillary tangle-bearing neurons of Alzheimer's disease. With a similar approach we also demonstrated prominent aluminum accumulations in tangle-bearing neurons of cases of ALS/parkinsonism-dementia complex of Guam. Studies using laser microprobe mass analysis demonstrated increased concentrations of iron and aluminum within the neurofibrillary tangle, itself. We suggest that aluminum is a requisite constituent of the neurofibrillary tangle, binding to negatively charged sites on its constituent proteins and acting as a cross-linking stabilizer.

Historical Perspective

Introduction

The association of aluminum and Alzheimer's disease has a long and controversial history. In 1965, it was first reported that direct exposure of rabbit cerebral cortex to aluminum-containing compounds produced dramatic neurofibrillary degeneration. Aluminum had long been known to be highly neurotoxic to cats and rabbits, where local application of aluminum-containing compounds was used experimentally to induce

focal seizures. Klatzo and coworkers (1965) as well as Terry and Peña (1965) reported simultaneously the induction of apparent neurofibrillary changes in cerebral cortical, brainstem and spinal cord neurons which were initially considered by light microscopic criteria to be comparable to the neurofibrillary tangles encountered in cases of Alzheimer's disease. Treated animals showed evidence of short-term memory deficits (King et al., 1975, Solomon et al., 1990) and other cognitive abnormalities. Subsequent studies have shown that the aluminum-induced tangles were composed primarily of straight filaments, rather than the paired helical filaments of human neurofibrillary tangles (Wisniewski et al., 1976).

Based on these findings, Crapper (McLachlan) and colleagues (1973) reported evidence of a 2 to 3-fold increase in the concentration of aluminum in brain specimens derived from 4 individuals with Alzheimer's disease, when compared to normal controls. Aluminum, although ubiquitous in the environment as the most abundant metallic element in the earth's crust, is not considered essential to life and is present in very small amounts in most tissues of the body. This is particularly true of the brain where less than 2 μg/g dry weight of cerebral cortex is now considered a normal concentration. Subsequent studies by Crapper (McLachlan) and colleagues (1976) analyzed a greater number of specimens and reported regional differences which appeared to follow the extent of neurofibrillary tangle formation in the tissues being analyzed. These studies employed atomic absorption spectrometry to determine bulk aluminum concentrations in the individual tissue samples. The total aluminum concentration in normal brain tissue was extremely small and although the increases reported were relatively large, nevertheless, the total amounts being determined were still quite small.

Microprobe Approaches to the Question of Aluminum and Alzheimer's Disease

The atomic absorption spectrometric approach measures total aluminum content of the entire sample being analyzed and is essentially destructive in nature. This technique cannot address the question of localization of the element on the cellular or subcellular level. In a situation in which the element was concentrated focally within a lesion that only comprised a small portion of the overall tissue mass, such bulk analytic approaches might miss the differences between normal and pathologic samples. Recognizing this problem, Perl and Brody (1980) turned to a microprobe analytic approach. Using the technique of scanning electron microscopy in conjunction with energy dispersive X-ray spectrometry, they reported evidence of aluminum accumulation within the hippocampal neurofibrillary tangle-bearing neurons of cases of Alzheimer's disease. Adjacent tangle-free neurons in the Alzheimer's disease cases and normal appearing neurons in aged controls did not show a similar degree of aluminum accumulation. Additional evidence was provided of focal aluminum accumulation using linear scans through the neurofibrillary tangle-bearing neurons identified in the specimens and analyzing the emerging X-ray signal by wave-length dispersive analysis.

This study suggested that most of the aluminum-related peaks were localized in the tangle-bearing neurons to the nuclear region. However, it should be recognized that the study employed cryostat tissue sections that were relatively thick (20 μm) and

therefore precise localization was virtually impossible. The tangle-bearing neurons were identified on sections that had been stained by a silver impregnation technique, similar to that originally used by Alzheimer, with the tangles being visualized by back-scattered electron imaging. Free floating tissue sections were stained with silver and then mounted on pure graphite stubs, which served as a substrate to support them in the scanning electron microscope. Marked differences in the depth of penetration of the electron beam and the relative efficiency of the emitted X-rays to leave the surface of the section, coupled with imprecise histologic detail in the surface and backscattered images all combined to severely limit the ability to identify the subcellular location of the aluminum accumulations being identified.

The use of the silver staining approach raised concern by some that the introduction of large amounts of silver in the staining protocol (and potentially other constituents within the stain reagents) might have introduced the aluminum we had identified merely through its inadvertent addition in the processing of the tissues prior to analysis. However, when other dense silver deposits were probed on the same tissue sections, aluminum-related peaks were not identified. Since, in even the most severe case of Alzheimer's disease, only a minority of hippocampal neurons demonstrate a neurofibrillary tangle, it had been impossible to carry out the determinations in the absence of some way to identify which neurons contained neurofibrillary tangles and which did not. Because the sections were stained with silver, there was considerable variation in the intensity of the emission of non-specific background X-rays (bremstrallüng). With the instrumentation available at that time, this variability precluded any meaningful attempt at quantitative analysis of peak densities (even following background subtraction) or of elemental mapping.

The next major contribution of this group to this question dealt with similar studies of the neurofibrillary tangles associated with amyotrophic lateral sclerosis/parkinsonism-dementia complex (ALS/PDC) of Guam. ALS/PDC of Guam is an unique neurodegenerative disorder of inordinately high prevalence among the native Chamorro population living on the island of Guam in the western Pacific (Wiederholt, 1999, Lilienfeld et al., 1994). Clinically and neuropathologically, it has features of all three age-related neurodegenerative disorders, namely amyotrophic lateral sclerosis (motor neurone disease), Parkinson's disease and Alzheimer's disease. In addition, the brains of victims of this disorder show large numbers of neurofibrillary tangles. Ultrastructurally, histochemically and biochemically, these neurofibrillary tangles are identical, in all ways in which they have been tested, to the neurofibrillary tangles of Alzheimer's disease (Piccardo et al., 1988, Shankar et al., 1989, Buee-Scherrer et al., 1995). In 1982, Perl working with Garruto and colleagues (Perl et al., 1982) published evidence of dramatic aluminum accumulation in the neurofibrillary tangle-bearing neurons of specimens derived from cases of ALS/PDC of Guam. The ALS/PDC cases chosen for study had such severe involvement by neurofibrillary tangle formation in the CA1 region of the hippocampus that virtually all pyramidal neurons in that portion of the brain demonstrated that lesion. These cases were compared with specimens derived from Guam natives that were virtually free of neurofibrillary tangles. Since all identifiable neurons in the affected cases contained a neurofibrillary tangle the specimens could be examined as unstained sections. Furthermore, because all of the specimens were handled in identical fashion and no staining procedure was used, this effectively avoided any potential controversy regarding

post-mortem introduction of aluminum prior to the analysis. The results showed dramatic aluminum-related peaks obtained from the tangle-bearing neurons when compared to that obtained from the cells in the tangle-free cases.

The finding of prominent aluminum accumulation in the tangle-bearing neurons of ALS/PDC of Guam represents an important observation and extends our previous observations made on cases of Alzheimer's disease. The concentration of aluminum present in the Guam ALS/PDC cases is clearly greater than is seen in Alzheimer's disease by almost two orders of magnitude. This difference has made it considerably easier for others to replicate these findings using other techniques. The identification of increased aluminum concentrations in the tangle-bearing neurons of cases of ALS/PDC of Guam has now been replicated using a wide range of different technologies, including wave-length dispersive X-ray imaging, secondary ion mass spectrometry (SIMS), laser microprobe mass analysis and even histochemical methods (Garruto et al., 1984, Linton et al., 1987, Piccardo et al., 1988, Perl et al., 1986).

Identifying excess aluminum in the neurofibrillary tangle-bearing neurons seen in association with Alzheimer's disease is quite different since the actual concentrations present are frequently at or below the detection limits for most available microprobe instruments. It is likely that this has been responsible for many of the negative studies that have been reported over the years. While Arnold Brody had no training in the study of brain tissues, he was a pioneer in the use of microprobe analysis for the examination of pneumoconiosis cases related to mineral dust exposure. He was able to maximize the combined analytic and morphologic performance of our equipment and this was clearly instrumental in our initial successes. Without his expertise and involvement, these early studies would likely have failed.

The Introduction of Laser Microprobe Mass Analyzer (LAMMA) to Alzheimer's Disease Research

With the need for achieving lower detection limits for microprobe analysis and hoping to provide better localization, in collaboration with Paul Good, I turned to the analytic approach of laser microprobe mass analysis to extend this line of research. The laser microprobe mass analyzer or LAMMA combines a high resolution optical microscope with a time-of-flight mass spectrometer and employs a focussed high energy pulsed Nd : YAG laser to ionize a minute portion of tissue sample. The LAMMA typically analyzes a fixed tissue sample which has been embedded in plastic (in a similar fashion as that employed for routine electron microscopy) and is sectioned at 0.75 μm thick. These sections are stained with toluidine blue to reveal cellular detail and are mounted on a 3 mm in diameter circular mesh electron microscopy grid. The grid is placed in the LAMMA specimen holder, allowing x-y maneuvering and is inserted in the instrument where it is sealed behind a quartz cover glass and is subjected to high vacuum.

The specimen mounted in the LAMMA instrument can be transluminated and be examined histologically with the optical microscope that is part of the apparatus. This allows the operator to identify a cell of interest and then target its subcellular constituents for analysis. The LAMMA is equipped with two collinear lasers, a low power He : Ne

laser which serves as the aiming laser and the high power pulsed laser which perforates the specimen and ionizes the contents of the perforation. Both lasers are directed into the optical pathway of the observing microscope and are focused on the tissue section by its objective lens. The 100× objective lens is capable of focussing the laser down to a perforation measuring approximately 1 μm in diameter, which is the ultimate resolution of the instrument. Through this focussing of the laser the power that can be directed to this minute spot becomes greatly magnified and is sufficient to ionize the contents down to its individual elemental constituents. The positively charged ions produced by the laser perforation are attracted and accelerated by a series of charged ion lenses and are introduced into a 1.8 meter long time-of-flight mass spectrometry column. Within this elongated column the ions demonstrate differing acceleration based on differences in their relative atomic masses. The various individual elemental masses are thus separated and detected individually, based on the relative time it takes for them to reach the detector at the end of the column.

The LAMMA instrument produces a mass spectrum with simultaneous detection of virtually all of the positively changed elements throughout the entire atomic table. The instrument has a minimum detection limit for most elements of less than ten parts per million. In addition, as we and others have shown, the relative intensity of the spectral signal obtained for each element is proportional to the concentration present within the sample. Accordingly, quantitative data can be obtained on the concentration of specific elements present within subcellular compartments of biologic tissues at physiologic concentrations. The ability to observe a stained histologic plastic-embedded section allows for unequivocal identification of the structures being selected for elemental analysis. In the context of this research, neurons can be readily and specifically identified and evaluated on the basis of whether they contain a neurofibrillary tangle or not. The resolution of the probe site is sufficiently small that both the tangle, itself, non-tangled cytoplasm, nucleus and other surrounding tissues can be selected individually for multipoint elemental analysis. With this capability available to us, Paul Good and I approached using LAMMA to better identify the nature of the deposits in the neurofibrillary tangle-bearing neurons of cases of Alzheimer's disease which had previously been detected using X-ray microprobe (Good et al., 1992).

The cases we employed were consecutively accessioned specimens derived from an ongoing study of clinicopathologic correlates of Alzheimer's disease. All cases employed satisfied NINCDS–ADRDA clinical criteria for the disorder (McKhann et al., 1984) during life and, at autopsy, fulfilled the so-called Khachaturian criteria for the neuropathologic diagnosis of Alzheimer's disease (Khachaturian, 1985). Four controls were also included and consisted of individuals who had been free of clinical evidence of cognitive impairment during life (although they had not been prospectively evaluated neuropsychologically prior to death) and were free of significant numbers of Alzheimer's disease-related lesions at autopsy. Samples of the CA1 region of the hippocampus were removed from each case and embedded in plastic. Sections were cut at 0.75 μm, stained with toluidine blue and mounted on 3 mm in diameter electron microscopy grids. The laser microprobe sampling protocol was to identify a tangle-bearing neuron containing a nucleus on the section. This neuron would be probed in the following fashion. A total of 5 probe sites each, would be placed in the nucleus, the neurofibrillary tangle, the

non-involved cytoplasm and the adjacent neuropil. Following this, the nearest non-tangled neuron containing a nucleus that was identified in the section would be chosen for probing. For this cell, 5 probe sites would be made in the nucleus, the cytoplasm and the adjacent neuropil. Probing of both tangle-bearing and non-tangled neurons would continue in alternating sequence until at least 10 pairs of neurons had been analyzed. Once a neuron had been chosen for analysis all resultant spectra would be collected and placed into the computerized data system.

The data system for the LAMMA instrument provides integrated peak intensities related to individual mass numbers as identified by their time-of-flight. The resultant spectra can be evaluated for the integrated mass intensity of 250 atomic mass numbers, many of which relate to the presence of almost all of elements in the atomic table. Using an analysis of variance, the mean peak intensity for each atomic mass number was compared for the 7 probe sites obtained from the ten neurons of each type (tangle-bearing vs. non-tangled) sampled. As such, the seven sites to be compared were neurofibrillary tangle, non-tangle cytoplasm in a tangled neuron, nucleus in a tangled neuron, neuropil adjacent to a tangled neuron and nucleus in a non-tangled neuron, non-involved cytoplasm in a non-tangled neuron and neuropil adjacent to a non-tangled neuron. It should be noted that for this study the comparisons were made only within each individual case. In essence, each case served as its own control. This served to remove variables such as postmortem interval, time in fixation and other issues involving pre-mortem and post-mortem events.

Using this approach, we found that in all 10 cases there were only two mass numbers in which the mean peak intensity for the probe sites directed to the neurofibrillary tangle were significantly different than the other 6 probe sites. These two mass numbers were 27 (aluminum-related) and mass 56 (related to iron). In other words, in all 10 Alzheimer's disease cases, the probe sites directed to the neurofibrillary tangles of the hippocampus consistently revealed a significantly greater aluminum-related and iron-related signal when compared to the probe sites that were directed to the other sites (see Fig. 1). We also noted considerable case-to-case variability in the relative intensity of the aluminum and iron-related signals obtained. Nevertheless, in all cases, the tangles themselves demonstrated the greatest aluminum and iron-related signal. Finally, the mean peak intensity of the aluminum and iron-related signal for the probe sites in the normal control subjects were significantly lower than the non-tangled neurons of the Alzheimer's disease cases. This difference, however, was not emphasized in the paper.

As can be seen from Table 1, the increase in aluminum and iron-related signal in all 10 cases examined was highly significant, despite the multiple comparisons being made. Only a few other elemental signals showed significantly increased intensity in the neurofibrillary tangle-directed probe sites (e.g., lithium increased in 2 cases, tungsten increased in one case, etc.). However, there was no consistency in these results across the entire series of cases. The finding of increased lithium in 2 cases was of interest and we tried to inquire if these particular cases had been treated with a lithium-containing compound. Unfortunately, we were unable to obtain specific data regarding that issue. Nevertheless, this emphasizes the sensitivity and specificity of this microprobe analytic technology and further reinforces the consistency of the iron and aluminum-related data that we obtained.

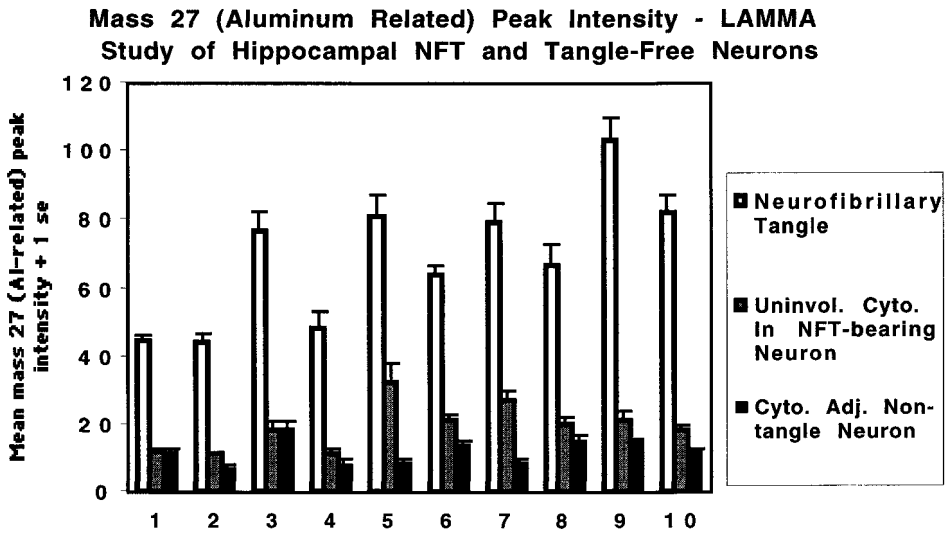

Fig. 1. Mean mass 27 (aluminum-related) peak intensity obtained from probe sites directed to the neurofibrillary tangle (NFT), non-tangle cytoplasm of NFT-bearing neurons and cytoplasm of the nearest normal appearing hippocampal neurons of 10 consecutive Alzheimer's disease cases. Note the prominent increase in the aluminum-related signal obtained from the probe sites of the NFTs. (From Good et al. *Annals of Neurology* 31: 286–292, 1992.)

Table 1. Analysis of Variance (ANOVA) Comparing Neurofibrillary Tangle-derived data with other cellular compartments in NFT-bearing neurons. F calculations for aluminum and iron-related peak intensities are shown along with other masses with a p value <0.05. (from Good, et al. *Annals of Neurology* 31: 286–292, 1992)

Alzheimer disease Case	Aluminum (mass 27) F value, p value	Iron (mass 56) F value, p value	Other elements corresponding to masses detected with $p < 0.05$
1	$F(1,60) = 113$, $p < 0.005$	$F(1,60) = 40$, $p < 0.005$	Zr
2	$F(1,40) = 213$, $p < 0.005$	$F(1,40) = 55$, $p < 0.005$	Pt
3	$F(1,36) = 79$, $p < 0.005$	$F(1,36) = 29$, $p < 0.005$	
4	$F(1,20) = 29$, $p < 0.005$	$F(1,20) = 5.6$, $p < 0.05$	Li, Ti, Zr
5	$F(1,32) = 72$, $p < 0.005$	$F(1,32) = 28$, $p < 0.005$	
6	$F(1,36) = 33$, $p < 0.005$	$F(1,36) = 79$, $p < 0.005$	K
7	$F(1,44) = 132$, $p < 0.005$	$F(1,44) = 29$, $p < 0.005$	Li, Si, P, S
8	$F(1,32) = 51$, $p < 0.005$	$F(1,32) = 7.4$, $p < 0.01$	
9	$F(1,32) = 394$, $p < 0.005$	$F(1,32) = 20$, $p < 0.005$	F, La, Hf, Ta
10	$F(1,36) = 68$, $p < 0.005$	$F(1,36) = 20$, $p < 0.005$	W

In conjunction with these studies we attempted to dispel concerns that the aluminum-related signals we obtained might reflect inadvertent contamination by this rather ubiquitous element in the tissue fixation, and processing. Accordingly, 5 μm thick cryostat frozen sections of the pyramidal layer of the hippocampus of Alzheimer's disease cases were obtained from specimens that had been snap frozen in liquid nitrogen cooled

isopentane but not subjected to fixation or plastic embedding. These cryostat sections were mounted on Formvar-coated grids, air-dried in a covered container and stained for 10 seconds with cold toluidine blue prior to LAMMA analysis. Pyramidal neurons were identified in the CA1 region and subjected to laser microprobe analysis along with the adjacent neuropil. In these studies, prominent mass 27 aluminum-related signals were obtained from neurons identified in the cryostat sections which were not present in the adjacent neuropil. Based on this result, we concluded that the aluminum accumulations we had identified could not be the result of exogenous contamination related to the postmortem processing of the tissues and that the presence of the element was as a consequence of the biologic process that had produced the disorder.

Once again, I was fortunate to have as a collaborator, Paul Good, who became very proficient in the operation of the laser microprobe instrument. We quickly learned that proper tuning and alignment of the laser was necessary to optimize the ionizing power delivered to the tissue specimen and that this was important for consistent data collection. Good developed protocols which standardized the operation of our microprobe approach and permitted a minimum of day-to-day variability. This was clearly important in our obtaining the highly significant differences we reported. When the Kentucky group attempted to replicate our findings (Lovell et al., 1993), it was clear to us that substantial variability was present in their data and they had yet to learn these lessons (Good & Perl, 1993b). Later, Markesbery (1997) would agree that iron, and likely aluminum, were present in excess amounts in the neurofibrillary tangles of cases of Alzheimer's disease.

Informed Opinion

Why Has It Been so Difficult for a Potential Role for Aluminum in the Pathogenesis of Alzheimer's Disease to Be Widely Accepted?

Over the years there have been a few individuals who have been persistent critics of the concept that aluminum plays a potential role in the pathogenesis of Alzheimer's disease. Curiously, at the same time they were providing these criticisms, some also were serving as paid consultants to the aluminum industry. At the time, rules regarding disclosure of financial ties and potential conflicts of interest were not yet in force for authors and speakers at meetings and, for the most part, few were aware of this serious breach in the impartiality of this vocal portion of the scientific community. Indeed, over the years, to my knowledge, none ever publicly acknowledged their close ties to this industry. The aluminum industry has had a considerable economic stake in how this question is interpreted by the scientific community and by the public. This can be appreciated by the following observation. With little direct evidence regarding the potential sources of the aluminum accumulations we observed, based on a single letter to the editor of the prestigious New England Journal of Medicine (Levick, 1980), aluminum pots and pans were actively discarded and virtually disappeared from use in many households. Some have argued that I should have been more vocal about the fact that paid consultants for the aluminum industry served as consistent and vocal critics of our findings. I always felt, perhaps naively, that our data were properly collected, honestly and completely reported

and were essentially correct. Accordingly, I have felt that the truth would eventually be known and be ultimately accepted.

Another distracting factor had been the conflicting data regarding aluminosilicate deposits within senile plaques. In 1986, Candy and coworkers (1986) reported evidence of dramatically high concentrations of aluminosilicates within the cores of senile plaques of cases of Alzheimer's disease. The senile plaque, along with the neurofibrillary tangle, represents the other cardinal neuropathologic feature of Alzheimer's disease. The analytic techniques used and the nature of the signals being obtained by Candy and colleagues suggested that senile plaque cores contained virtually pure aluminosilicate deposits. For several decades it had been known that these cores were composed of an amyloid protein, now referred to as β-amyloid. The biology of the finding of aluminosilicate concretions was difficult to understand and their origin was difficult to imagine. The colocalization of a proteinaceous structure that had been well-characterized biochemically, histochemically and ultrastructurally was not consistent with its colocalization with an insoluble mineral deposit. We attempted to replicate Candy's findings but were unable to identify such deposits using scanning electron microscopy-X-ray energy dispersive analysis (Stern et al., 1986). Subsequent studies using LAMMA, where individual senile plaques could be identified histologically and extensively probed, also failed to reveal evidence of excess silicon. Small concentrations of aluminum were noted in the distrophic neurites at the edge of some plaques (presumably coming from concentrations of paired helical filaments within these structures) but were not seen within the central cores.

Subsequent studies of senile plaques derived from Alzheimer's disease cases were reported by Landsberg and coworkers (1992) at the Department of Physics at Oxford. This group used a focussed 3 MeV ion beam nuclear microprobe with particle induced X-ray analysis (PIXE). These workers failed to identify the presence of significant amounts of aluminum within identified senile plaques cores in their specimens. The nuclear microprobe study of the Oxford group was carefully performed using a highly sophisticated and relatively sensitive microprobe approach. Unfortunately, when this study was prominently published in Nature, the authors used their negative data regarding senile plaque content to claim that aluminum could not play any role in Alzheimer's disease pathogenesis. As we subsequently pointed out (Good & Perl, 1993a), their study dealt only with senile plaques and contained no data regarding the elemental content of neurofibrillary tangles.

An additional concern has been raised about the absence of neurofibrillary tangle formation in cases of dialysis encephalopathy or dialysis dementia. Dialysis encephalopathy is a disorder that typically occurs in patients with renal failure who are undergoing chronic renal dialysis. It is characterized by confusion, dyspraxia, myoclonus and generalized seizures. The brain tissues of affected patients show markedly elevated aluminum content with gray matter concentrations frequently more than 10 times greater than normal levels. The aluminum accumulation is linked to ingestion of large amounts of aluminum-containing phosphate-binding gels or to aluminum contamination of the dialysate fluids, themselves. Dialysis encephalopathy is generally fatal within 6 months, although cases with a lesser degree of aluminum accumulation can survive for years, particularly when treated with chelating agents, such as desferrioximine.

The brains of affected individuals may show a non-specific increase in astrocyte number but otherwise, these cases demonstrate little in the way of histopathologic

alteration. Most specifically, they fail to show evidence of neurofibrillary tangle formation. Harrington and coworkers (1994) reported studies of 15 dialysis patients and compared changes in *tau* and β-amyloid on the basis of high and low brain aluminum content. None of the patients had shown features of dialysis encephalopathy during life or significant numbers of neurofibrillary tangles on neuropathologic evaluation. Nevertheless, the cases with high aluminum content showed immunochemical evidence for the presence of a truncated form of *tau*, similar to that of Alzheimer's disease. Despite their relatively young age, two of these cases revealed the presence of protease-resistant paired-helical filaments in the frontal lobes, consistent with neurofibrillary tangle formation. These findings suggest that aluminum can participate in inducing changes in relevant cytoskeletal proteins that mimic those seen in Alzheimer's disease.

The absence of tangle formation has been used as an argument that since such cases have extremely high brain aluminum content, the element cannot cause this change in Alzheimer's disease. We have considered this argument to be fallacious, since it confuses etiology with pathogenesis. As will be discussed below, we do not consider aluminum to cause Alzheimer's disease or possibly neurofibrillary tangle formation, itself. On the other hand, since we find aluminum to be present in conjunction with neurofibrillary tangle formation in many setting, such as Alzheimer's disease, Guam ALS/parkinsonism-dementia complex, adult onset Hallervorden–Spatz disease (Eidelberg et al., 1987), Down syndrome, etc. we believe that aluminum plays a critical role in the pathogenesis of its formation. Clearly, many other steps must be taken in order for tangle formation to take place and the presence of aluminum, in and of itself, is not likely to be sufficient for this pathologic lesion to form. Accordingly, the absence of tangles in cases of dialysis dementia fails to speak to the question of pathogenesis, in the setting of Alzheimer's disease. Finally, using LAMMA analysis of a small number of cases, we identified elevated aluminum concentrations in cerebral cortical astrocytes and vascular endothelial cells (Good & Perl, 1988). In this study neuronal levels of aluminum were not significantly elevated. This would suggest that in this particular encephalopathic entity, aluminum accumulates in a different cellular compartment than we see in the neurodegenerative disorders associated with neurofibrillary tangle formation.

Recent Evidence Regarding the Association of Aluminum with the Neurofibrillary Tangles of Alzheimer's Disease

Our work over the years has provided a continuing series of data suggesting that aluminum is bound to the constituents of the neurofibrillary tangle and that the metal's presence represents a functional step in the biology of the formation of this important pathological inclusion. Due to the high charge and small ionic radius of aluminum, one would expect that the element would bind tightly to appropriate negatively charged sites on proteins (likely hyperphosphorylated regions) and to induce cross-linking stabilization of these proteins, thus yielding them relatively insoluble. The neurofibrillary tangle is known to consist primarily of an aggregation of the microtubule-associated cytoskeletal protein called *tau* (Lee et al., 1991). The *tau* protein constituents of the neurofibrillary tangles are known to be hyperphosphorylated and form a highly insoluble aggregate.

Fasman and colleagues (Hollosi et al., 1994, Hollosi et al., 1992) have pointed out, small concentrations of aluminum have the property of inducing a beta-pleated sheet configuration when added to a 13 amino-acid polypeptide repeat (rich in phosphorylation sites) that is present at the C-terminal phosphylation sites of both the medium and heavy chain of the neurofilament protein. In this regard, it is interesting to note that the neurofibrillary tangle demonstrates Congo red birefringence and is one of the few examples in human pathology of an intracellular amyloid.

Recently Murayama and colleagues (1999) studied the association of aluminum and neurofibrillary tangles in Alzheimer's disease tissues. They showed that Morin staining of the neurofibrillary tangles (a histochemical method for identifying aluminum in tissues) could be abolished by pretreatment of the tissue sections by the harsh treatment of boiling in desferioxamine, a potent aluminum chelator. Further, they showed that pretreatment of the sections by aluminum-containing solutions abolished immunostaining of the contained neurofibrillary tangles using antibodies to the hyperphosphorylated epitopes. When the aluminum-treated sections were then exposed to desferioxamine, the immunoreactivity returned, indicating that aluminum binding favored these hyperphosphorylated sites. Finally, they demonstrated that *in vitro* aluminum treatment caused aggregation of paired helical filaments and that this was reversed by dephosphorylation of the tangle preparation. All this indicates that aluminum binding to the neurofibrillary tangle is, indeed, part of the biology of Alzheimer's disease, and not a postmortem artifact. Furthermore, it represents an independent confirmation that a) aluminum is present on the neurofibrillary tangles of cases of Alzheimer's disease and b) that aluminum binding is directed at the phosphorylation sites of *tau*, causing cross-linking stabilization of this important constituent protein. This important study reinforces mechanisms we hypothesized several years ago. While it has been very gratifying to see another group extend ones original concepts, these important findings have yet to be taken up in more general discussions of the pathogenesis of this disorder.

Final Thoughts

It is now over 20 years since we first reported microprobe evidence that aluminum selectively accumulates in the neurofibrillary tangles of cases of Alzheimer's disease. This finding has generated considerable controversy and discussion. Along the way there have been negative studies which some have passionately embraced. However, such negative data should be viewed with caution since the study in question must be able to demonstrate that if the substance being sought were indeed present in increased concentration, it would have been detected by the instrumentation used. By and large, this important prerequisite has not been followed. This must be weighed against the fact that excess aluminum has now been repeatedly demonstrated in association with the neurofibrillary tangle, especially in the setting of Guam ALS/PDC, using a wide variety of analytic procedures which rely upon vastly different physical principles.

If aluminum is indeed an integral component of the neurofibrillary tangle, as we have argued, then what role might it play in the etiology or pathogenesis of this important pathologic component of neurodegenerative disorders, including Alzheimer's disease? It

is highly unlikely that aluminum represents an etiologic agent for neurodegeneration. However, based in part on the data reviewed above, it has seemed to me that it is highly likely that aluminum does represent a requisite constituent for neurofibrillary tangle formation and, as such, plays a role in the pathogenesis of this pathologic process. The concept that aluminum only serves a role in the pathogenetic cascade does not lessen the element's potential importance since, other than infectious diseases, most approaches to treatment and prevention of diseases relate to an understanding of pathogenetic mechanisms rather than direct approaches to etiologic factors.

Over the past 10 or more year, there has been a dramatic increase in our understanding of the genetic factors which play a role in the development of Alzheimer's disease. Nevertheless, it is highly unlikely that this extremely common disorder of late life is wholly genetic in etiology. It should be anticipated that a disease like Alzheimer's disease represents, in a similar fashion to other common disorders encountered late in life, such as cancer and atherosclerosis, a combination of the interaction of both genetic and environmental factors. Relevant etiologic factors are difficult to identify with certainty and likely interact with various genetically based factors that govern relative susceptibility and/or resistance to these environmental agents. Interestingly, as we know from the examples of cancer and atherosclerosis, when these disorders occur relatively early in life, genetics has had a more major role, whereas, occurrence in late life reflects more the effects of environmental factors. Alzheimer's disease is likely to operate in a similar fashion. It is important to remember that our current approach to the prevention of cancer and atherosclerosis mainly involves the use of an understanding of the relevant environmental factors (such as diet, smoking, exercise, etc.) and their modification by the patient at risk. This has certainly worked effectively in instances of advanced atherosclerosis leading to myocardial infarction and in dealing with the relationship of smoking and lung cancer (or atherosclerosis). Unfortunately, we have have only scant data related to environmental factors which may have relevance to an understanding of who gets Alzheimer's disease. Despite over 20 years of research, whether and how aluminum in our environment might play a role in the induction of this important disorder remains unanswered.

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Aluminium and Alzheimer's Disease
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CHAPTER 7

Aluminum and Gene Transcription in the Mammalian Central Nervous System — Implications for Alzheimer's Disease

Walter J. Lukiw *

LSU Neuroscience Center and Department of Ophthalmology, Louisiana State University School of Medicine Health Sciences Center, 2020 Gravier Street, Suite 8A8, New Orleans, LA 70112-2272, USA

* email: wlukiw@lsuhsc.edu

Abbreviations: AD – Alzheimer's disease; ALS – amyotrophic lateral sclerosis; Alu – Alu repetitive element; ANOVA – analysis of variance (p); GFAP – glial fibrillary acidic protein; HIF-1 – hypoxia inducible factor 1; M – adenine or cytosine **; nM – nanomolar; N – any nucleotide **; NIH/NIA–CERAD – National Institutes of Health/National Institute of Aging – Consortium to Establish a Registry for AD; NF-κB – nuclear factor for kappa B; NFL – neurofilament light chain (gene or protein); R – any purine **; RDA – recommended daily allowance for trace elements (US Government NIH guidelines); ROS – reactive oxygen species; TF – transcription factor.

** IUPAC codes.

Summary

Aluminum has been repeatedly implicated in the etiopathology of several human neurodegenerative disorders including Alzheimer's disease. Due largely to the extensive euchromatization of normal brain cell neuronal nuclei and their high intrinsic rate of transcription, one prime target for aluminum appears to be within the nucleic acid compartments of the central nervous system. Generally, the high positive charge density of aluminum ($Z^2/r = 17.65$) acts as a pervasive repressor and demodulator of neural activity. This chapter will review our current knowledge concerning aluminum as a genotoxic 'dementing' cation, particularly as it applies to neural-specific gene transcription and transcription factors in the human brain.

Historical Perspective

Introduction

Aluminum, averaging 81.3 mg/gm of the earth's crust, is the most abundant neurotoxic metal in the biosphere. Once aluminum has bypassed important gastrointestinal, physio-

logical and blood–brain barriers, it has been shown to adversely affect the physiological, biochemical and genetic integrity of the mammalian central nervous system (CNS). Due largely to (a) the extensive delocalized electron fields of mammalian brain cell nucleic acids, (b) a neuronal intranuclear phosphate content approaching 60 mM, (c) the high rates of intrinsic neural metabolic activity and (d) the remarkable rapidity of brain-specific genetic processes such as transcription, one particular target for aluminum toxicity, once it gains access to the CNS, are within the brain's genetic compartments. CNS nuclei appear to act as high affinity, high-capacity 'sinks' to attract and trap aluminum within the nuclear matrix. In general, this extremely high charge density trivalent cation ($Z^2/r = 17.65$) can be thought of as acting as a pervasive repressor of normal brain genomic activity, although there are important exceptions. Interestingly, these exceptions include the super-activation of pro-inflammatory transcription factors (TFs) which are ultimately detrimental to normal patterns of brain gene expression. The question is not 'if' aluminum behaves as an extremely potent genotoxic agent, but rather 'why' specific genetic sub-compartments are selectively targeted for interaction with aluminum. This may help explain the remarkable specificity of brain gene dysfunction in the presence of trace amounts of aluminum. One emerging hypothesis is that aluminum is strongly attracted to the phosphates of nucleic acids (and nucleotide triphosphates) especially rich in adenine (A) and thymine (T) residues, so brain RNA and DNA sequences containing a high molar percentage of A + T nucleotides may be particularly at risk for the deleterious effects of this 'dementing' cation. Importantly, many of the molecular genetic mechanisms involved in aluminum neurotoxicity towards neuronal and glial cell nuclei and brain-specific gene transcription remain unclear. This chapter will review our current knowledge concerning aluminum genotoxicity, particularly as it applies to gene transcription and TFs utilized within the CNS.

Aluminum Associates with the Genetic Material of Eukaryotic Cells

Historically, aluminum's potential for interaction with the genetic material was first noted in the affinity of chromogenic or fluorescent aluminum-containing dyes for the nuclei and genetic material in diverse species and cell types. One of the first reports of an aluminum induced impairment on the flow of genetic information expression came from the studies of Weser and Koolman (1969 & 1970) who in experimental aluminum neurotoxicity studies (EANTS) demonstrated the repressive and 'damping' effects of aluminum on nuclear-directed protein biosynthesis in rat liver nuclei. Since nuclear-directed RNA message interaction with ribosomal RNA and protein lies at the heart of the cellular translation machinery, these experiments were in fact among the first describing a mechanistic specificity for aluminum toxicity in derailing normal nucleic-acid driven cytoplasmic processes. Similar investigations on nuclear function soon followed, including the reports of Estable-Puig et al. (1971) who showed the induction in brain glial cells of a marked condensation of genetic material after aluminum hydroxide exposure. The condensatory power of aluminum on chromatin, which encapsulates and regulates the expression of the genetic material, was found to be particularly exceptional when compared to other neurotoxic transition metals, and experiments complementary to

Estable-Puig's have been replicated many-fold since their original description in a wide variety of mammalian brain cells and nervous systems, both in vivo and in vitro (see for example, De Boni et al., 1974; Lukiw et al., 1987; Walker et al., 1989; Bolla et al., 1992; McLachlan et al., 1995a,b; Lukiw et al., 1998b, 2001b).

While the detrimental effects of aluminum on normal physiological and biochemical systems are decidedly large, over the last 30 years, a considerable body of scientific evidence has emerged concerning the deleterious effects of aluminum on the structural and biochemical function of the genetic apparatus. Clearly, multiple adverse biological effects of aluminum on nucleic acid structure and function have been repeatedly demonstrated, and instances of highly specialized interactions with nuclear genomic structures, such as those encountered in brain cell neurons, illustrate one prime example of specific aluminum targeting within the neural cells of the CNS. In particular, the high content of intranuclear phosphate (approaching 60 mM; Lukiw et al., 1987, 1989a,b), the normally decondensed or extensive euchromatization characteristic of neocortical neuronal nuclei, their intrinsically high rate of transcriptional output and their generation of complex RNA message populations required for normal brain cell function may specifically target neural cells for many of aluminum's genotoxic effects. Here, an array of multiple deleterious effects of aluminum have been associated with, or intimately linked functionally to, induced abnormalities in DNA strand-stabilization (Sarkander et al., 1983; Walker et al., 1989), base pair stacking and nucleic acid helix intercalation (Stockert 1979; Karlik et al., 1980, 1989; Llorente et al., 1989), the structure, function and mobility of chromosomes (Stockert, 1979; Sanderson et al., 1982; Ganrot, 1986; Kobayashi et al., 1987; Lukiw & McLachlan, 1995; Lukiw & Bazan, 1999), both β- and Z-forms of the DNA double helix (Karlik et al., 1980, 1989; Hanas and Gunn, 1996), euchromatin, the H1-class of linker histones, heterochromatin, rough endoplasmic reticulum and ribosomes, mitochondria, the nucleolus, nuclear organizer region, TFs, both general and specific, DNA and RNA polymerase enzyme systems (please refer to Table 1 and the references therein). In most cases however, the exact molecular mechanisms involved in aluminum's genotoxicity towards neurobiological systems, and how aluminum may act in concert with other neurotoxic transition metals such as zinc remain incompletely understood.

Aluminum is Implicated in Sporadic Human Neurodegenerative Disease

The presence of aluminum has been implicated in the etiopathogenesis of numerous human neurodestructive disorders in part due to its association with disease-related lesions in CNS tissues. These include amyotrophic lateral sclerosis (ALS) and Parkinson's dementia (PD; Perl et al., 1982; Yoshida et al., 1991; Yasui et al., 1992, 1997), progressive myoclonic encephalopathy of dialysis (Sabouraud et al., 1978; Itzhaki, 1994; Berend et al., 2000), deficient renal function (McLachlan et al., 1995; Lukiw & McLachlan, 1995a; 1995b; van der Voet, 1998; Berend 2000), status epilepticus (Hantson et al., 1995; Hoang-Xuan K et al., 1996) and in particular the sporadic form of AD (De Boni & McLachlan, 1980; Crapper et al., 1980; Perl & Pendlebury 1984; Ganrot, 1986; Kobayashi et al., 1987; McLachlan et al., 1988, 1995a, 1995b; Ehmann & Markesbery, 1994; Lukiw et al., 1987, 1992, 1994a, 2000c; Bouras et al., 1997; Lukiw, 1997, 1998; Yumoto et al.,

Table 1. Aluminum interacts with eukaryotic nuclear and genetic material — chronological listing

Condition	Analytical method	Compartment	Reference
EANTS	ADD	rat liver nuclei	Weser and Koolman, 1969
EANTS	ADD	rat liver nuclei	Weser and Koolman, 1970
EANTS	LM	glial nuclei	Estable-Puig et al., 1971
EANTS	MS/LM	brain nuclei	De Boni et al., 1974
EANTS	ADD/EPM	thyroid nuclei	Truchet 1976
EANTS	GS	epithelial nuclei	Lillie et al., 1976
EANTS	^{28}Al	liver nuclei	Kushelevsky et al., 1976
EANTS	LM/EM	rabbit nuclei	Kadota and Kadota, 1978
EANTS	AH	chromatin	Stockert 1979
EANTS	MS/LM	brain nuclei	De Boni et al., 1980
AD	EAA	neocortical nuclei	Crapper et al., 1980
AD	EAA	neocortical nuclei	De Boni and McLachlan 1980
Guam PD	EDXS	human brain nuclei	Perl et al., 1982
EANTS/AD	ADD/LM	hippocampal nuclei	Sanderson et al., 1982
EANTS	CV	plant root nuclei	Niedziela and Aniol 1983
AD	XMA	human hippocampal nuclei	Perl and Pendlebury 1984
EANTS	CD/FA	calf thymus DNA	Karlik et al., 1980
EANTS	ADD/ROTA	rabbit brain nuclei	Sarkander et al., 1983
EANTS	EDXS	rabbit brain nuclei	Uemura 1984
AD	AA	heterochromatin	Ganrot 1986
EANTS	ADD/EMSA	H1–DNA binding	Lukiw et al., 1987
EANTS	ADD/EPM	rat liver nuclei	Truchet et al., 1987
AD/Balints	XMA	human brain nuclei	Kobayashi et al., 1987
EANTS	ADD/NB	cytoskeletal genes	Muma et al., 1988
AD	NA/EAA	neurofilament promoter	Crapper McLachlan et al., 1988
EANTS	EAA	neocortical nuclei	Lukiw et al., 1989a
EANTS	EM	calf thymus DNA	Karlik et al., 1989
EANTS	LM/FA	avian chromatin	Llorente et al., 1989
AD	EAA	human brain nuclei	Lukiw et al., 1989a, 1989b
EANTS	ADD/UCA/NA	brain chromatin	Walker et al., 1989
EANTS	EAA	rabbit brain nuclei	Bertholf et al., 1989
EANTS	LM/XMA	avian nuclei	Del Castillo et al., 1990
EANTS	ADD/EMSA	H1–DNA binding*	Oikarinen et al., 1991
ALS	EELS	heterochromatin	Yoshida 1991
EANTS	ADD/EPM	hepatocyte nuclei	Jeantet et al., 1992
AD	EAA	human brain euchromatin	Lukiw et al., 1992
EANTS	ADD	H1–DNA binding	Tarkka et al., 1993
AD	LAMMA	neuronal nuclei	Lovell et al., 1993
AD	INAA/GFAAS/LAMMA	neocortical nuclei	Ehmann & Markesbery 1994
aging	LM/MS	human brain nuclei	Shimizu et al., 1994
EANTS	EAA	human brain nuclei	Lukiw et al., 1994a
AD	EAA	human brain nuclei	Lukiw & McLachlan, 1995
EANTS	EAA/EPM/XMA	rat liver nuclei	Spencer et al., 1995
EANTS	CF/SS	nucleus/nucleolus	Zhang 1995
ALS	PIXE	nucleolus/RER	Yoshida et al., 1995
EANTS	FTIRS	calf thymus DNA	Ahmad et al., 1996
EANTS	PCR	human genomic DNA	Chen et al., 1996
EANTS	ADD/DNAFP	TFIIA–DNA binding	Hanas and Gunn 1996
EANTS	INAA/MT	brain nuclei/MC	Amano et al., 1996
EANTS	ADD/DCPES	hippocampal nuclei	Julka et al., 1996
EANTS	LM/EM	hippocampal nuclei	Somova et al., 1997

Table 1 (continued)

Condition	Analytical method	Compartment	Reference
AD	LAMMA	human hippocampal nuclei	Bouras et al., 1997
EANTS	ADD/ROTA	human neocortical nuclei	Lukiw 1997
EANTS	ADD/MA	rat brain nuclei	Nayak & Chatterjee, 1998
EANTS	ADD	neuronal genes	Parhad et al., 1989
AD	EDXS/PIXE	human hippocampal nuclei	Yumoto et al., 1998
EANTS	ADD/ROTA	human brain nuclei	Lukiw et al., 1998a, 1998b
EANTS	ADD	cGMP pathway (neurons)	Cucarella et al., 1998
EANTS	ADD/ROTA	NFL RNA message	Lukiw and Bazan, 1999
EANTS	ADD	ATP nucleotide	Exley, 1999
EANTS	EMSA/NB	transferrin receptor RNA	Oshiro et al., 2000
EANTS	FA/CLSM	root tip nuclei	Silva et al., 2000
EANTS	EMSA/NB	glial/neuronal nuclei	Oshiro et al., 2000
EANTS	MS/FA/LM	glial/neuronal nuclei	Levesque et al., 2000
EANTS	^{26}Al-AMS	rat brain nuclei	Yumoto et al., 2000
EANTS	BM/WIB	cGMP pathway (glia)	Corbalan et al., 2000
EANTS	XMA/FA	astrocyte chromatin	Haghparast, 2000
EANTS	ADD/EMSA	human TFs	Lukiw et al., 2001a
EANTS	ADD/EMSA	TFs	Campbell and Bondy, 2001
EANTS	HDARRAY	human hippocampus	Lukiw et al., 2001b

Abbreviations for Condition/Analytical Method: ^{26}Al-AMS = ^{26}Al accelerator mass spectrometry; ^{28}Al = ^{28}Al isotopic uptake; AA = atomic absorption (flame); AD = Alzheimer's disease; ADD = addition experiments; AH = aluminum hematoxylin; BM = brain microdialysis; CD = circular dichroism; CF/SS = carbol fuchsin/silver stain; CLSM = confocal laser scanning microscopy; CV = catechol violet stain; DCPES = direct current plasma emission spectroscopy; DNAFP = DNA footprinting analysis; EAA = electrothermal atomic absorption (flameless); EANTS = experimental aluminum neurotoxicity studies; EDXS = energy dispersive X-ray spectroscopy; EELS = electron energy loss spectrometry; EM = electron microscopy; EMSA = electrophoretic mobility shift assay; EPM = electron probe microanalysis; FA = fluorescence assay; FTIRS = Fourier transform infrared spectroscopy; GFAAS = graphite furnace atomic absorption spectrometry; GS = Gallo blue stain; Guam PD = Kii Peninsula of Japan amyotrophic lateral sclerosis (ALS) and Parkinson dementia (PD) complex with neurofibrillary degeneration; HD = high density cDNA arrays analysis; LM = light microscopy; INAA = instrumental neutron activation analysis; LAMMA = laser microprobe mass analysis; LMMS = laser microprobe mass spectroscopy; MA = metabolic analysis; MC = mitochondria; MS = morin stain; MT = multitracer technique; NA = nuclease accessibility; NB = Northern blotting; NFL = neurofilament light chain; PCR = polymerase chain reaction; PD = Parkinson's disease; PIXE = particle-induced X-ray emission spectroscopy; RER = rough endoplasmic reticulum; ROTA = run on transcription assay; SIMS = secondary ion mass spectroscopy; TF = transcription factors; TFIIA = TF factor IIA; UCA = ultracentrifugation (analytical); WIB = Western immunoblot; XMA = X-ray microanalysis.

1998; Lukiw & Bazan, 1999, 2000, 2001b). Epidemiological data from diverse human populations shows that all forms of AD accounts for more than 50 percent of all cases of dementia and severe cognitive impairment in the aging population, thereby representing the most prevalent chronic form of human neural degeneration (Selkoe, 2000; St. George Hyslop, 2000). However, these studies have yet to identify a single common cause for either familial or the much more common sporadic forms of AD. Genetic defects have been linked to mutations in the familial AD genes encoding the polytopic transmembrane proteins presenilin-1 and -2, β-amyloid precursor protein and the apolipoprotein E4 allele

to human chromosomes 14q24, 1q42, 21q21 and 19q13, respectively, and further putative genetic linkage of AD to chromosomes X, 1, 3, 8, 10. 12 and several others (Rogaev et al., 1995; St. George Hyslop., 1995; Tanzi et al., 1996; Zubenko et al., 1998; Hong et al., 1999; Czech et al., 2000; Yu et al., 2000; Selkoe, 2000; St. George Hyslop, 2000). These data underscore the fact that the genetic basis for familial AD is decidedly heterogeneous (St. George Hyslop, 2000; Lukiw et al., 2000b, 2000c). Furthermore, all of the known familial AD mutations account for at most 5 to 10 percent of all autopsy-confirmed AD cases suggesting the involvement of yet unidentified mutant genes, genetic modifying processes and other non-genetic or environmental factors in the development of sporadic AD (Bolla et al., 1992; Lukiw et al., 1994a, 1994b; St. George Hyslop et al., 1995; Van Leeuwen et al., 1998; Yu et al., 2000; Selkoe, 2000; St. George Hyslop, 2000; Lukiw and Bazan, 2000). Tragic cases of accidental aluminum poisoning in the human population have repeatedly illustrated aluminum's causative role in the induction of various acute aluminum-induced encephalopathies and its potential role as a 'dementing' ion once it gains access to the CNS (Crapper et al., 1980; Perl et al., 1982; Guy et al., 1991; Hantson et al., 1995; Berend et al., 2000). In addition, abundant data now link the amount of aluminum in drinking water with an increased risk for the later development of AD (Flaten et al., 1996; Harris et al., 1996; Forbes and Gentleman, 1998; Levesque et al., 2000; Flaten, 2000; Rondeau et al., 2000; Desroches et al., 2000).

Aluminum-Cellular and -Nuclear Interactions in Experimental Aluminum Neurotoxicity Studies (EANTS) Mimic Analogous Defects Found in AD Brain

It should at first be pointed out that EANTS most often apply an acute dose of aluminum to an experimental system when compared with the more chronic accumulation of aluminum observed with neurodegeneration, such as that observed in aged AD brain. Indeed, few EANTS have incorporated this important time factor. In EANTS, aluminum targets numerous cytoplasmic and nuclear structures at equivalent concentrations to those reported in human neurodegenerative disease (typically at 10–100 nM; Table 1). In each case, similar cytoplasmic and nuclear sub-compartments are adversely affected. To cite several important cytoplasmic examples, the classical neuropathological lesions of AD-afflicted brain include twisted, atrophic neurites termed neurofibrillary tangles (NFT) and dense, insoluble aggregations of amyloid beta (Aβ) peptides termed senile plaque (SP) deposits (Alzheimer et al., 1995; Singer et al., 1997; Yasui et al., 1997; Lukiw & Bazan, 2000). Aluminum is associated both with the NFT in AD affected neocortex (Tokutake et al., 1995; Bouras et al., 1997) and has been shown to induce, in a dose dependent fashion in EANTS, pathological structures morphometrically analogous to NFT. Importantly, these lesions are immunologically indistinguishable from the NFTs found in AD brain neocortex (Guy et al., 1991; Somova et al., 1997; Singer et al., 1997). Aluminum has also been found both at the core of the SP in AD brain and has the ability in vitro to nucleate Aβ peptides thereby seeding SP formation (Bertholf et al., 1989; Evans & Harrington, 1998; Exley et al., 1993; Exley & Korchazhkina, 2000).

At the level of gene transcription, again, there are remarkable similarities between EANTS on brain gene deactivation and that which is observed in AD association

neocortex and hippocampal regions. For example, a single stereotactic injection of 100 μl of a 1 per cent aluminum lactate solution into rabbit cerebral ventricles results, within 3 hours, in decreased levels of RNA messages encoding the key cytoskeletal components α-tubulin, β-actin and the light chain of the neuron-specific neurofilament (NFL) protein. This is a pattern remarkably similar to the down regulated cytoskeletal RNA message pools observed, by several independent laboratories, in AD afflicted neocortices containing similarly elevated aluminum levels (Kadota & Kadota, 1978; McLachlan et al., 1988; Muma et al., 1996; Troncoso et al., 1990; a., 1994, 1996). Selective neural deficits in α-tubulin, β-actin and NFL gene expression have been hypothesized to contribute to the bizarre cytoarchitecture characteristic of AD-afflicted neocortical neurons (Hoffman et al., 1987; Lukiw, 1997; Lukiw et al., 1998a, 1998b). Interestingly, more recent studies examining the incorporation of very high specific activity [α-^{32}P]-UTP (>3000 Ci/mM) into newly synthesized RNA message pools into isolated short post-mortem interval human brain nuclei using run-on gene transcription (Thompson, 1973; Fei & Drake, 1993) suggest that transcription in the presence of nM aluminum (importantly at concentrations which have been reported in neurodegenerative disorders such as AD; Crapper et al., 1980; Lukiw et al., 1992; Shimizu et al., 1994; Bouras et al., 1997) impairs brain-specific cytoplasmic gene transcription from human neocortical genes, and especially those containing a high molar A + T per cent in their promoters (such as the human NFL gene; Lukiw et al., 1998a, 1998b). Moreover, studies examining this effect using the Alu repetitive element (Schmid & Jelinek, 1982), NFL and GFAP RNA messages and RNA polymerase II genetic output (Gelles & Landick, 1998; Fig. 1) are again remarkably similar when compared to the effects of aluminum salts on the kinetics of rabbit neocortical nuclei transcription (Thompson, 1973; Sarkander et al.,1983). Therefore, despite a rabbit–human evolutionary divergence at least 6.7 × 10^7 yr (Pesole et al., 1991), the effects of aluminum on brain gene transcription suggest similar mechanisms of RNA polymerase II inhibition are a highly conserved phenomenon across the brain genetic material of diverse mammalian species.

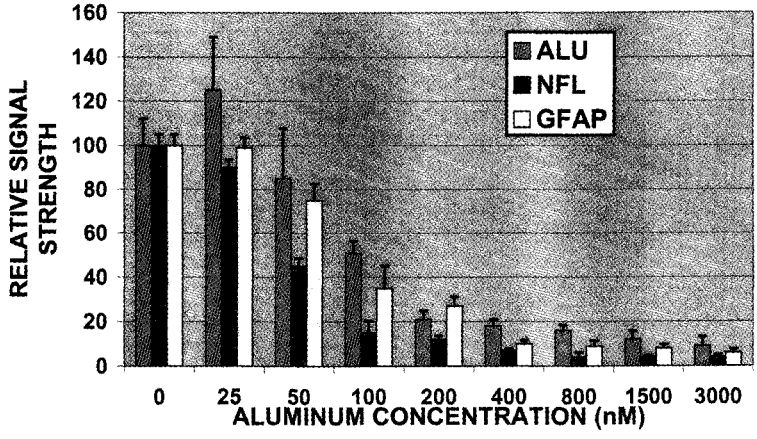

Fig. 1. Aluminum and run-on gene transcription. Impairment in the readout of genetic information by RNA polymerase II.

Informed Opinion

The Presence of Aluminum is an Impediment to Normal Brain Gene Function

From the previous section, it should be apparent to the reader that 10–100 nM concentrations of aluminum salts are detrimental to many vital aspects concerning the structure and function of the mammalian genetic apparatus. For example, the key transcriptional DNA 'readout' enzyme complex RNA polymerase II during active transcription can be considered as a mechanistically complex molecular motor powered by DNA helix separation, the free energy liberated by ATP hydrolysis and the *de novo* polymerization of ribonucleotides into newly synthesized RNA message species (Roeder & Rutter, 1970; Kasas et al., 1997; Gelles & Landick, 1998). In a graded dose–response, aluminum has been shown (a) to significantly increase the melting temperature of the DNA template and thereby enhance DNA stability (Karlik et al., 1980; Karlik et al., 1989; Chen et al., 1996; Hanas and Gunn, 1996), (b) to strongly inhibit ATPase and/or ATP hydrolysis (Martin, 1992; Schindelin et al., 1997; Ryle & Seefeldt, 2000; Zatta et al., 2000) and (c) to inhibit RNA polymerase II translocation activities at 10–100 nM concentrations (Sarkander et al., 1983; Lukiw, 1997; Lukiw et al., 1998b, 2001b; Fig. 1). Thereby, all three key biophysical parameters known to fuel RNA polymerase II activity are targeted for impairment by aluminum, and importantly at concentrations found in human brain neurodegenerative conditions such as AD.

The following three sections will reflect on several more recent observations on (i) the interplay of aluminum, oxidative stress, TFs and neurodegenerative disease (with specific reference to AD), (ii) the analysis of broad spectrum gene expression in AD hippocampus and aluminum chloride treated normal human neural progenitor (NHNP) cells using high density cDNA arrays and (iii) the current involvement of zinc in human brain dysfunction and gene transcription and possible interplay with trace amounts of aluminum. Emphasis will be placed as to what this current information is telling us about an emerging role for aluminum in the impairment of genetic activity in the human brain during neurodegeneration.

Aluminum, oxidative stress, transcription factors (TFs) and neurodegenerative disease

In recent years abundant research has clearly pointed to oxidative stress as an important event in the induction and proliferation of human neurodegenerative disease including AD pathogenesis. However, the precise mechanism by which reactive oxygen species (ROS) are generated and redox balance are altered have remained incompletely defined (Mundy et al., 1997; Mattson et al., 1998; Butterfield et al., 1999; Campbell and Bondy, 2000; Lukiw & Bazan, 2000; Christen, 2000; Varadarajan et al., 2000;). For example, Fe^{2+}–Fe^{3+} chemistry in redox active states appear to be increased in both NFT and Aβ deposits where Fenton chemistry drives the production of ROS (Smith et al., 2000; Varadarajan et al., 2000). These processes are intimately linked to the proliferation, in AD, of a brain-specific neuroinflammatory response (Lukiw & Bazan, 2000). Aluminum, which also accumulates within neurons containing NFT and within mature Aβ and SP deposits, can stimulate iron-induced lipid peroxidation and play a

role in generating ROS, especially during the 'activation' of microglia which surround mature Aβ and SP deposits (Oteiza, 1994; Zhu & Bunn, 1999; Lukiw & Bazan, 2000). Accompanying AD neuropathology, a flood of ROS in the neuron has been shown to directly affect the activation and nuclear availability of several oxygen-sensitive TFs including hypoxia inducible factor-1 (HIF-1) and the nuclear factor encoding NF-κB (O'Neill & Kaltschmidt, 1997; Lukiw & Bazan, 1998). Both HIF-1 and NF-κB are currently implicated in various aspects of human brain degeneration and inflammatory disease (Butterfield et al., 1999; Lukiw & Bazan, 1998; Semenza et al., 2000; Mattson et al., 2000; Lukiw et al., 2001a). Because both of these pro-inflammatory TFs have been shown to be upregulated in AD brain (Kitamura et al., 1997; O'Neill & Kaltschmidt, 1997; Lukiw & Bazan, 2000; Christen, 2000; Lukiw et al., 2000c) their molecular biology as it pertains to aluminum and gene transcription will be discussed below in more detail.

Ubiquitously expressed in rodent and human tissues, HIF-1 is a relatively rare TF whose DNA binding activity (recognizing the promoter DNA sequence 5′-RCGTG-3′; Trifonov, 1991; Wang & Semenza, 1995; Haring & Kypr, 1999; Lukiw et al., 2000a, 2000b, 2001a) is directed toward oxygen sensitive gene expression, including activation of oxygen-responsive target genes that function in erythropoiesis, neovascularization, glycolysis and energy metabolism, glucose transport, the neuroinflammatory response and other genetic functions that include inducible nitric oxide synthase and β-amyloid precursor protein gene activation (Lukiw et al., 1994b, 2001a). Rapidly induced by hypoxia, the magnitude of the HIF-1 activation response is inversely proportional to the levels of available cellular oxygen or concentration of ROS (Wang & Semenza, 1995; Semenza, 1998, 2000). The primary molecular oxygen sensor for HIF-1 activation appears to be an iron-containing flavoheme protein in the plasma membrane, and interestingly, the divalent transition metals cobalt, manganese, nickel and zinc, but not magnesium, also activate HIF-1 presumably by augmenting intracellular redox conditions and ROS concentrations to simulate an intracellular hypoxic condition (Chun et al., 2000; Mukhopadhyay et al., 2000). Interestingly the PS1 and PS2 genes implicated in AD have been clearly shown to be under inducible transcriptional control by HIF-1 (Lee et al., 1996; Sato et al., 1999; Lukiw et al., 2000, 2001a, unpublished observations). Neural fluctuations in oxygen-sensitive NF-κB-DNA binding activities are also responsive to ROS concentrations, and these may play an important role in the proliferation of a brain-specific inflammatory response in neurodegenerating brain tissues (Lukiw and Bazan, 2000; Smith et al., 2000; Varadarajan et al., 2000; Christen, 2000). Indeed, recent studies support the hypothesis that aluminum may super-induce both HIF-1- and NF-κB–DNA binding to further fuel proinflammatory gene cascades in the AD brain (Campbell and Bondy, 2000; Lukiw et al., 2001b, unpublished observations). For example, the effects of magnesium, zinc and aluminum (all as chlorides at 100 nM) on HIF-1–DNA binding activation in monkey retinal cells are shown in Fig. 2 (Lukiw et al., 2001b; manuscript in preparation). After 48 hours of metal induction, zinc and aluminum both induce HIF-1-DNA binding approximately 3- and 5-fold respectively. It is tempting to speculate that aluminum may knock out and occupy key iron, magnesium and zinc binding sites necessary for normal molecular genetic function of TF proteins and thereby play an ancillary role for further disrupting gene transcription mechanisms. As discussed further below, this may be especially important for the rather large gene family encoding

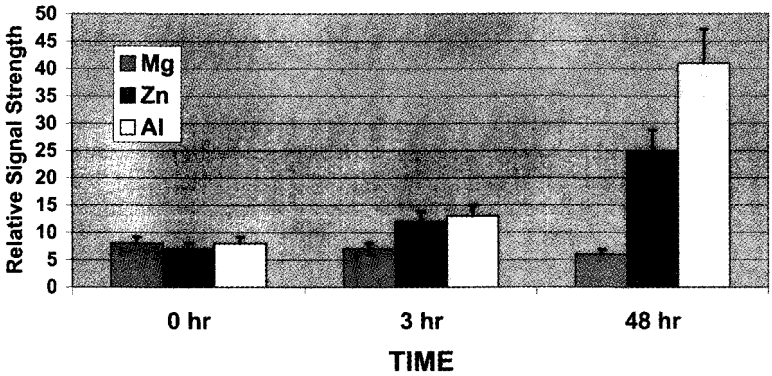

Fig. 2. Superactivation by zinc and aluminum chloride (100 nM) of HIF-1–DNA binding in monkey retinal cells in primary culture.

'zinc finger' TFs which are abundantly transcribed from the human genome (Pavletich & Pabo, 1991; Imanishi et al., 2000; Frederickson et al., 2000; Molkentin, 2000).

Analysis of broad spectrum gene expression in AD hippocampus and aluminum-treated normal human neural progenitor (NHNP) cells using high density cDNA arrays

The fact that there are broad-spectrum deficits in RNA message abundance in senescent cultured neural cells in vitro, in aging normal human brain and in particular in AD hippocampus and association neocortex suggest that in both aging and in neurodegeneration there are alterations in the regulation of brain genes and the mechanisms responsible for controlling their expression. As previously discussed, altered expression of TFs and signal transduction elements have been widely implicated in AD and age-related syndromes. To further understand alterations in TF gene expression associated with normal aging and proliferative human brain neurodegeneration, in one recent study from this laboratory, 1184 poly A+ RNA message levels were analyzed in broad-spectrum cDNA array panels (Clontech Panel 7850, Clontech Labs, Palo Alto, CA) and were interrogated using ^{32}P-labelled cDNA probes (specific activity > 3000 Ci/mM) derived from pooled human hippocampal CA1 RNA message populations (Lukiw et al., 2000b). These cDNA array panels are typically subdivided into 7 (A–G) theme-targeted sectors each containing a 14 × 14 dot matrix of genomic DNA, and focus on neural pathways involved in (A) cell cycle regulation (B) signal transduction (C) apoptosis (D) TF-DNA signaling (E) cell surface receptors (F) chemokine and cytokine signaling and (G) 9 housekeeping genes that include ubiquitin, cytosolic phospholipase A_2, hypoxanthine-guanine phosphoribosyl-transferase, glyceraldehyde 3-phosphate dehydrogenase, brain-specific α-tubulin, the HLA class I histocompatibility antigen C-4 α-subunit, β-actin, 60S ribosomal protein 13A and 40S ribosomal-associated protein 9 (Fig. 3). RNA message populations used for probing were isolated from a carefully selected group of 5 control and 5 AD brains. Importantly there were no significant differences in age (69.2 ± 1.5 vs 68.8 ± 1.9 yr, $p = 0.75$, ANOVA), death-to-brain-freezing interval (2.1 ± 0.7 vs 2.0 ± 0.7 hr, $p = 0.93$), drug or neurogenetic history between AD and control brains, and all AD brains

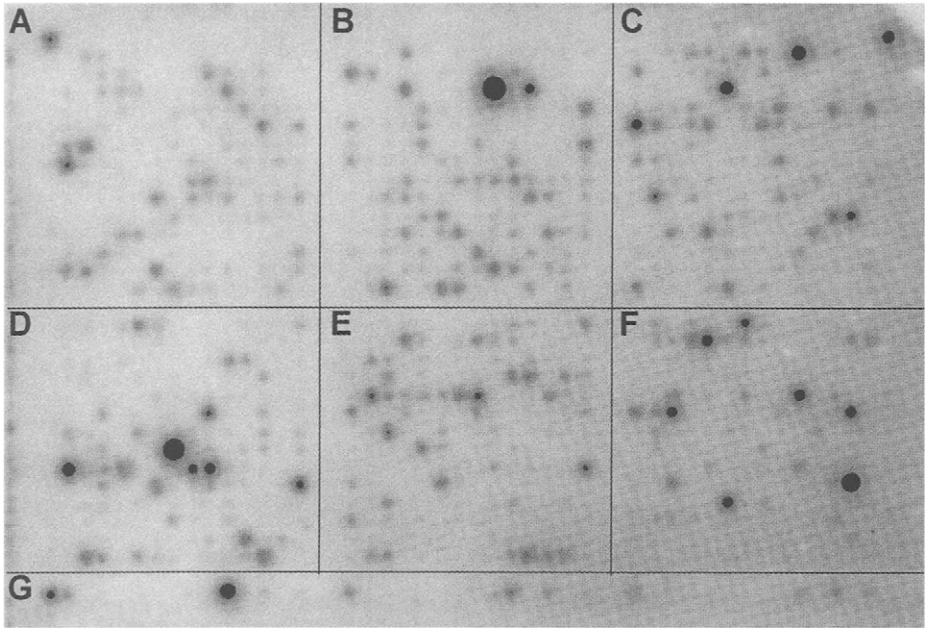

Fig. 3. Typical signal output in a broad spectrum cDNA array probed with [^{32}P-ATP] labeled cDNA isolated from normally aging human hippocampal CA1.

were classified as sporadic by NIA–CERAD criteria (Jellinger, 1998). In summary, AD samples showed statistically significant differences by a factor of 3-fold ($p < 0.05$), either increased or decreased, in the intensity of reporter signals for 11 genes encoding TFs and those defects correlated well with the observed, generalized down-regulation of gene expression in AD neocortex. Of the 9 TF RNA message levels found to be decreased in this study (TF factors in Sector D, Fig. 3; negative signals in Table 2), 8 of these (MTF-1, LYL-1, GATA-4, PCAF-associated 65β, GLUR repression factor 1, GATA-2, basic TF 62-kDa subunit (BTF2) and BRCA1-associated BARD1) require zinc as an essential metal ion cofactor (Table 2; Lukiw et al., 2000b, 2001a, 2001b). It is remarkable that the genes encoding these TFs have in their promoter DNA binding sites for the same TFs they encode, enabling positively reinforcing TF generation when these gene expression cascades are rapidly activated. Interestingly, the zinc-requiring metal regulatory element MTF-1, essential for neural response to metal ion load and the maintenance of the cellular redox state (Aschner, 1996) was found to be down-regulated in AD brain. Even more interesting, the only TFs found to be increased in this study were the pro-inflammatory NF-kappaB p52 subunit and HIF-1, key players in the proliferation of the inflammatory

Table 2. Deficits in RNA messages encoding zinc-requiring TFs in Alzheimer hippocampal CA1 using a human high density cDNA array panel (Clontech Panel 7850)

Rank	Signal strength	Protein/gene	Transcription factor	Metal requirement
1	−26344	metal-regulatory TF (MTF-1)	xxxxx	Zn++
2	−25560	LYL-1 TF HLH protein)	xxxxx	Zn++
3	+18192	NF-kappa-B p100/p52 subunit	xxxxx	Zn++
4	−53732	TF GATA-4	xxxxx	Zn++
5	−37376	P2X purinoceptor 3 (ATP receptor)	xx	?
6	−52348	PCAF-TF P65β	xxxxx	Mg++/Zn++
7	−25308	GLUR repression factor 1	xxxxx	Zn++
8	−21816	endothelial TF; GATA2	xxxxx	Zn++
9	−20820	basic TF 62-kDa subunit (BTF2)	xxxxx	Zn++
10	−30580	BRCA1 TF (BARD1)	xxxxx	Zn++
11	+24880	hypoxia inducible factor-1 (HIF-1)	xxxxx	?

response in neurodegenerating brain tissues (Lukiw and Bazan, 2000). Note that the NF-kappaB p52 apparently does not require the presence of a zinc atom to complete its own structure for activity, per se, but rather, zinc is required for the phosphorylation of its cytoplasmic inhibitor IKK which promotes the liberation of the transcriptionally active form of NF-κB (O'Neill & Kaltschmidt, 1997; Lukiw and Bazan, 1998).

Given the significantly higher abundance of aluminum over zinc in the biosphere, its smaller size, unchanging valence at 3+, significantly higher charge density, absence of a known or essential biological function, and its persistence as an unusually neurotoxic non-essential element when compared to zinc (RDA; recommended daily allowance for trace elements, US Government NIH Guidelines; Table 3) and the potential for aluminum to displace zinc from several key biological reactions, it is again tempting to speculate that aluminum is capable of knocking out or subverting the function of key zinc-requiring

Table 3. Alzheimer's disease from aluminum to zinc — biophysical characteristics

	Aluminum	Zinc
Rank abundance in biosphere:	Aluminum	Zinc
(as a metal)	1	18
(as an element)	3	24
(per cent mass)	8.8%	0.02%
	(average = 81,300 μg/gm; ~3 M)	(average 70 μg/gm; 440-fold less)
Atomic No. (weight)	13 (26.98)	30 (65.39)
Specific Gravity	2.7	7.1
Ionic structure	$1s^2 2s^2 2p^6 3s^2 3p^1$	$1s^2 2s^2 2p^6 3s^2 3p^6 3d^{10} 4s^2$
Element Group	IIIb	IIb
Charge	3+	2+ (+1)
Ionic radius (pm)	51	74
Z^2/r	17.65	5.4
Essential element	no	yes
Biological Function	??	several
RDA (NIH, USA)	??	5–25 mg

TFs (Pavletich & Pabo, 1991; Imanishi et al., 2000; Lukiw et al., 2000b, 2000c) and thereby attenuate normal brain gene expression profiles. This might be expected to play some role in the loss of normal brain transcriptional homeostasis and contribute to neural degenerative or inflammatory processes. Preliminary data from our laboratory suggest that using high density cDNA array analysis, aluminum treated normal human neural progenitor cells (NHNP; Clonetics CC-2599) in primary culture show remarkably similar deficits in TF RNA message abundance patterns to those found in AD hippocampal CA1 when compared to non-demented, age-matched controls (Fig. 3; Lukiw et al., 2001b; Lukiw & Bazan, manuscript in preparation).

An interplay between zinc and aluminum, gene transcription and human brain dysfunction?

Just as for the trivalent element aluminum (group IIIa), the roles of essential divalent transition metals such as zinc (group IIb; Table 3) in normal brain genetic function are not well understood. After calcium and iron, zinc is the third most abundant trace element in the mammalian brain, and has been receiving a lot of attention lately because of its potential role in the development of human neuropathology (Frederickson et al., 2000; Huang et al., 2000; Imanishi et al., 2000; Lukiw & Bazan, 2000; Molkentin, 2000; Rulon et al., 2000; Weiss et al., 2000; Yang et al., 2000). Synaptic zinc release at hippocampal excitatory glutaminergic synpases, and its correlation with neural activity was first demonstrated some twenty years ago (Assaf & Chung, 1984; see Weiss et al., 2000) however, the involvement of zinc in several common neurological dysfunctions has only recently become appreciated (Lukiw & Bazan, 2000; Frederickson et al., 2000; Weiss et al., 2000; Rulon et al., 2000). Excessive synaptic zinc release and postsynaptic zinc accumulation may contribute to neuronal loss associated with certain acute brain injury conditions including transient global ischemia and epilepsy. In the normal brain, zinc is thought to provide highly specialized neuromodulatory, neuroprotective and neurosecretory functions and is intimately involved with structural, regulatory and enzymatic proteins that provide these functions. However, imbalances in zinc homeostasis accompanying pathophysiology have been shown to be involved with, for example, Aβ aggregation and SP formation in AD (Yang et al., 2000), and the inhibition of cysteine proteases, normally catabolic and degradative enzymes which have a role in the clearance from the neuropil of potentially neurotoxic peptide accumulation (Weiss et al., 2000). In summary, alterations in brain zinc homeostasis in AD have been implicated in accelerated Aβ aggregation, increased ROS generation, impaired mitochondrial function and oxidative metabolism and altered neurotransmitter release (Christen, 2000; Frederickson et al., 2000; Huang et al., 2000; Lukiw & Bazan, 2000).

As previously outlined, one yet unexplored region for potential malfunction in zinc metabolism in the brain is in their critical function in the maintenance of TF structure and their ability to bind to promoter DNA, interact with other TF proteins and promote gene transcription. In many TFs zinc performs the essential role of coordinating specific cystine and histidine residues, thereby shaping amino acid sequence into 3-dimensional 'zinc finger' TF motifs. These are encountered, for example, in the brain-rich TFs SP1 and *zif-268*, which are capable of directly contacting the major groove of β-DNA and

wrapping part way around the DNA double helix (Pavletich & Pabo, 1991; Imanishi et al., 2000). Interestingly, bioinformatics analysis of newly sequenced human DNA from the human genome project suggests that up to 1 per cent of total human coding DNA, or up to 1000 genes, encode zinc-requiring TFs (Frederickson et al., 2000; Bentley, 2000). Zinc requiring TFs factors through the 'zinc-finger' motif therefore tend to be amongst the most common TF class known (Frederickson et al., 2000; Molkentin, 2000), and therefore may play an as yet unprecedented role in regulating patterns of brain-specific gene expression patterns in both health and disease. Investigations into the interactions at the molecular level of transition metals such as zinc in combination with aluminum and their roles in regulating and deregulating key aspects of brain-specific gene expression, and thereby their participation in neurodegenerative, neuroinflammatory and neuopathological events are in their infancy. Further studies addressing the hypothesis that aluminum may usurp normal transition metal-requiring genetic functions in the human brain are currently underway in our laboratory.

Conclusions

In summary, aluminum is by far the most abundant neurotoxic metal in the biosphere of the earth. Once it has bypassed highly selective and normally effective physiological barriers, aluminum has been shown to adversely affect cellular and membrane biochemistry, ionic homeostasis and in our studies the genetic function of nervous tissue within the aluminum 'infected' host. As aluminum has a particular attraction for the human CNS leading to normal nervous tissue functional impairment and dysfunction in several neurodegenerative disorders, it can be considered as a pervasive 'dementing' cation. The biophysical character of the highly reactive transcription apparatus unique to the evolutionarily recent genetic structures of the human brain neocortex may predispose this genetic compartment to aluminum's multiple genotoxic actions. Our current research suggests that aluminum has an unexplored potential to interact with important zinc-containing elements of the brain's gene expression system which may further impair the efficient transfer of genetic information in the CNS.

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Aluminium and Alzheimer's Disease
The Science that Describes the Link
C. Exley (Editor)

CHAPTER 8

Behavioral Studies in Animals: Past and Potential Contribution to the Understanding of the Relationship between Aluminum and Alzheimer's Disease

Mari S. Golub *

Department of Internal Medicine, University of California, Davis, CA 95616, USA

* Corresponding author; email: msgolub@ucdavis.edu

Abbreviations: Al – aluminum; Pb – lead; Hg – mercury; Ca – calcium; F – fluoride; Mg – magnesium; Fe – iron; NFT – neurofibrillary tangles; AD – Alzheimer's disease; CNS – central nervous system

Summary

Only a small portion of research directed at the Al–AD link has been undertaken in animal models using behavioral endpoints. Neurotoxicology studies of Al using drinking water administration, long term treatment duration, and animal models that develop NFT provide some additional relevant information. Functional CNS studies need to be integrated with neuropathological, genetic and biochemical approaches to adequately assess the Al–AD link. Such studies should test a set of behavioral endpoints specific to AD cognitive deficits and use Al exposure forms, routes and durations relevant to humans.

Historical Perspective

Introduction

Early studies administering aluminum (Al) and measuring behavioral variables in animals were focused on the possible relationship between Al and Alzheimer's disease (AD). More recently, Al has been studied as a neurotoxicant independent of a potential link with AD. Because children are most susceptible to classic heavy metal neurotoxicants (Pb, Hg), studies with developmental Al exposure have been undertaken. Some, but not all, of this collected literature is directly or peripherally relevant to the Al/AD issue. This perspective focuses on the following areas:

I. Behavioral studies in animal models that develop histopathological changes characteristic of AD.
II. Behavioral studies with long term or lifelong exposure to Al that simulate chronic human exposure.

III. Behavioral studies with administration of Al in drinking water that simulate human exposure via drinking water.

In addition a brief summary of findings is presented for the following areas:

I. Studies with short term (subchronic) treatment with Al by the oral route.
II. Studies with developmental treatment with Al.

Prior to review of these studies, some general considerations are presented.

General Considerations in Conducting/Evaluating Behavioral Studies with Al

Secondary effects on behavioral endpoints

In this review, the term 'neurobehavioral' is used for unlearned and spontaneous behaviors, and 'cognitive' is used for learned behaviors or behaviors modified by experience. Behaviors of most interest for AD are cognitive (learning, memory). It is important for studies with cognitive endpoints to evaluate sensory and motor function as well as activity level, body weight and general health. Deficits in these areas can secondarily influence performance of cognitive tasks. Also, changes in food motivation or response to stress can secondarily affect cognitive performance in tasks that use food reward or negative reinforcement.

In general, behavioral studies with oral Al dosing are not seriously complicated by secondary effects. Decreased food intake and weight loss or failure of weight gain are not characteristic of Al toxicity in the lower range of oral doses that influence behavior. In the case of Al, major target organs affected at toxic doses besides the CNS are bone (mineralization) and blood (anemia and immune effects). Secondary effects on behavior are not prominent from these sources. However, interpretation of studies that do not include information on health and growth is hindered, particularly if Al is administered by injection. As regards effects secondary to motivation, we have found that Al can enhance food motivation in mice (Golub & Germann, 1997). Al effects on stress-responsive systems (e.g. adrenocortical hormones) have not been studied.

Doses, routes and forms of Al

Studies with in vivo administration of Al are complicated by the high concentration of Al in commercial grain-based laboratory animal diets (Gawlick et al., 1987). Concentrations between 60 and 8300 μg/kg have been recorded (ATSDR, 1999); in our laboratory we routinely detect Al concentrations of 150–300 μg Al/g diet in commercial mouse diet. The Al may come from various components of the diet formula, including addition of minerals (Ca, Mg) from natural sources that also contain Al. This 'background' Al may be a considerable percent of the total amount ingested each day. For this reason we routinely use purified diet with an Al concentration (7 μg Al/g diet) more similar to that found in human diets as a 'background' diet. Many researchers now report the Al concentrations in the commercial diet that they use, so that a total daily Al intake (background plus administered) can be calculated. However, the relative bioavailability of the Al in commercial diet is not known, so that the appropriateness of totaling background

and added Al is not clear. Unless purified diets are used, it is difficult to specify the total daily intake of Al accurately.

Many routes of administration are relevant to potential Al exposures in humans. Gavage studies with Al would simulate exposures via medication or contaminated drinks. Feed and drinking water studies simulate exposure to food additives/contaminants and Al used in water purification. Injection studies parallel Al exposure in dialysis, parenteral fluids and vaccines. Al is also injected intracerebrally, usually in the ventricles, in order to induce neuropathology resembling neurodegenerative disease. Because of the low gastrointestinal absorption of Al and dietary factors controlling its uptake and retention (Domingo et al., 1993), extrapolation of behavioral effects across routes is particularly limited. This adds to the difficulty of integrating results of findings that use different routes of administration.

Several forms of Al are also used. Al sulfate and hydroxide are relevant to water purification and antacid exposures. Al lactate is often used as a representative organic form of Al with enhanced gastrointestinal absorption. Al chloride has been employed in food, drinking water, and injection studies. While administered doses can be compared across studies in terms of mg Al/kg body weight (bw), differences in bioavailability of Al forms leads to variability in the actual internalized dose.

The relevance of the relatively high doses used in animal experiments to human exposures is often called into question. Higher oral doses may be required in animals to achieve the same effects seen at lower doses in humans because of differences in diet. It is important to note that laboratory animals have a higher 'background' Al exposure due to presence of Al in commercial animal feeds (Gawlick et al., 1987). While humans reportedly consume about 0.1 mg Al/kg body weight/day (ATSDR, 1999), laboratory mice and rats (and rabbits) regularly eat 100 mg Al/kg body weight/day (based on the range of Al content of commercial diets). Thus a 100 mg Al/kg bw/day dose would double the rodent intake, equivalent to the percent increase in intake of a much smaller 0.1 mg Al/kg dose in humans. In addition, research indicates humans are at a higher risk for amount of Al absorbed than laboratory animals because of diet. Animal diets always contain the recommended dietary concentrations for essential elements like Fe, Ca and Mg, whose deficiencies are known to increase Al uptake (Allen, 1987; Van der Voet & De Wolff, 1987; Provan & Yokel, 1990; Brown & Schwartz, 1992; De Voto & Yokel, 1994), while few humans ingest optimal amounts of these elements daily. Also, grain based rodent diets, unlike human diets, lack appreciable citrate, lactate and gluconate, which are found in fruit, dairy products and meat and which can increase Al uptake (Molitaris et al., 1989; Domingo et al., 1993; De Voto & Yokel, 1994), and contain considerable phytate, which can inhibit Al uptake (Glynn et al., 1999). In addition, impaired renal function, which promotes Al retention, is present in human populations but rare in the optimally healthy rodent populations used in research.

Species

For the most part, Al neurotoxicology studies have used outbred rat and mouse strains traditional in this area of research. No particularly sensitive strains have been identified and no mutant mice have been tested. Because of the Al/AD issue, there is also a

fairly large literature on rabbits, a species that forms NFT in response to Al injection. Cross-species and cross-strain comparisons of brain Al concentrations after oral exposure may be valuable in determining sensitive animal models. However, we cannot confidently select animal models based on whole brain Al accumulation until we identify the cellular and molecular target of Al that might be important to AD pathogenesis.

Behavioral Studies in Animal Models that Develop Histopathological Changes Characteristic of AD

Although no animal models are known to develop AD-like neuropathology spontaneously in aging, rabbits and cats can be induced to produce neurofibrillary tangles (NFT) in response to Al intracerebral or subcutaneous injection (Petit, 1983). Shortly after this phenomenon was discovered in the 1960s, the NFT were described as 'similar but not identical' to those reported in Alzheimer's disease. Al-induced NFT are currently being investigated with modern techniques to determine similarities and differences relative to NFT of AD and other neurodegenerative diseases (Huang et al., 1997). Because they develop NFT, rabbits and cats provide a potential model of Al induction of AD by virtue of a similar histopathology, and studies of cognitive function could be undertaken to extend comparison of the syndromes. It should be noted that no animals are known to develop NFT or amyloid plaques in response to *oral* Al administration.

Several rabbit and cat studies in the 1970s and 80s used direct intracerebral Al administration (Petit, 1983). This procedure leads to a progressive encephalopathy syndrome with onset of motor signs days to weeks after Al injection, and ultimately to seizures and death. Behavioral testing was typically conducted in a period before onset of severe motor symptoms, but with a delay to allow formation of NFT. Marked impairment of function was seen in the behavioral tests. Crapper and Dalton (1973) found deterioration of a previously learned delayed response task, impaired learning of a conditioned avoidance response, and disruption of operant behavior rewarded by intracranial stimulation after intracerebral Al administration to cats. Petit et al. (1980) found impaired acquisition and retention of a passive avoidance task when training was conducted 10 days after intracerebral Al administration to rabbits. Because encephalopathy progresses more slowly in young rabbits than in older ones, 15 day old rabbits were injected with 50 μL 1% Al chloride (Rabe et al., 1982). Increased errors and time to learn a water maze were found when training was conducted 10 days after treatment.

These studies attempted to draw a link between NFT formation and cognitive decline, but there were many obvious difficulties with using the intracerebral injection/progressive encephalopathy model. Sensory or motor dysfunction caused by the brain damage could secondarily interfere with the ability to perform behavioral tests, apart from any disruption of the basic ability to learn and remember. Also, effects of Al injection on brain other than formation of NFT could be involved. An attempt to address these issues was made in a study that included both neuropathologic (NFT) and behavioral measures (Pendlebury et al., 1988; Solomon et al., 1988). The behavioral method, eyeblink conditioning, is less subject to interference by motor, sensory and motivational components of the

neurodegenerative syndrome than the active and passive avoidance tasks used previously. In the classical conditioning paradigm, a tone and air puff to the eye are repeatedly paired until the tone alone elicits a blink response. The motor component (blinking) is a simple behavior to perform, the sensory aspect only requires the ability to hear a tone, and no reward is involved. Aluminum (as Al chloride, 100 μL of 1% solution) was administered intraventricularly either before (Pendlebury et al., 1988) or after (Solomon et al., 1988) eyeblink conditioning (n = 10 for the Al group and 4 for the two controls, saline and HCl). Rabbits trained after Al injection failed to learn the conditioned response over a 4-day period when controls reached a performance level of 70%. When training was conducted before injection, and continued after injection and a recovery period, performance was reduced to about half of the control level. The authors found the deficits in learning and retention to be correlated with ranking of degree of NFT formation, but not with scoring of clinical, motor, or sensory changes caused by the injection. The authors (Pendlebury et al., 1988; Solomon et al., 1988) concluded that retention of the conditioning was disrupted by the Al chloride injections and NFT formation. Although this experiment is supportive of a relationship between NFT and cognitive impairment, disentangling of cognitive decline from other aspects of this clearly severe neurological syndrome remains problematic. While studies of Al injection and NFT formation in the rabbit continue, cognitive behavioral components have not been included.

A link between NFT and cognitive function would receive support from studies showing that intracerebral injection of Al in an animal species not forming NFT would not interfere with learning and memory. A study in rats failed to identify either cognitive impairment or neurodegenerative syndrome after intracerebral injection of Al (King et al., 1975). In contrast to most intracerebral injections, the rats were injected directly into hippocampal tissue, rather than into the ventricles or cisterna magnus. No NFT were seen in histological analysis of 7 rat brains 10 days after testing and no signs of neurodegenerative disease were seen in 3 rats followed for 12 additional months. In addition, no effects on learning of an avoidance task 7 days after injection were found using a trials to criterion measure (n = 6 to 9/group). However, Al group means were higher and there was considerable intra-group variability, suggesting that effects might have been detected with a larger group size or more sensitive measure. Another experiment was able to elicit a progressive neuropathy syndrome in rats with intracerebral Al (Lipman et al., 1988). Al tartrate (1.9 mg in 10μL) was injected intracerebrally (ventricles) and a progressive syndrome ending in seizures and death was produced. There was early impairment of active avoidance learning (fewer avoidance responses and more intertrial crossings in a shuttlebox). NFT were not examined in this study.

It is interesting to emphasize that no animal model has been developed for dialysis encephalopathy. Such a model would involve long term intermittent administration of Al by the i.v. or other parenteral route. The experiment of Lipman et al. (1988), described above, which produced a progressive encephalopathy in rats with i.c.v. injection, was intended as such a model. Apparently no further behavioral studies were conducted after the model was described, although an intraperitoneal dialysis model for uremic encephalopathy was also developed. Another possible model was provided recently with a study employing daily intraperitoneal injections of 10 mg Al/kg bw/day (as Al lactate) (Julka et al., 1995). The behavioral tasks employed were one trial passive

avoidance with a 24 h retention trial and 7 days of shuttlebox active avoidance training (n = 8/group). Testing was presumably conducted immediately after exposure was discontinued. Latencies for the passive avoidance retention trial and the number of avoidance trials were stated to be lower than for controls, indicating poorer performance. Unfortunately, the weight and general condition of the animals was not sufficiently described to evaluate the link between behavioral performance and cognitive impairment. Rotorod and spontaneous motor activity were not affected, but control variability was very high for these tests and the sample size may have been inadequate to detect effects. The further progression of the syndrome was not described as the rats were killed for brain biochemistry studies.

In addition to studies in rabbits with intracerebral Al injections, a model of induction of progressive neuropathy and NFT with subcutaneous injection was developed (Forrester & Yokel, 1985). Rabbits treated with a month-long series of injections developed abnormal gait and posture, limb splaying and seizures. The behavioral studies of Al in this model have used the conditioned eyeblink response which, as mentioned above, is less subject to interpretations of performance deficit based on impaired motor, sensory or motivational factors. It also differs from shock avoidance tasks typically used with intracerebral model in that conditioning is classical rather than instrumental. In classical conditioning, learning establishes a relationship between a previously neutral stimulus and a stimulus that reliably elicits a response from the animal. In instrumental conditioning the animal learns to produce a response for reinforcement.

More recently, Yokel and colleagues described new and previously published data from their laboratory using the conditioned eyeblink method in the rabbit and oriented toward understanding the relationship between Al and AD (Yokel et al., 1994). The studies used a series of 20 daily subcutaneous Al injections of 400 μmol/kg (Al as Al lactate), which had previously been shown to produce NFT in the rabbit (Forrester and Yokel, 1985). The experiments examined three issues: the effect of aging; reversal of behavioral deficits in rabbits by agents effective in human AD patients; and the occurrence of defective eyeblink conditioning in human AD patients.

In the age-dependent experiments, Al was administered to prenatal, early postnatal, adult or aged rabbits (Yokel et al., 1994). The rabbits treated prenatally and postnatally were trained either shortly after treatment or when they were more mature. The aged rabbit received a series of 200, rather than 400 μM Al injections because they showed mortality at the higher dose. Only the adult Al-treated rabbits (6 months old) showed a deficit in acquisition of the conditioning compared to age-matched controls. There was no effect on retention. Aged rabbits did not condition as well as the adults; the data from the aged rabbits (2–3 years old) were interpreted as showing a deficit from control although group differences were not significant. Pre and postnatally treated rabbits showed no conditioning deficit. Differences in dose, age at testing and baseline performance need to be taken into account in interpreting this data. In particular the authors mention that the 'aged' group consisted of 2–3 year old females, while rabbits have a life span to 7–8 years. However, no clear enhancement of cognitive effects of Al in aging was seen that would encourage further use of the aging rabbit as a model for AD.

In a second experiment directed at Al–AD relationships, aminopyridine was administered to Al treated rabbits prior to each conditioning session (Yokel et al., 1994).

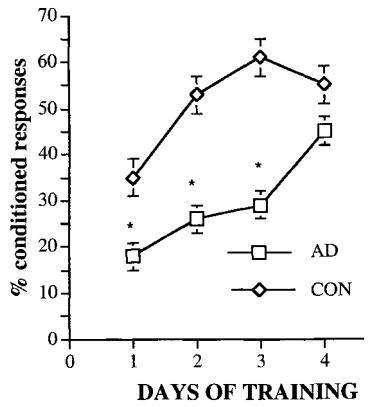

Fig. 1. Learning of the conditioned eyeblink response in ten AD patients and ten age and education matched controls. * Statistically different from control, $p<.05$. From Solomon et al. (1995).

Aminopyridine is a cholinesterase inhibitor has been used to enhance acetylcholine action and improve cognitive function in AD patients. The rate of conditioning was statistically improved for the aminopyridine-injected Al-treated rats relative to controls. This study is suggestive, although aminopyridine alone and Al alone groups would be needed for full interpretation.

The third experiment compared eyeblink conditioning in patients with AD vs controls. Characteristics of the groups other than diagnosis were not described. The AD group was highly impaired in acquisition of the eyeblink conditioning and did not reach the performance level of controls. Retention was not apparently influenced. A more recent study (Solomon et al., 1995) found that AD patients, although initially impaired, can reach the same performance level as controls, indicating that motor/sensory/motivational changes do not prevent acquisition (Fig. 1).

The final experiment was intended to assess cholinergic system changes typical of AD brain pathology in Al-treated rabbits. ACh was measured via microdialysis in the extracellular fluid as an index of impairment of ACh reuptake. The rabbits received a single intraventricular injection of 5 μmol Al (as Al lactate). After stimulation of neuronal activity with potassium ion, a decrease in extracellular ACh was found relative to controls. Further, when ACh was measured in dialysates collected during eyeblink conditioning sessions, a failure of both conditioning and ACh stimulation was seen. Together, this series of experiments provides interesting links between the brain pathology of AD and that induced by Al injection in rabbits. The experiments were exploratory and further work is needed for full interpretation.

Studies with Long-Term or Lifelong Aluminum Exposure and Behavioral Measures

A few studies of rats and mice are now available with chronic exposure to Al over a major portion of the lifespan (6 months or greater). There were numerous limitations of these experiments that preclude definitive statements about neurotoxic effects of chronic

Al. However, these studies have not opened new avenues for approaching the Al/AD issue by showing more striking effect with extended exposure, or with exposure during aging.

A 6.5 month exposure of Al in drinking water (50 and 100 mg Al/kg/day as Al nitrate nonahydrate) was conducted in male rats (Domingo et al., 1996). Citric acid was added to the water to promote Al absorption. The exposures were initiated in young (21 days of age), adult (8 months of age) and old (16 months of age) rats. They were tested after discontinuation of exposure for activity (open-field, 5 min period) and learning (passive avoidance, one-trial with 24 h retention trial). No effects of Al on either test were noted at any age. Group size was not stated but may have been 13–14 if the same rats were used in both tasks or 6–7 if different rats were used. Aluminum concentrations were 2–3 fold higher in several areas of young brains (hippocampus, cerebellum, thalamus, olfactory bulb, brain stem) but only in the brainstem of adults and the thalamus of the aged rats.

However, another 6 month study did report behavioral consequences of Al chloride administered in drinking water. Using a dose of 50 mg Al/kg bw/day (estimated from information in the article), several behavioral effects were demonstrated after both 90 and 180 day exposures (n = 10 male rats/group) (Lal et al., 1993). The effectiveness of the Al exposure was indicated by slower growth of the rats, which were probably immature at the time the exposure began, resulting in 10% lower body weights. Statistically lower activity counts in the open field and impaired learning over 5 days of active avoidance learning, as well as impaired extinction and relearning were found. This study is valuable in illustrating that extension of exposure from 90 to 180 days did not appreciably increase the degree of behavioral impairment. Al concentrations were about 50% higher in several brain areas (cerebral cortex, cerebellum, hippocampus, corpus striatum) at the end of the 180 day exposure period.

In an 11 month study, male Sprague Dawley rats were fed 1000 mg Al/kg diet as Al Cl_3 in a commercial chow diet (Commissaris et al., 1982). Spontaneous activity during a one hour session was lower in the Al-treated group than in controls (N = 14/group). In addition, acquisition (trials to criterion) of the shock motivated avoidance task was impaired, whereas extinction and retention were not. Body weights were not reported. The authors concluded that chronic Al ingestion "alters rat behavior in subtle ways".

In a 2 year study, minimally reported in the literature, rats were also exposed via diet (Clauberg & Joshi, 1992). The dose was stated as 100 μM $AlCl_3 \cdot 6H_20$, or 2700 μg/g diet. Body weights and hematocrit were reported to be lower in the treated rats. Morris maze testing suggested longer escape latencies during learning and shorter latencies during long term memory testing, but statistical group differences were not established (group size was not stated).

Our laboratory has recently completed a set of small studies with lifelong Al exposure in mice (Golub et al., 2000). The purpose of these experiments was to explore the mouse as a possible model for neurodegeneration in aging with chronic Al exposure. The animals received 1000 μg Al/g diet as Al lactate, a dose that approximates 100 mg Al/kg bw/day over the lifespan (conception to 2 years of age). This dose does not produce overt toxicity in adult mice with short term exposure. The basal diet, a purified diet, contained 7 μg Al/g diet. Swiss Webster mice (an albino outbred strain) and C57 BL/6J mice (a pigmented inbred strain) responded similarly to the high Al diet.

In this same study, biochemical measures as well as behavioral measures produced some counter-intuitive findings, which were, however, consistent with a hypothesized relationship between brain Al accumulation and adverse effects on behavior. When Al, Fe and Mn were measured in brain and spinal cord, brain Al was somewhat *lower* in the Al-treated mice than controls. TBARS (thiobarbituric reactive substance, a measure of lipid peroxidation), both with and without in vitro addition of Fe, was *lower* in the homogenate and microsomes of Al-treated mice than controls; Al had no effect on mitochondrial fraction. Protein content of brain homogenate, as well as membrane fractions (microsomes and mitochondria) was significantly higher in Al-treated mice than controls. Thus, at least in this model, the hypothesis that Al would accumulate in brain and promote oxidative damage with aging was not supported. The data are, however, consistent with findings of Domingo et al. (1996) that Al does not accumulate in brain of older rats, as it does in younger rats, and with the finding that lower brain Al concentration is associated with lower lipid peroxidative potential. The lower Al accumulation in older brains may be due to aging effects on brain constituents that reduce Al binding sites.

Mice were tested in the Morris maze between 18 and 24 months of age (n = 6 to 9/group). Al-treated mice showed *shorter* escape latencies (an index of better learning) than controls. Swiss Webster latencies were shorter on the second session of the 5 day learning period and C57BL/6J latencies were shorter on the second session after relocation of the visual cue used in the task (Fig. 2). Although these shorter latencies may be secondary to some cognitive abnormality in the mice, the experiment clearly did not show a general deficit in learning, memory or cue utilization.

A final mouse study looked at a potential interaction between dietary Al and MPTP injection. Both younger (66 days of age) and older (235 days of age) adult male Swiss Webster mice were given MPTP injections at a dose known to influence striatal dopamine in this strain (3 daily i.p. injections of 30 μg MPTP/g). This dose led to increased forelimb and hindlimb grip strength which was more pronounced in the younger mice. Al did not alter the effect of MPTP on grip strength.

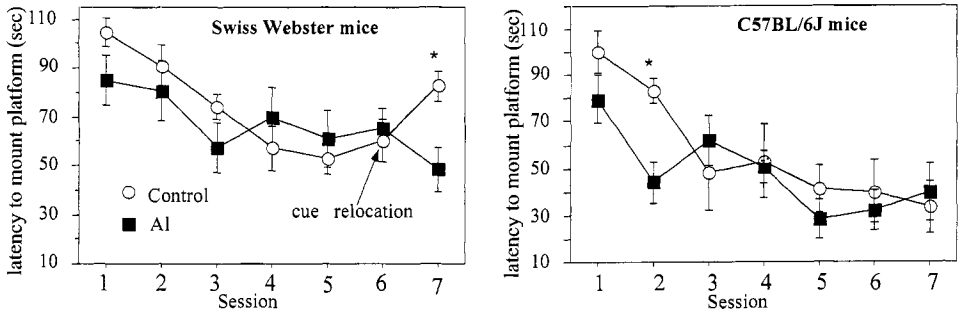

Fig. 2. Assessment of learning and memory in the Morris maze after lifelong exposure to a high aluminum diet. Mice were fed a diet containing 7 (control) or 1000 μg Al/g diet from conception to 18 months of age. Al-exposed mice performed better than control in the initial learning (C57BL/6J) or after cue relocation (Swiss Webster). * Statistically different from control. From Golub et al. (2000).

Table 1. Studies with Al administration in drinking water and neurobehavioral measures

Study	Agent, route, species (sex)	Doses, duration	Behavioral effects		No behavioral effect	Non-behavioral measures
			neurobehavioral	learning and memory		
Colomina et al., 1999	Al nitrate, drinking water, mice (male)	0, 300, 600 mg/kg/day, 14 days	open field, rotorod	–	active avoidance	↓body weight, ↓ water intake
Connor et al., 1988	Al sulfate, drinking water, rats (male)	0, 270 mg Al/kg/day, 30 days	not tested	passive avoidance extinction	active avoidance, radial arm maze, open field activity	body weights not reported
Domingo et al., 1996	Al nitrate, drinking water (with citrate), rats (male)	0, 50, 100 mg Al/kg/day, 6.5 months	not tested	–	open-field activity, passive avoidance learning, retention	body weights not reported
Lal et al., 1993	Al chloride, drinking water, rats (male)	0, 48 mg Al/kg/day [a], 90, 180 days	motor activity	avoidance, learning, extinction, relearning	Y-maze relearning	↓ body weights (after 90 days), brain lipid peroxidation
Sahin et al., 1995	Al chloride, drinking water, mice (male)	1 mg Al/kg/day [a], 100 days	rotorod	not tested	–	body weights not reported
Varner et al., 1994	Al fluoride, drinking water, rats (male)	0.05, 0.5, 5 mg Al/kg/day [a], 4–45 weeks	gait pattern, olfactory discrimination, balance test	–	open field, spontaneous alternation, Morris maze	high mortality in the 0.5 ppm group (8/10), body weight not reported

[a] Calculated for this review from information in the article.

Studies with Aluminum Administered in Drinking Water

Because epidemiological studies show a link between drinking water Al concentration and incidence of AD, animal studies using drinking water administration are particularly relevant to discussion of the relationship between Al and AD. These studies are outlined in Table 1.

Two of these studies (Lal et al., 1993; Domingo et al., 1996), conducted in rats, were described above. A 6.5 month study with drinking water found no behavioral effects with relatively brief testing protocol and doses of 50 and 100 mg Al/kg bw/day (Al as Al nitrate) (Domingo et al., 1996). In contrast, a 6 month study administered a dose of 50 mg Al/kg bw/day (Al as Al chloride), used a more extended testing protocol and found effects on activity, learning and memory after 6 months of exposure (Lal et al., 1993).

A rat study using a shorter exposure (30 days) but a higher dose (about 400 mg Al/kg as Al chloride) and a more extensive test battery (active avoidance, passive avoidance, radial arm maze) found Al effects only on passive avoidance extinction (Connor et al., 1988). A follow-up study reported that this effect could be reversed by administration of the chelator desferrioxamine (Connor et al., 1989). A mouse study with a short exposure period (14 days) used doses of 300 and 600 mg Al/kg bw assessed open field activity, avoidance learning and rotorod performance (Colomina et al., 1999b). For the open field, total distance traveled and vertical movement were both influenced by Al (dose groups were not compared individually with controls). For rotorod, the 600 mg Al/kg bw group fell off the rod sooner. No effects were found on an active avoidance learning task. In another drinking water study with Al chloride (1% solution from birth to postnatal day 60) learning and memory of active avoidance were impaired in mice; a similar effect was found with a 2-day treatment with Al chloride i.p. or s.c. (Yen-Koo, 1992).

Two studies using drinking water administration stand out because of the low doses of Al. For these experiments the administered Al dose would be estimated to be lower than the Al consumed in diet each day. The point is often made that daily Al exposure via drinking water is only a fraction of exposure via diet in humans, and thus differences in drinking water Al content are not important to Al toxicity. However, others argue that Al in drinking water may be more bioavailable.

In a study of mice, Al was administered in drinking water for 100 days at a dose of 1 mg Al/kg bw/day as Al chloride (Sahin et al., 1995). This dose was calculated from information in the article stating that mice drank 5 mL water/day containing 4.4 μg Al/mL. Assuming the mouse diet contained 200 μg Al/g, the mice would receive about 50 mg Al/kg bw/day from diet and the increase from drinking water would be about 2%. The study used a motor endpoint, the percent of the group ($n = 20$) that were able to complete a 2 min trial on a rotorod. This endpoint was reported to be significantly lower in Al-treated group than in controls after 38 and 90 days of exposure. While it may not seem reasonable that a 2% increase in Al via drinking water could be effective, another recent paper reported effects on dopamine content of the hypothalamus in mice given 1 mg/kg Al in drinking water in addition to 25 mg Al/kg that was received through diet in controls (Tsunoda & Sharma, 1999). No effects were noted at higher doses (4.6 and 23 mg Al/kg bw).

In a study of Al fluoride (F) in drinking water, Long Evans rats received 0.5, 5 or 10

ppm AlF_3 (mixture of $AlCl_3$ and NaF, 1:6 Al:F) in drinking water for 11 months (Varner et al., 1994). The authors estimated that the drinking water led to increase in total daily Al intake of <1%, 7.5% and 75%. The rats were given behavioral tests (gait and balance, olfactory preference, open field, spontaneous alternation, Morris maze) at various times ranging from 5 to 40 weeks of exposure. Interpretation of the study is complicated by the death of a number of animals during the evaluation period; group sizes at the time of testing are not stated. Potential F toxicity must also be taken into account. Additional work with this Al fluoride treatment are described elsewhere (Varner et al., 1993; Isaacson et al., 1997; Varner et al., 1998). The only group difference reported was on an olfactory task which tallied the amount of time the rats spent in compartments containing banana or lemon odor cues. Controls preferred the banana cue, while AlF_3 groups showed no preference. The authors suggest that joint Al-F exposure is more toxic than either alone, an issue relevant to the use of both Al and F in drinking water preparation. In an experiment related to this issue, nephrectomized rats injected subcutaneously with Al and F in a molar ration of 1:3 had reduced spontaneous motor activity compared to controls, while those injected with only Al or only F did not (Stevens et al., 1987). However, the rats in Al/F group suffered weight loss during the 30 day time period when control gained weight and none of the Al treated rats demonstrated a significant increase in brain Al.

Subchronic Studies

Behavioral studies with exposures shorter than six months have also been conducted. Shorter studies using a drinking water route are reviewed above (Connor et al., 1988; Yen-Koo, 1992; Lal et al., 1993; Sahin et al., 1995). These studies have been helpful in demonstrating that effects of Al on behavior can be produced after relatively short exposures (30–90 days) as demonstrated with neurobehavioral measures. Cognitive tests were seldom used with these short exposures.

A number of short term studies using diet or gavage examined neurobehavioral endpoints. Our group has conducted 3 studies of Al given in diet to mice at doses of approximately 50 or 100 mg Al/kg bw/day. After a 6 week exposure to 100 mg Al/kg body weight, spontaneous motor activity was lower than in controls (Golub et al., 1989). After 5 or 7 week exposures to 100 mg Al/kg bw/day, air puff startle, fore- and hindlimb grip strength were lower than controls (Oteiza et al., 1993). After a 13 week exposure to 100 mg Al/kg bw/day, airpuff startle, motor activity and hindlimb grip strength were lower than in controls (Golub et al., 1992a). Effects of subchronic exposure on activity have also been reported by other investigators. Activity was reduced in rats fed 100 mg Al/kg body weight for 12 weeks (Commissaris et al., 1982). In addition, a negative correlation was found between activity and brain Al concentration in male rats fed Al chloride at 0, 75, 125 or 175 mg Al/kg body weight/day for 60 days (Thorne et al., 1986). Significant negative correlations with brain Al were also reported for passive avoidance and learning of a shock-motivated discrimination task. Younger (weanling) rats given the same treatment were not affected in tests of activity, passive avoidance or radial maze learning (Thorne et al., 1987). Several studies have employed the rotorod test of balance and motor coordination. Rotorod performance was correlated with brain Al concentration after 4 weeks

of gavage with Al chloride at doses of 40, 80, or 120 mg Al/kg bw/day (Bowdler et al., 1979). No rotorod effects were reported with 12 week exposure to 100 mg Al/kg bw/day in diet (Commissaris et al., 1982). A rotorod effect at a very low reported dose (1.1 mg Al/kg bw/day) and 90 days of exposure was described previously (Sahin et al., 1995).

Developmental Al Studies

Developmental Al toxicity has been an area of concern due to exposure of infants to Al in formula, parenteral fluids, antacid uremia therapies, as an adjuvant in vaccination, from pregnant women's use of antacids, and from children's exposure through soil (Golub & Domingo, 1996). In a recent controlled experimental study, administration of parenteral nutrition fluids with high Al content to premature infants for 10 days or more led to a delay in behavioral development at 18 months of age (Bishop et al., 1997).

A number of studies have used Al treatment during pregnancy in rats and mice and examined behavior as part of a more general evaluation of Al developmental toxicity (Domingo, 1995). Injection (intraperitoneal or subcutaneous) or gavage were commonly used routes. Some studies looked primarily at behavioral development prior to weaning (Bernuzzi et al., 1986; Bernuzzi et al., 1989a; Bernuzzi et al., 1989b; Müller et al., 1990). Frequently behavior was also tested in adulthood to determine whether long-term or permanent effects were induced by these early treatments. Common endpoints in adults were spontaneous locomotor activity, rotorod, avoidance learning, and radial maze (Cherroret et al., 1992; Santucci et al., 1994; Gonda & Lehotzky, 1996; Gonda et al., 1996; Gonda et al., 1997; Alleva et al., 1998; Colomina et al., 1999a). Modest growth retardation was part of the syndrome in many studies. Effect levels were on the order of 200–400 mg Al/kg bw/day for oral exposures. Lower effective doses of 5–10 mg Al/kg bw/day were seen with subcutaneous injection of Al lactate (Gonda & Lehotzky, 1996; Gonda et al., 1996; Gonda et al., 1997) and endpoints of avoidance learning and memory. Reports of effect on tasks with positive reinforcement have been described in mice using 200 mg Al/kg bw/day i.p. injection of Al sulfate (Santucci et al., 1994; Alleva et al., 1998). Exposure by injection in these studies was often limited to the period of organogenesis. These studies have a potential relevance to AD in showing that developmental Al can influence brain function and that these effects can be long-lived or permanent, thus possibly setting the stage for enhanced susceptibility to neurodegenerative disease in adults. However, no hypotheses concerning the importance of early insult in AD have been forthcoming.

Our own studies in this area used longer term dietary exposure from conception to weaning (21 days) or puberty (35 days) in mice to parallel general human environmental exposures (Donald et al., 1989; Golub et al., 1992b; Golub et al., 1994; Golub et al., 1995). In testing cognitive function of adults we did not use shock motivated testing and failed to find Al effects on food rewarded operant tests of learning and memory (Golub et al., 1995). However, more recently learning effects have been detected with Morris maze testing (water escape reward) (Golub & Germann, 2000). We also found long term effects of developmental exposure on grip strength and auditory startle (Golub et al., 1994; Golub et al., 1995).

Informed Opinion

It is important to note that no major long term study of the Al/AD issue with behavioral endpoints has been undertaken. In particular, no attempt has been made to formulate and evaluate a set of behaviors modeled after AD cognitive deficits. It is also important to keep in mind the lack of availability of funding for such a project.

However, considerable information has been gathered through smaller scale studies of Al and the behavioral toxicology literature on Al. At the current time, several questions can be put forward for consideration.

Are Animal Behavioral Studies Needed in Al/AD Related Research?

A negative answer would seem to be provided by survey of current Al/AD research, which focuses on continued work at the cell biology level to recreate the neuropathological hallmarks of AD in animal models or in vitro systems. Neuropathology is also the focus on mutant mouse work that seeks to identify genes important to AD pathogenesis. The goal of this work is to create an animal model of Alzheimer's that can be used to develop preventive and therapeutic approaches, as well as an understanding of mechanism.

However, obtaining an exact model for human AD neuropathology through genetic manipulation may become complex (Van Leuven, 2000). Further, the relationship between neuropathological and functional changes is not yet understood. With this in mind concurrent evaluation of neuro and behavioral pathology would be helpful in determining the adequacy or value of the particular animal model. Behavior is probably a more sensitive endpoint of AD than neuropathology. Models that include behavioral endpoints also need to be available for use in developing prevention and therapy.

Eventually, animal studies with behavioral endpoints will be needed for scientific understanding of this disease. Tracing the pathway between damage at the cellular level and functional impairment will have to be done in animals and is important for rationally identifying points for potential intervention.

What Are Appropriate Behavioral Methodologies for Studying the Al/AD Issue?

None of the techniques used to date in Al behavioral research were built around the symptomatology of AD. The succession of behavioral methodologies in Al behavioral studies reflect borrowing of tools in common use for studying other problems. The early use of active and passive avoidance tasks reflected methods used for screening of anti-anxiety and anti-psychotic drugs in the psychopharmacology era. Eyelid conditioning was used to identify brain substrates of learning and memory because of simplicity of sensory and motor components and opportunity for tight experimental control. Toxicology has behavioral test batteries that are accepted as methods for screening chemicals for neurotoxicity. Morris maze is common in screening transgenic mice (Crawley, 1999). Any of these tests is probably able to reflect brain dysfunction in an animal with brain pathology as severe as that seen in AD. However, none of them are specific to AD.

It is probably inappropriate to use any available cognitive test in animal Al/AD research; an attempt should be made to assess the type and severity of cognitive deficit typical of AD. Eyelid conditioning remains a valuable tool because it is possible to separate motor, sensory and motivational deficits from learning and memory deficits. Although most commonly conducted in rabbits, techniques have been developed for use in mice (Aiba et al., 1994) and rats (Stanton & Freeman, 1994). However, eyelid conditioning lacks face validity as a parallel to cognitive deficits of AD, which are recognized in humans in declarative memory, verbal understanding and recall, and organizing and manipulating information. Spatial memory tests like the radial maze and the Morris maze are a better parallel to human cognitive dysfunction in AD, but extensive work needs to be done to control for potential motor, sensory and motivational deficits. Recognition memory tasks based on novelty response and not dependent on locomotion or positive or negative reinforcement could be valuable (Dodart et al., 1999).

What Is the Appropriate Species for Studying Al/AD Relationships?

At the moment it is not clear that an adequate animal model for Alzheimer's disease can be developed. In attempting to establish such a model, attention is focused on recreating the characteristic neurohistopathological changes seen in postmortem brains from Alzheimer's patients. With this in mind, the rat, cat or transgenic mouse are the most appropriate species. If it is determined that β-amyloid toxicity to the nervous system can occur without the formation of plaques, other animal models could be used. It is also possible that models lacking plaque and tangle morphological changes will be acceptable once the pathogenesis of onset of AD is better understood. Finally, it is possible that AD is a distinctly human disease and animal models will play only a peripheral role in research in this area.

Do Al Behavioral Toxicology Studies Tell Us Anything about the Relationship of Al to AD?

Al behavioral toxicology studies can only provide a framework and foundation for approaching the Al/AD issue. For instance, these studies have failed to demonstrate that high oral doses of Al for extended periods of time can in themselves induce neurodegenerative conditions in mice and rats. They also contain extensive information about the relationship of brain Al accumulation to behavioral dysfunction; whole brain and regional Al are seldom elevated more than 5 fold in studies where behavioral change is indicated; Al accumulation is lower in aged than adult or developing rats and mice. Details of protocols for in vivo administration of Al and measurement of affected behaviors are available. Ranges of minimally toxic doses for rats and mice are known. Very long exposures do not appear to be necessary. Developmental exposures have permanent effects. It is clear that spontaneous activity and motor ability are readily altered by Al and this could complicate learning and memory studies. Rats and mice do not differ substantially in response to Al. Such information is valuable in planning studies

and may some day be relevant to understanding AD, but has not provided direction for focused hypothesis-based research on AD.

What Is on the Horizon for Al/AD Behavioral Studies?

In vivo oral Al exposure needs to be studied in animal models with modified expression of genes relevant to AD pathology (APP, presenilins, ApoE). To date, mice are the only species available with these genetic manipulations. Some behavioral studies have been conducted with most of these transgenic models that could be used as a basis for studying the modifying effects of Al. However, a strong negative finding would not be obtained from such studies because the critical role of the particular gene expressed has not yet been determined, and the similarity between particular behavioral endpoints and cognitive dysfunction of AD is not established. Another obvious experiment, based on the growing evidence of amyloid peptide neurotoxicity, would combine in vivo Al exposure (at doses previously shown to influence behavior) with beta amyloid treatment. Finally, behavioral studies should be conducted in rabbit models in which AD neuropathology has been induced by injection of highly soluble forms of Al. While these studies should be informative, a negative finding has limited implications because none of these approaches has firmly established a model of AD.

Continuing research on pathogenesis of AD and neurotoxic mechanism of Al may identify converging pathways for investigation in animal models. These might for example, involve common lesions at the cellular level, like oxidative damage or deranged metabolism. However, while behavioral tasks are often able to target damage to specific neurotransmitter systems or brain areas, they are usually not specific to different types of cellular damage.

The difficulties of designing research in this very complex area must be weighed against the potential importance of the research. A very exciting prospect for understanding interaction between environmental and genetic factors in human disease is presented by research on Al and AD. Although limited, research on gene-environment interaction is more extensive for Al and AD than for many other neurodegenerative disease. State-of-the-art behavioral research that is integrated with state-of-the-art genetic approaches may establish an important precedent.

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Aluminium and Alzheimer's Disease
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CHAPTER 9

Aluminum as an Experimental Neurotoxicant: The Neuropathology and Neurochemistry

Michael J. Strong *

Neurodegeneration Research Group, The John P. Robarts Research Institute, and the Department of Clinical Neurological Sciences, The University of Western Ontario, Room 7OF 10, UC-LHSC, 339 Windermere Road, London, N6A 5A5, Canada

* Corresponding author, email: mstrong@julian.uwo.ca

Abbreviations: NFL – low molecular weight neurofilament protein; NFM – intermediate weight neurofilament protein; NFH – high molecular weight neurofilament protein; ALS – amyotrophic lateral sclerosis; NF – neurofilament

Summary

Although no single experimental paradigm has, as yet, recapitulated all of the neuropathological and neurochemical processes of Alzheimer's disease, the use of aluminum as an experimental neurotoxicant can, at one time or another, give rise to virtually every process observed to be aberrant in Alzheimer's disease. Variations in the speciation of aluminum utilized, the age of the host animal, the in vitro conditions, the duration of the aluminum exposure, or the analytical methods utilized all contribute to this observation. Hence, as will be reviewed in this chapter, although the aetiology of Alzheimer's disease remains unknown, paradigms of aluminum toxicity remain potent tools in understanding this process.

Historical Perspective

Introduction

Although aluminum has been implicated in the pathogenesis of a number of human neurodegenerative disorders including Alzheimer's disease, dialysis encephalopathy and the Western Pacific variant of amyotrophic lateral sclerosis (ALS), the direct evidence linking aluminum to the genesis of these disorders is scant. However, in the century since the first pathological description of experimental aluminum toxicity by Dölken (1897), there has been increasing evidence that the administration of either organic or inorganic aluminum compounds, by a variety of routes, leads to disease states bearing many of the hallmarks of these disorders (Strong, 1994). This was highlighted by

the studies of Terry and Peña (1965) and Klatzo et al., (1965) in which aluminum compounds applied directly to the cerebral cortex induced the formation of dense, argentophilic aggregates and a profound encephalopathy. Although these aggregates are now known to consist primarily of the intermediate cytoskeletal neurofilament proteins, depending on the speciation of aluminum utilized and the host, such aggregates can also contain aberrantly phosphorylated microtubule associated *tau* protein (Huang et al., 1995; Savory et al. 1995; Singer et al., 1997), amyloid precursor protein, α_1-antichymotrypsin, and ubiquitin–protein conjugates (Huang et al., 1997). In the models developing predominantly neurofilamentous aggregates, the neuropathological features bear striking similarity to those observed in amyotrophic lateral sclerosis (Wakayama et al., 1996), while the latter constellation of findings is more consistent with the neuropathology and neurochemistry of Alzheimer's disease.

This dichotomy serves to highlight one of the key aspects of aluminum neurotoxicity — that the determinant of the neuropathological phenotype is as critically linked to the formulation of aluminum utilized as it is to the species/host under examination (Exley et al., 1996). This chapter will highlight this by examining the spectrum of neuropathological and neurochemical effects of aluminum across a number of experimental paradigms. The neurotoxic effects of aluminum in the rabbit will, however, be discussed only indirectly given the discussion by Savory (see Chapter 10 of this volume).

Neuropathology and Neurochemistry of Aluminum Toxicity

Although *in vivo* the response of a host to aluminum exposure can be arbitrarily defined as either the development of neurofilamentous aggregates with an associated encephalomyelopathy (as seen in rabbits, cats, ferrets and nonhuman primates) or as the development of neurochemical changes in the absence of neuropathology (e.g., rats, mice) (Strong et al., 1996), this differentiation obscures the extensive neurobehavioral and neurophysiological effects of aluminum. Nowhere is this more evident than in the observation of striking functional, neurobehavioral and motor impairment in the absence of obvious cytoskeletal pathology as illustrated by rodents fed either organic or inorganic aluminum compounds (King et al., 1975; Bowdler et al., 1979; Thorne et al., 1986; Connor et al., 1988; Cherroret et al., 1992; Golub et al., 1992; Yen-Koo 1992). Indeed, the effects of aluminum are protean — a feature that belies the utility of aluminum as a tool for understanding the pathogenesis of neurodegeneration.

Perturbations of the neuronal cytoskeleton

As will be discussed, aluminum can induce metabolic disturbances beginning at the level of gene expression through to modifications in protein–protein interactions that can be incriminated in the formation of intraneuronal aggregates (Table 1). Which, if any, of these processes is integral to the pathogenesis of human neurodegeneration remains a critical research issue.

At the molecular level, aluminum alters the normal stoichiometry of neurofilament (NF) protein gene expression. In human temporal neocortical tissue, there is an association

Table 1. Perturbations in cytoskeletal structure and protein interactions

Modifications of cytoskeletal protein gene expression	
Increased affinity of linker histone to DNA	(Karlik et al., 1980; Lukiw et al., 1989; Tarkka et al., 1993)
Chromatin condensation	(Lukiw et al., 1987; Okarinen et al., 1991)
Inhibition of DNA repair mechanisms	(Hamlin et al., 1989)
Reduced rate of DNA synthesis	(Berlyne et al., 1972)
Increased DNA replication errors	(DeBoni et al., 1980)
Inhibition of hormone-induced chromosomal puffing	(Sanderson et al., 1982)
Inhibition of chromatin-dependent RNA synthesis	(Sarkander et al., 1983)
Reduction in RNA pool	(Mitsumoto et al., 1988)
Suppression of cytoskeletal mRNA steady state levels	(Muma et al., 1988) (Parhad et al., 1989; Strong et al., 1994)
Cytoskeletal protein modifications	
Increased microtubule associated protein (MAP-2) phosphorylation	(Johnson and Jope, 1988; Johnson et al., 1990, 1992)
Elevations in cAMP, cGMP	(Johnson and Jope, 1987; Johnson, 1988)
Inhibition of endogenous phosphatase activity	(Shea et al., 1992a; Shetty et al., 1992; Strong and Jakowec, 1994)
Inhibition of calmodulin binding	(Putterill and Gardner, 1988)
Failure of inhibition of calmodulin interaction with phosphodiesterase	(Richardt et al., 1985)
Impairments of neurofilament/microtubule interactions	(Cunningham et al., 1997)
Cross-linking of NF subunits	(Lefebvre and Mushynski, 1988; Troncoso et al., 1990; Hollósi et al., 1992, 1994; Leterrier et al., 1992, 1993; Shea et al., 1992b)
Neurofilament resistance to calpain-mediated proteolysis	(Nixon et al., 1990)
Reduced α-antichymotrypsin inhibitor activity	(Clauberg and Joshi, 1993)
Preserved proteolytic properties	(van Ginkel et al., 1993)
Inhibition of axonal transport	(Kashiwagi et al., 1998)
Tau protein aggregation	(Abdel-Ghany et al., 1993; Savory et al., 1995; Savory et al., 1996; Singer et al., 1997)
Aggregation of amyloid β-protein	(Kawahara et al., 1994)

between the presence of aluminum and chromatin condensation via tighter binding of the linker histone H1, thereby reducing chromatin sensitivity to nucleases and reducing transcriptional rates for selective proteins (Lukiw et al., 1987, 1989; Oikarinen et al., 1991; Walker et al., 1989). The low molecular weight NF protein (NFL) has been postulated to be the most sensitive to this process. This is of considerable interest given a close parallel to transgenic mice in which the selective alteration of NF stoichiometry through deletions of individual NF subunit protein genes leads to neurofilament aggregate formation and the occurrence of motor neuron disease (reviewed in Strong, 1999). The alterations in gene expression induced by aluminum have been most extensively studied in the rabbit, a host that is exquisitively sensitive to the effects of aluminum. Unfortunately, the results of various laboratories have differed significantly, including the findings of either a widespread and non selective suppression of mRNA levels (Parhad et al., 1989), a selective suppression of the NFL mRNA (Muma et al., 1988; Strong et al., 1994), or no effect whatsoever (Savory et al., 1993). In part, the discrepancy

between these observations relates to the route of aluminum administration, the aluminum species utilized, and the age of the host rabbit. Critical to this however is the method of analysis, an issue addressed by Chambers and Muma (1997). While the previous studies utilized Northern blot analysis and thus can be argued to be insensitive to specific individual neuronal responses to aluminum exposure, *in situ* hybridization confirmed a significant alteration in neurofilament stoichiometry with suppression of high molecular weight NF protein (NFH) steady state mRNA levels restricted to neurons containing neurofilamentous aggregates. Intermediate weight NF protein (NFM) steady state mRNA levels were not affected and NFL levels were not studied.

To some extent, this may be a mute point. Using the sciatic axotomy paradigm of inducing suppressions in NF gene expression, we demonstrated that even in the presence of a robust suppression of all NF gene expression, neurofilamentous aggregates still formed within axotomized spinal motor neurons in young adult New Zealand white rabbits (Strong and Gaytan-Garcia, 1996). This implies that, in the rabbit, aluminum-induced neurofilamentous aggregate formation can occur independent of alterations in NF gene expression. Indirectly, this observation also implies that a post-translational protein modification in one or more cytoskeletal proteins might underlie the formation of neurofilamentous aggregates.

Ex vivo, aluminum has been demonstrated to induce the cross-linking of NF proteins, suggesting that such a process may underlie aggregate formation *in vivo* (Leterrier et al., 1992). In addition to this cross-linking, we have shown that following the intracisternal inoculation of $AlCl_3$ in young adult New Zealand white rabbits, that the co-sedimentation properties with tubulin of NFH isolated from the cervical spinal cord of inoculated rabbits are strikingly modified (Cunningham et al., 1997). Exhaustive dephosphorylation induced a marked increase in co-sedimentation, opposite to the findings of NFH derived from control inoculated rabbits. Whether this cross-linking of cytoskeletal proteins is at the basis of the inhibition of axonal transport observed both *in vivo* and *in vitro* following aluminum exposure is unknown. However, it is clear that aluminum induces impairments in a number of aspects of axonal transport, including slow axonal transport (SCa), and the transport of several proteins of fast axonal transport (Troncoso et al., 1985; Kashiwagi et al., 1998). Utilizing hippocampal neurons *in vitro*, Wakayama and colleagues extended these observations to demonstrate that aluminum also retards dendritic transport (Wakayama et al., 1997). The latter authors postulated that inhibitions in dendritic transport would have a profound effect on MAP2 synthesis, and thus a reduction in the number of microtubules in distal dendrites. The net effect of the latter would be anticipated to be a reduction in the complexity of the dendritic tree similar to that observed in Alzheimer's disease (Schmidt et al., 1994).

Aluminum will also perturb the post translational processing of cytoskeletal proteins, giving rise to alterations in phosphorylation state, binding characteristics to calmodulin and the attendant calmodulin mediated proteolysis of proteins. In total, these observations have led us to propose that aluminum can induce an intraneuronal pool of proteolysis-resistant, aberrantly phosphorylated neurofilament, aggregating on the basis of increased protein cross linking properties. The incorporation of both *tau* protein and β-amyloid into these structures might easily follow (Shin et al., 1994).

Alterations in membrane stability and signal transduction

Distinct from its effect upon the cytoskeleton, aluminum induces significant alterations in membrane fluidity and signal transduction (Table 2). Increased lipid peroxidation will directly affect membrane integrity and, through alterations in membrane fluidity, impact directly on Ca^{2+} signalling (Bolotina et al., 1989). Of relevance to our understanding of Alzheimer's disease, the modification of intracellular Ca^{2+} homeostasis can occur through a number of mechanisms. This includes the activation of the Na^+/K^+-ATPase pump, altering the Na^+ gradient, and thereby enhancing intracellular Ca^{2+} levels. At micromolar concentrations, Al^{3+} alters Ca^{2+} uptake into the endoplasmic reticulum, accelerates Ca^{2+} release from mitochondria and inhibits Ca^{2+} efflux by the inhibition of

Table 2. Alterations in membrane stability and signal transduction

Increased membrane rigidity	
Adsorption to phosphatidyl surfaces	(Vierstra and Haug, 1978; Akeson et al., 1989; Deleers et al., 1986, 1987; Sorek and Meiri, 1992)
Oxidative injury	
Increased lipid peroxidation	(Gutteridge et al., 1985; Fraga et al., 1990; Chainy et al., 1993; Oteiza et al., 1993)
Enhanced iron-induced lipid peroxidation (*in vitro*)	(Fleming and Joshi, 1987; Ohyashiki et al., 1993; Oteiza, 1994; Xie et al., 1996; Xie and Yokel, 1996; Bondy et al., 1998)
Cholinergic effects	
Decreased choline acetyltransferase (ChAT) activity	(Gulya et al., 1990; Bilkei-Gorzo, 1994; Cherroret et al., 1994;)
Absence of effect on ChAT activity	(Connor et al., 1988; Johnson and Jope, 1987)
Reduced choline uptake	(Johnson and Jope, 1986; Zubenko and Hanin, 1989)
Reduced acetylcholinesterase activity	(Marquis and Lerrick, 1982; Moraes and Leite, 1994; Zatta et al., 1994)
Alterations in signal transduction	
Decreased inositol phosphate (IP_3)	(Haug et al., 1994; Johnson et al., 1990, 1992; Jope, 1988; Mundy et al., 1995; Shafer et al., 1993; Shafer and Mundy, 1995)
Decreased glutaminergic neurotransmission	(Provan and Yokel, 1992; Platt et al., 1994; Cucarella et al., 1998; Llansola et al., 1999)
Enhanced monoamine oxidase activity (A/B)	(Zatta et al., 1998)
Alterations in calcium homeostasis	
Enhanced Ca^{2+} uptake (mechanism not determined)	(Anghileri, 1992)
Inhibition of voltage gated Ca^{2+} channels	(Koenig and Jope, 1987; Büsselberg et al., 1993, 1994; Platt et al., 1993)
Inhibition of Ca^{2+}/Mg^{2+} ATPase	(Kodavanti et al., 1993; Mundy et al., 1994)
Inhibition of Ca^{2+} ATPase	(Vig et al., 1989) *(inhibition via a calmodulin dependent pathway)*; (Julka and Gill, 1996)
Inhibition of endoplasmic reticulum Ca^{2-} pump	(Gandolfi et al., 1998)
Activation of pro-apoptotic pathways	
Caspase III activation	(Guo-Ross et al., 1998)

Ca^{2+}-ATPase activity (Gandolfi et al., 1998; Julka and Gill, 1996). A direct consequence of increased $[Ca^{2+}]_i$ is an increased level of lipid peroxidation (Julka and Gill, 1996; Xie et al., 1996). Potentially, a self-propagating process can thus arise in which the initial insult of Al^{3+} induces alterations in Ca^{2+} homeostasis, which in turn alters membrane phospholipids and membrane fluidity, resulting in further alterations in Ca^{2+} signalling.

In addition, elevated $[Ca^{2+}]_i$ can trigger apoptosis. To some extent, this is supported by the observation of enhanced caspase III activity in homogenates derived from the brains of aluminum treated rats (Guo-Ross et al., 1998). *In vivo*, however, aluminum induced motor neuron cell death appears to be via an non-apoptotic pathway (He and Strong, 2000), an observation supported *in vitro* by the failure to find apoptosis in aluminum-induced cerebellar granule cell death (although apoptotic astrocytic cell death was observed) (Suárez-Fernández et al., 1999). The discordance in these observations may well relate to the previously discussed concern of aluminum speciation, the experimental paradigm utilized, and the host characteristics.

Distinct from the induction of cell death, aluminum will induce significant metabolic perturbations. These include the inhibition of hexokinase (Womack and Colowick, 1979), the inhibition of G6PD (Cho and Joshi, 1988, 1989), and regional reductions in glucose metabolism (Johnson and Jope, 1986; Clauberg et al., 1994).

The effect of aluminum on non-neuronal cells

As noted above, when young adult New Zealand white rabbits are administered $AlCl_3$ intracisternally at monthly intervals, a chronic motor neuron degeneration bearing ultrastructural similarity to ALS ensues (Strong et al., 1991; Wakayama et al., 1996). Of interest was the observation that this process was reversible upon cessation of aluminum exposure, and that this process correlated with the extent of microglial proliferation (He and Strong, 1999; Strong et al., 1995). Utilizing the immortalized microglia cell line (BV-2), we have now demonstrated that both organic and inorganic aluminum compounds can inhibit the activation, migration and proliferation of microglia cells *in vitro*, potentially suggesting that the extent of aluminum-induced neuronal injury may also be dependant on the extent of microglial activation (He and Strong, 2001).

The concept that aluminum neurotoxicity can be modulated by glia is not without experimental support. Aluminum-induced cerebellar granular cell death *in vitro* is enhanced by astrocytic co-culture (Suárez-Fernández et al., 1999). Isolated glial cultures, exposed to aluminum, develop a variety of neuropathological changes, including a decrease in GFAP immunoreactivity. Compared to isolated neurons, glia appear to be more sensitive to the neurotoxic effects of aluminum *in vitro* (Campbell et al., 1999) although the presence of glia within the culture will enhance the neurotoxic effect of aluminum (Sass et al., 1993). It is less clear what the net effect of aluminum upon astrocytic function may be *in vivo*. Gliosis, with an attendant increase in GFAP has also been documented in aluminum injected rabbits and following the application of aluminum to guinea pig or monkey cortex (Hoeppner and Morrell, 1986; Yokel and O'Callaghan, 1998). Conversely, the intraperitoneal or intraventricular injection of aluminum to rats leads to a reduction in the amount of GFAP (Guo-Ross et al., 1999). The failure to observe a significant increase in GFAP in glial cultures following aluminum exposure suggests that the observed increase

in GFAP *in vivo* may relate to a response to neuronal injury induced by aluminum. Regardless, it is clear that the neurotoxicity of aluminum cannot be viewed in isolation from the attendant glial reaction.

Informed Opinion

Regardless of the human neurodegenerative process under examination, the individual researcher is hampered by the realization that we are, at any given time, looking only at a 'snapshot' of a disease process. In general, this 'snapshot' is taken at the terminal stages of the disease when that which remains for us to observe represents the final marker of a disease process. The real value of aluminum as an experimental neurotoxicant is in its utility as a tool with which to induce neurodegeneration in a variety of hosts and rather than obtain restricted 'snapshots' of a disease process, gain an intimate knowledge of the evolution of the disease. Amongst such insights gained has been the determination that a seemingly simple trigger such as the administration of a single trace metal can give rise to a plethora of individual aberrant biological processes that ultimately yield a rather homogenous appearing clinical phenotype.

As highlighted in the previous sections, the impact of aluminum within an individual cell is at multiple levels. However, it is overly simplistic to consider that, for any given cell, only one effect of aluminum might be evident. For instance, alterations in NF expression ratios, thereby altering the normal stoichiometry of NF subunit protein should be sufficient to induce NF aggregates. Altering the normal process of NF dephosphorylation and inhibiting NFH dissociation from microtubules should add further to this process by taking such aggregates and rendering them largely insoluble. As discussed, the generation of axostasis by aggregates will ultimately lead to Wallerian degeneration and cell death, the rate of which may well be further enhanced by aluminum induced alterations in membrane fluidity and calcium influx. The participation of non-neuronal cells in this process, as a response to the degeneration of the neuron or as a distinct effect of aluminum, begins to expand this degenerative process outside of the realm of a single cellular biochemistry.

Such an all-inclusive approach is not without human parallels. The neuropathological hallmarks of Alzheimer's disease consist of a variety of neuronal and non-neuronal cytoskeletal changes, including the presence of senile plaques, amyloid deposition and neurofibrillary tangle formation. Neurofibrillary pathology can be observed within neuronal perikaryal and as neuropil threads or dystrophic neurites. In addition, considerable evidence now exists that microglial activation is also a significant component of not only Alzheimer's disease, but additionally of the neurofibrillary degeneration of the Western Pacific parkinsonism-dementia complex (McGeer et al., 1993; Schwab et al., 1996). The neurochemical pathology of Alzheimer's disease reflects primarily the loss of acetylcholine activity. In this sense, the neuropathological and neurochemical features of Alzheimer's find significant parallels in the neuropathological and neurochemical features of both acute and chronic aluminum neurotoxicity. Unfortunately, given the considerable effort at either proving or disproving that aluminum toxicity is the aetiology of AD, the fact that aluminum produces these parallel effects is often lost or disregarded.

Future studies should be directed to further understanding the mechanisms by aluminum induces alterations in protein–protein interactions, and specifically how cross-linking is induced and maintained. Given the widespread effects on intracellular signalling pathways, further understanding alterations to this pathway, and how this relates to the induction of cell death should remain a priority. It would seem, at minimum, that aluminum intoxication should be considered as a suitable experimental paradigm for understanding the biology of both ALS and AD, rather than relegating it to the vast wasteland of discarded etiological concepts.

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Aluminium and Alzheimer's Disease
The Science that Describes the Link
C. Exley (Editor)

CHAPTER 10

The Rabbit Model System for Studies of Aluminum-Induced Neurofibrillary Degeneration: Relevance to Human Neurodegenerative Disorders

John Savory [1,2,*], Othman Ghribi [1], Michael S. Forbes [1] and Mary M. Herman [3]

Departments of [1]Pathology, [2]Biochemistry and Molecular Genetics, University of Virginia, Charlottesville, VA 22908, USA; [3] IRP, NIMH, NIH, Bethesda, MD 20892, USA

* Corresponding author, email: js2r@virginia.edu

Abbreviations: Al – aluminum; NFA – neurofibrillary aggregate; AD – apical dendrite

Summary

Rabbits are particularly sensitive to aluminum neurotoxicity and develop severe neurological changes, especially if the metal is administered directly into the central nervous system. Other routes of administration determine the degree of severity of such clinical symptoms, with oral administration exhibiting minimal effects. The complex chemistry of aluminum must be understood before designing these experiments in order to avoid formation of insoluble aluminum hydroxide complexes. However, by selecting appropriate aluminum compounds to be administered to the animals, neuropathological and biochemical changes bearing similarities to those seen in Alzheimer's disease are observed, thus supporting the hypothesis that aluminum is involved in the pathogenesis of this disease.

Historical Perspective

Introduction

Studies using the rabbit may be particularly relevant to the investigation of human disease since, according to the sequences of 88 proteins, this animal belongs to the mammalian order Lagomorpha, a group which has been reported to resemble primates more closely than rodents (Graur *et al.*, 1996). The first experiment suggesting that aluminum (Al)-induced neuronal changes might have relevance to Alzheimer's disease was that of Klatzo *et al.* (1965) who reported that the intracisternal administration of Al phosphate to New Zealand white rabbits produced intraneuronal protein aggregates which, with silver staining, appeared remarkably similar to the neurofibrillary tangles

of Alzheimer's disease. This was a serendipitous finding, since the experiment was designed to study the immune response of the central nervous system, and an antigen had been administered intracerebrally to rabbits in Holt's adjuvant (I. Klatzo, personal communication, 1997), the latter containing Al phosphate. For almost 70 years, makers of vaccines have used either Al sulphate, Al hydroxide or Al phosphate as adjuvants to improve the body's immune system, in order to favor an earlier response to an antigen (Malakoff, 2000). In the pioneering experiment of Klatzo, the rabbits developed severe neurological symptoms within 2 days of the injection, and had to be sacrificed. Examination of brain tissue from these animals revealed the characteristic Al-induced neurofibrillary degeneration; that is, silver-impregnated (argyrophilic) fibrillary inclusions found predominantly in the neuronal cell bodies (perikarya) and proximal neurites (dendrites and axon hillock) (Klatzo *et al.*, 1965). Al-induced neurofibrillary aggregates at the light microscopic level closely resemble neurofibrillary tangles, one of the histologic hallmarks of Alzheimer's disease.

Other histopathologic hallmarks of Alzheimer's disease, such as neuritic plaques, were not present in this Al-induced encephalopathy. Interestingly, an abundance of neurofibrillary tangles coupled with a relative paucity of neuritic plaques characterizes the amyotrophic lateral sclerosis/parkinsonism-dementia complex of Guam, indicating that widespread neurofibrillary tangles (without plaques and/or significant Aβ deposition) may be the cellular correlate of neurological decline, as reviewed in Mawal-Dewan *et al.* (1996). Often neuritic plaques show a perivascular predilection, suggesting that the dystrophic neurites and the attendant amyloidogenic accumulation may be pathogenetically linked to vascular (or microvascular)-associated factor(s). Cerebrovascular pathology may be relevant in the pathogenesis of naturally-occurring Alzheimer's disease, and may account for increased Al accumulation during central nervous system damage. Conversely, in experimental Al-induced neurofibrillary degeneration, the factor of cerebrovascular pathology is not present, at least with respect to the non-senescent laboratory rabbit. Also, in the first experiments of Klatzo *et al.* (1965) the mode of Al delivery in the central nervous system was direct (intracerebral or intracisternal), thereby effectively bypassing the blood–brain barrier. Thus, it is not surprising that the type and distribution of neurofibrillary degeneration in rabbits treated in this manner was in some respects different from the more naturally-occurring neurofibrillary tangles in Alzheimer's disease in humans. Also, the formation of neuritic plaques appears to be species-dependent, being found in few mammalian species and then only in the aged (Martin *et al.*, 1994; Satou *et al.*, 1997). As is developed below, these differences are less compelling when compared to the broad array of immunochemical similarities which exist between experimental Al-induced neurofibrillary degeneration in rabbits and the various neurofibrillary lesions of the neurodegenerative disorders in humans.

The first experiments of Klatzo *et al.* (1965) and many subsequent studies, employed the direct injection of an Al compound into the rabbit central nervous system, using the intracisternal, intraventricular or intraparenchymal routes of injection. These direct administrations simplify the experimental system since the blood–brain barrier is bypassed. However, the better control achieved in the animal system is countered in part by the acute neurotoxicity which is usually produced, while Alzheimer's disease is of course a chronic disease. Monthly intracisternal injections of Al salts have been used to induce

experimental chronic neurodegeneration in the rabbit (Strong *et al.*, 1991; Strong and Garruto, 1991a; Strong and Garruto, 1991b), as will be discussed later. Both the systemic and oral routes of Al administration to rabbits have been proposed as a means for studying changes pertaining to Alzheimer's disease *in vivo*. All routes of administration will be discussed in detail later in this review.

Aluminum Speciation Issues

A complicating factor in assessing neurotoxic effects of Al in rabbits (and other experimental animals) has been the variety of Al compounds used in the experiments. The chemistry of Al is extremely complex, as has been discussed in detail by Martin (1986; 1988; 1990; 1991; 1992; 1997a; 1997b). As we have observed in a recent review of experimental Al encephalomyelopathy (Rao *et al.*, 1998), an understanding of the chemistry of Al is of considerable importance in the experimental design of studies related to Al toxicity. An understanding of the complex hydrolysis chemistry of Al as a function of pH is important and has been reviewed (Martin, 1997a). Below pH 5.0, Al (III) exists as an octahedral hexahydrate ($Al(H_2O)_6^{3+}$), usually abbreviated as Al^{3+} . As the solution becomes acidic, $Al(H_2O)_6^{3+}$ undergoes deprotonation to yield $Al(OH)^{2+}$ and $Al(OH)_2^+$. In neutral and alkaline solutions, $Al(OH)_3$ precipitates and soluble $Al(OH)_4^-$ is formed. Aluminum speciation in the stock solutions must be evaluated, since it hydrolyzes readily; at pH 7.0 there is a strong tendency for precipitation of $Al(OH)_3$ which makes the preparation of Al stock solutions difficult. Calculation of the molarity of Al solutions cannot be made by just adding a known quantity of an Al compound to water, without taking hydrolysis reactions into account. Martin (1991) has provided an example of how such properties of Al affect the design and interpretation of biological experiments. When an Al chloride solution of 0.01 M is added to tissue at pH 7.0 the permissible free Al^{3+} is only $10^{-10.3}$ M and that of all soluble forms is 2 μM. Unless the remainder of the added Al(III) has been complexed by other ligands then insoluble $Al(OH)_3$ will be formed. Al maltolate is particularly suitable for toxicological studies because of its defined molecular structure in solution and its neutral charge, high solubility and hydrolytic stability at pH 7.0 (Finnegan *et al.*, 1986), and for this reason it serves to reduce the formation of insoluble Al salts maintaining a much higher proportion of Al in solution in a bioavailable form.

Several studies have been carried out in the authors' laboratory using this compound for both in vitro (Hewitt *et al.*, 1991) and in vivo (Bertholf *et al.*, 1989; Wills *et al.*, 1993a; Katsetos *et al.*, 1990; Savory *et al.*, 1993; 1994; 1995a; 1995b; 1996) experiments via the oral, intravenous, and intracerebral routes of administration. With direct administration of as little as 2 μmole of Al injected directly into the central nervous system of rabbits, the effect of Al maltolate is dramatic, even at regions distant to the injection site.

Direct Injection of Al into the Central Nervous System of Rabbits

Direct injection of Al compounds into adult rabbit brain has constituted the most common route of administration for studies of the neurochemical characteristics of Al-induced

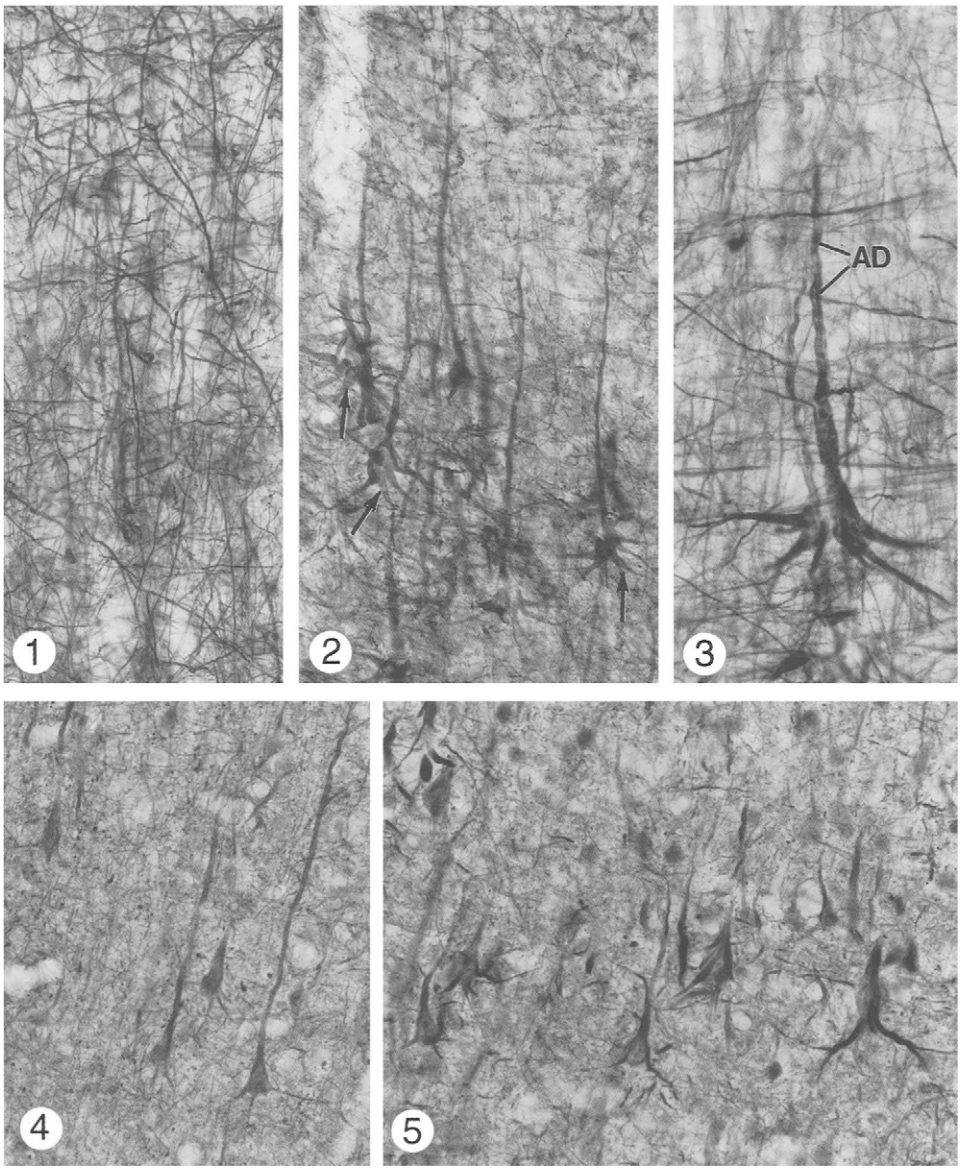
neurodegeneration. Intracranial Al administration has been carried out by several routes: either directly into the brain parenchyma, intraventricularly using a stereotaxic frame, or intracisternally. Klatzo *et al.* (1965) injected Al phosphate into the anterior portion of the left cerebrum, and reported for the first time epileptogenic effects and neurofibrillary degeneration. Pendlebury *et al.* (1988), using an intraventricular route of administration of Al chloride, found similarities between the distribution of neurofibrillary aggregates in rabbit brain and the formation of neurofibrillary tangles in Alzheimer's disease. In the same study, phosphorylated neurofilaments were shown to accumulate in neuronal perikarya containing the neurofibrillary aggregates, and double-labeling techniques suggested that the projection type neurons were primarily affected. With this route of Al chloride administration, the Al-induced neurofibrillary aggregates differed antigenically from the neurofibrillary tangles seen in Alzheimer's disease (Kowall *et al.*, 1989), although studies from our laboratory would suggest that these differences are less than thought originally (Huang *et al.*, 1997). Perhaps the development of improved reagents over the past decade could explain the differences. Nevertheless, using the intraventricular route of administration, Kowall *et al.* (1989) observed that many neuronal subsets that are particularly susceptible to Alzheimer's disease are also affected by Al-induced neurofibrillary degeneration. Indeed, in this mode of administration, widespread perikaryal neurofibrillary degeneration, characterized by positive Bielschowsky's silver impregnation and by immunostaining with the monoclonal antibody SMI-31 to detect phosphylated neurofilament proteins, was noted in the cerebral cortex, ventral hippocampus, basal forebrain, raphe nucleus, and brainstem nuclei, including the locus ceruleus (Kowall *et al.*, 1989). The thalamus and striatum were not involved, and only occasional Purkinje cells of the cerebellum showed these characteristic neurofibrillary changes. Studies from the same laboratory (Beal *et al.*, 1989), again using the intraventricular mode for treatment with Al chloride, demonstrated neurochemical changes that paralleled those seen in Alzheimer's disease, particularly a significant reduction of choline acetyltransferase activity in the entorhinal cortex and hippocampus. In contrast, there was no decrease in somatostatin and neuropeptide Y in the Al-treated rabbits, which differs markedly from observations in Alzheimer's disease patients (Beal *et al.*, 1989).

Forrester and Yokel (1985) compared the intraventricular administration of Al lactate to the subcutaneous route. Details of the systemic treatment experiments are given below. Rabbits treated via the intraventricular route were given either 5 or 10 μmole in 50 μL of solution. Typical clinical symptoms of hindlimb weakness, arching of the neck and seizures were observed. Surprisingly, no abnormalities in the righting reflex, which is the ability to quickly resume a normal stance after being forcibly turned onto one side, were seen. The symptoms appeared suddenly, following a symptom-free period. Clinical abnormalities were always associated with neurofibrillary changes in the hippocampus and frontal cortex, as detected by histological methods using the Bielschowsky's silver method and hematoxylin-eosin and cresyl violet staining. As discussed below, the effects in animals treated via the intraventricular route were remarkably similar to those treated subcutaneously (Beal *et al.*, 1989; Solomon *et al.*, 1990).

Intracisternal administration represents another route for drug delivery and is widely applied for the direct injection of Al compounds into the rabbit brain. Unfortunately, this mode of injection of Al compounds, besides producing neurofibrillary degeneration,

induces neurological signs involving the motor neuron system, and all of the rabbits develop seizures and die within 2 to 4 weeks. As has been demonstrated with other studies using this or similar modes of administration (Beal *et al.*, 1989), a severe encephalomyelopathy was observed in about 7 days, characterized by quadriparesis and weight loss (Beal *et al.*, 1989; Kowall *et al.*, 1989). Wisniewski *et al.* (1984), recognizing the limitations of the intracisternal route of administration in adult rabbits, suggested that Al-chloride should be injected into infants rather than adults, using the same technique. In an investigation applying this protocol, both young and old rabbits developed neurofibrillary degeneration consisting of intraneuronal neurofilamentous aggregates, but in the case of the infant animals, about 50% of them survived for many months and many did not develop neurological signs or seizures (Rabe *et al.*, 1982). The chronic intracisternal injection of Al chloride in rabbits has been shown to induce neurofibrillary tangle formation in cortical neurons (Wisniewski *et al.*, 1984) as well as profound neurofibrillary changes in neurons of the spinal cord and cerebrum (Wisniewski *et al.*, 1980). Troncoso *et al.* have demonstrated that intracisternal administration of Al chloride produces the accumulation of neurofilaments in axons and perikarya of motor neurons, associated with impaired axonal transport of neurofilament proteins (Troncoso *et al.*, 1986) and with axonal swellings (Troncoso *et al.*, 1982). Extensive dendritic degeneration, together with perikaryal neurofilamentous accumulation in motor neurons, has also been reported following the intracisternal injection of Al sulphate in rabbits (Wakayama *et al.*, 1993). Chronic administration of low doses of Al chloride, also by the intracisternal route, has been demonstrated to induce neurofilament inclusions in motor neurons (Strong *et al.*, 1991). In the young rabbits, co-administration of Al chloride and N-butyl benzenesulfonamide, a neurotoxin known to induce myelopathy, has resulted in a fulminant myelopathy, as well as striking behavioral changes (Strong and Garruto, 1991b). More recently, studies in the authors' laboratory with young adult New Zealand white rabbits have shown that the intracisternal administration of Al maltolate produces neurofilamentous aggregates possessing a number of similarities to the biochemical changes observed in Alzheimer's disease (Huang *et al.*, 1997). Examples of such features obtained in Al maltolate-treated rabbits are shown in Figs. 1–5. The photomicrographs shown in these figures are from an untreated New Zealand white rabbit and an animal subjected to the intracisternal injection of 100 μL of 25 mM Al maltolate and sacrificed after 7 days, by which time severe neurological symptoms were evident. Details of the clinical symptoms and methods of perfusion and tissue procurement have been reported by us previously (Savory et al, 1996). The photomicrographs shown in Figs. 1–5 depict pyramidal neurons in the parietal cortex of rabbit brain prepared by staining 50-μm sections cut on a vibrating microtome. Staining was with either Bielschowsky's silver impregnation or immunohistochemistry using the monoclonal antibody, SMI-31 (Sternberger Monoclonals Inc., Baltimore, MD) which recognizes phosphorylated epitopes on the high and medium molecular weight isoforms of neurofilament protein. Immunohistochemical detection was carried out with the ABC Elite kit (Vector Laboratories Inc., Burlingame, CA). Silver staining techniques are in common use for neuropathological diagnosis of Alzheimer's disease. In addition, one of the most significant findings from the authors' laboratory was the observation that microtubule-associated tau (tau) is a component of the Al-induced neurofilamentous

lesions (Savory *et al.*, 1995a,b); tau has been demonstrated to be a major constituent of the neurofibrillary tangles found in Alzheimer's disease (Iqbal *et al.*, 1994). Confirmation that tau is indeed a component of Al-induced neurofibrillary degeneration in rabbits has been provided by other workers (Singer *et al.*, 1997). Furthermore, the present authors also have reported Al maltolate-induced increased apoptosis in aged animals, as compared



to young adult rabbits and untreated controls of both ages (Savory *et al.*, 1999). These findings strongly support a key role of oxidative stress in the process of neurodegeneration in aging.

Systemic Administration of Al in Rabbits

One of the first studies using the systemic administration of Al into adult rabbit brain (exact age not stated) was that of De Boni *et al.*(1976) who injected Al lactate or Al tartrate subcutaneously daily for up to 30 days. An assessment of Al toxicity in rabbits with a variety of Al compounds, both inorganic and organometallic, was also carried out by the intravenous administration of Al maltolate into adult rabbits 3–5 times per week for 40–443 days (Favarato and Zatta, 1993; Fontana *et al.*, 1991). These authors pointed out the necessity of using a neutral, water-compatible Al complex as a tool for studying Al toxicity, and suggested that an aqueous solution of Al maltolate showed considerable promise for such studies (Favarato and Zatta, 1993; Fontana *et al.*, 1991). With the same compound, studies in the authors' laboratory have examined neuronal cytoskeletal changes in adult New Zealand white rabbits treated 3 times per week intravenously with Al maltolate for 8–30 weeks (Bertholf *et al.*, 1989; Katsetos *et al.*, 1990). Although no consistent neurological symptoms were observed in Al-treated animals, weight loss was noted. Neurofibrillary changes were found in the oculomotor nucleus in about one-third of the treated rabbits, as demonstrated by a monoclonal

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All photomicrographs depict pyramidal neurons in the parietal cortex of rabbit brain, prepared by staining 50-μm sections cut on a vibrating microtome.

Fig. 1. Untreated New Zealand white rabbit; Bielschowsky's silver impregnation. The intraneuronal cytoskeletal material is strongly argyrophilic and appears in the form of delicate, threadlike densities that mark the locations and orientations of the neuronal cell processes. × 210 .

Fig. 2. Section from an Al maltolate-treated rabbit sacrificed after 7 days, by which time severe neurological symptoms were evident. Orientation of section is comparable to Fig. 1. In contrast to the untreated control animal, the profiles of the Bielschowsky-stained neurons are evident and contain thickened opaque cords of cytoskeletal material ('neurofibrillary aggregates' or NFAs) that fill much of the cell body and both apical and basal dendrites (basal dendrites shown by *arrows*), the latter of which are especially prominent and darkly stained. × 210 .

Fig. 3. Higher magnification of a silver-stained pyramidal neuron in Al-treated rabbit cortex. The apical dendrite (*AD*) has a twisted profile. The NFAs in both apical and basal dendrites extend into the cell body and therefore obscure the nuclear profile. × 240.

Fig. 4. Normal cortex immunostained with a monoclonal antibody (SMI-31) directed against a phosphorylated epitope of neurofilament protein. Though neurofilaments are not present in great numbers in unchallenged neurons, the epitope is evidently present in substantial quantities (cf. Fig. 5) in a distribution similar to the NFAs seen in Al-treated brain. × 210.

Fig. 5. SMI-31 immunoreactivity in brain of an Al-treated rabbit. Distribution of the antigen in the pyramidal cortical neurons is similar to that shown in Fig. 4, despite the finding that in cells exposed to Al there is a massive increase in neurofilaments. × 210.

antibody to the 200 kDa subunit of neurofilament protein (Bertholf *et al.*, 1989). In a later study of tissue from these same animals, using the monoclonal antibodies SMI-31, -32 and -33, discrete focal perikaryal and proximal neuritic positivity in the form of spheroids and neuritic threads in a small number of pyramidal neurons were observed in the occipital striate cortex (Katsetos *et al.*, 1990). The monoclonal antibodies SMI-31, -32 and -33 recognize phosphorylated, non-phosphorylated/phosphate-dependent and non-phosphorylated/phosphate-independent epitopes of the high and medium isoforms of neurofilament proteins respectively.

More distinct evidence of neurofibrillary changes has been detected by Forrester and Yokel (1985) in a comparative study of subcutaneous and intraventricular administration of Al lactate to adult New Zealand white rabbits. For the subcutaneous study, rabbits received daily subcutaneous injections of 400 μmole/kg Al lactate, over a period of 28 days. Although no consistent clinical pattern of neurotoxicity was apparent, two of the animals displayed some evidence of motor abnormalities, as assessed by foot spread on landing, gait analysis and the righting reflex. Neurofibrillary changes, detected by Bielschowsky's silver method, hematoxylin-eosin, and cresyl violet stains, were evident in the hippocampus and frontal cortex of the affected animals. The neuronal perikarya and/or processes demonstrated positivity for neurofibrillary aggregates, and correlated with associated behavioral changes, such as seizures, postural changes, aggressiveness and excitability. An unexpected observation to us was the marked similarity in clinical and neuropathological abnormalities of animals treated subcutaneously with Al compared with those treated intraventricularly. In a later report aimed specifically at relating Al neurotoxicity to age, Yokel (1989) used the same mode of injection to older (2–3.4 years) New Zealand white rabbits; unexpectedly in the older animals, administration of 400 μmole/kg dose of Al lactate was lethal, so a lower dose of 200 μmole/kg was used. Tissue Al concentrations in the frontal gray cortex and hippocampus were significantly elevated. No histologic examination of tissue was performed.

Oral Administration of Al in Rabbits

Aluminum toxicity has been examined following the oral administration of Al citrate to young Japanese white rabbits fed a calcium and magnesium-deficient diet. This treatment induced neurofibrillary degeneration in the anterior horn of the spinal cord (Kihira *et al.*, 1994). Studies in the authors' laboratory following the oral administration of Al citrate to New Zealand white rabbits over a 3–12 month period has demonstrated no accumulation of Al in the brain and no evidence of neurofibrillary changes (Wills *et al.*, 1993a).

Neurobehavioral Effects Following Al Administration in Rabbits

To our knowledge, no behavioral studies on rabbits treated orally with Al have been performed. We have evaluated the long-term oral administration of Al maltolate to young adult rabbits (Hewitt *et al.*, 1992; Wills *et al.*, 1993a;b). Although decreased weight gain was noted, we performed no specific behavioral studies. Behavioral evaluation in

rabbits has been carried out following either the direct injection of Al compounds into the brain or systemic administration. Intracisternal administration of Al to New Zealand white rabbits of either sex has been reported to produce deficits in water maze acquisition (Rabe *et al.*, 1982). This water maze had three chambers with an escape ramp in the third chamber. Rabbits were trained by 3-spaced trials daily for 5 consecutive days. In this model, adult New Zealand white rabbits were injected intracisternally with 50 μL of 1% $AlCl_3$ (3.75 μmole) in physiological saline. Interestingly, administration of this amount resulted in neurological symptoms affecting the motor system within 1–3 weeks, and all animals died from seizures within 2–4 weeks. However, when $AlCl_3$ was injected intracisternally into infant rabbits on the 15th day of life, neurofibrillary changes were found in the cerebrum, brainstem and spinal cord, but there were no apparent clinical symptoms. Importantly however, this treatment of infant animals caused a significant learning deficit, as evaluated by the water maze test described above. Whether this deficit was due to the neurofibrillary changes or to some other effect of Al was unclear to the authors (Rabe *et al.*, 1982). It is indeed surprising that there has been little follow-up of this significant brief report.

The rabbit is not typically used for behavioral studies, but the classically conditioned-defensive eyeblink reflex is a useful tool and has been examined. This test appears to reflect the effects of Al on neural pathways and structures subserving simple forms of learning and memory; the hippocampus and cerebellum are structures that may be involved in these processes (Yokel, 1994). Using the intracisternal route of Al administration, Pendelbury *et al.* (1988) and Solomon *et al.* (1988) have demonstrated learning and memory deficits in 4–6 month old New Zealand white rabbits (sex not stated) by studying the acquisition or retention of the eyeblink reflex. In a series of experiments, Yokel applied the subcutaneous route of injection of Al lactate, and was able to demonstrate learning and memory deficits, but only in adult and aged (2–3.4 years) female New Zealand white rabbits (Yokel, 1983; 1984; 1985; 1987; 1989). This suggested, as we have also proposed (Savory *et al.*, 1999), that aging increases the susceptibility of the brain to Al toxicity, at least in rabbits. Yokel at al. (1994) related the Al-induced learning deficits in rabbits to patients with Alzheimer's disease by demonstrating that 4-aminopyridine, a compound reported to improve learning in Alzheimer's disease subjects, also reduced the severity of the Al-induced learning deficit in rabbits.

Informed Opinion

Does Al-Induced Neurodegeneration in Experimental Animals Support the Hypothesis that Al Might Play a Role in the Pathogenesis of Alzheimer's Disease?

There have been several animal models proposed as an aid in understanding Alzheimer's disease neuropathology, including transgenic mice (Sugaya *et al.*, 1997), rat, monkey, and dog (Brining *et al.*, 1996; Games *et al.*, 1995; Uno *et al.*, 1996). Transgenic mice have been used mainly to examine the process of Aβ deposition (Sugaya *et al.*, 1997), while individual events such as apoptosis, Aβ deposition, and neurofibrillary degeneration have been explored in other animals (Brining *et al.*, 1996; Games *et al.*, 1995;

Uno *et al.*, 1996). Yokel (1989) demonstrated over a decade ago that 2–3.4 years old aged female rabbits were more susceptible to Al following the subcutaneous route of administration than were young adults, and moreover also exhibited marked changes in behavior. Studies in the authors' laboratory have demonstrated that Al maltolate-treated rabbits (especially aged female animals, 3–4 year old), exhibit widespread formation of intraneuronal neurofilamentous aggregates which share significant immunochemical/antigenic characteristics with the neurofibrillary tangles found in the central nervous system of patients with Alzheimer's disease and in amyotrophic lateral sclerosis (ALS), a motoneuron disease. Typically these neurofilamentous aggregates contain hyperphosphorylated tau, amyloid precursor protein, Aβ, α-1-antichymotrypsin and ubiquitin (Savory *et al.*, 1996; Huang *et al.*, 1997). Interestingly, similarities have been reported in eyeblink classical conditioning between aged New Zealand White rabbits (up to 7 years of age) and patients with Alzheimer's disease (Woodruff-Pak and Trojanowski, 1996).

The relationship of Al-induced neurofibrillary degeneration in rabbits to human neurodegenerative disorders has been viewed with skepticism, because of apparent ultrastructural differences between the experimental lesions in rabbits and human neurofibrillary tangles. Compared to the tangles of Alzheimer's disease, which mostly contain paired helical filaments (Kidd, 1964; Terry, 1963; Wisniewski *et al.*, 1976), those produced by Al are predominantly straight, intermediate-like filaments (Dahl and Bignami, 1978; Wisniewski and Sturman, 1989). This difference in the ultrastructural properties of the intraneuronal neurofilamentous aggregates could be due to the different time course of their formation. In Alzheimer's disease, this time could be months or years, whereas in the acute animal experiments the time course is only a few days (Savory *et al.*, 1996). Supporting the argument that these protein aggregates might arise from a similar mechanism has been the observation that tau can form Alzheimer's-like filaments *in vitro* (Crowther *et al.*, 1994; Murayama *et al.*, 1999).

Aluminum-induced neurofibrillary degeneration is characterized predominantly by immunohistochemical staining with antibodies specific for the hyperphosphorylated epitopes of neurofilament protein (Katsetos *et al.*, 1990; Savory *et al.*, 1996; Wakayama *et al.*, 1996). The primary constituent of paired helical filaments in human neurofibrillary tangles is tau, although other proteins are present, including abnormally phosphorylated neurofilament proteins, ubiquitin, α1-antichymotrypsin, amyloid precursor protein and its derived peptide, Aβ. Studies in the authors' laboratory over the past five years, which now have been confirmed by others (Muma and Singer, 1996; Singer *et al.*, 1997), have shown that immunochemical similarities between the composition of Al-induced lesions and those found in Alzheimer's disease are far closer than was originally surmised. Rao *et al.* (2000) also have confirmed these observations.

Abnormally phosphorylated tau has been detected in these neurofilamentous aggregates (Huang *et al.*, 1997), using a variety of mAbs that recognize both nonphosphorylated and phosphorylated tau (Muma and Singer, 1996; Savory *et al.*, 1995b). Among the monoclonal antibodies applied for this immunostaining have been Tau-1, Tau-2, AT8, PHF-1 and Alz-50, indicating that both phosphorylated and nonphosphorylated tau are present. The time course of aggregation of these cytoskeletal proteins has been evaluated in the authors' laboratory (Savory *et al.*, 1996). The results indicate that the aggregates become detectable by silver staining within 24 hours following Al maltolate administration, and

that neurofilament proteins predominate; non-phosphorylated phosphorylation-independent epitopes as detected by mAb SMI-33 (Sternberger Monoclonals Inc. Baltimore, MD) are found first, followed by phosphorylated forms, immunostained by mAb SMI 31 (same vendor), at approximately 72 hours. Tau is also detectable by around 72 hours, although the characteristic epitopes of Alzheimer's disease as recognized by mAbs AT8 and PHF-1 are most distinct at 6–7 days following Al injection. It has been proposed that phosphorylation of cytoskeletal proteins drives the formation of the neurofilamentous aggregates, particularly in human neurodegenerative disorders. Since the aggregates are hyperphosphorylated, phosphorylation alone would render these protein accumulations unstable, because of the preponderance of negative charges on the phosphate groups. Thus, it is reasonable to propose that some positively-charged species constitute an inherent factor in the formation and stabilization of the neurofibrillary degeneration, both in Alzheimer's disease and in experimental Al-induced neurofibrillary degeneration; in the latter, Al^{3+} is an obvious candidate for this role.

We have proposed that aging in rabbits is an important factor regarding the susceptibility of neurons to oxidative stress and to subsequent apoptosis (Savory *et al.*, 1999); both processes have been observed in the Alzheimer's disease brain (Smith *et al.*, 1996; Su *et al.*, 1994). Protein changes controlling apoptosis, such as those of the *Bcl-2* family and caspases, also are altered in the Alzheimer's brain (Kitamura *et al.*, 1998). We have shown similar responses in aged rabbits treated intracisternally with Al maltolate (Savory *et al.*, 1999). Brain tissue from these aged animals exhibited intense intraneuronal silver positivity, indicative of the formation of neurofilamentous aggregates, together with oxidative stress. The changes occurred in the CA1 region of the hippocampus as well as in cerebral cortical areas. Apoptosis, assessed by the TUNEL *in situ* technique, colocalized with oxidative stress. Young animals treated with Al showed few of these alterations, while age-matched controls were essentially negative. Further studies on the time course of these and related changes have demonstrated that oxidative stress and redox-active iron accumulation in hippocampal neurons occurred very rapidly, within a period of 3 hours, and increased in intensity at 72 hours. Changes suggestive of apoptosis were apparent by 24 hours and were pronounced at 72 hours. In aged animals there was an initially intense immunopositivity at 3 hours for Bcl-2, with negative staining for Bax. By 72 hours, when apoptosis was strongly evident, Bcl-2 was negative and Bax strongly positive. In contrast to the aged rabbits, young animals treated similarly with Al exhibited much less oxidative stress with no apoptosis, and maintained Bcl-2 immunopositivity and negative Bax staining. Our findings strongly support the key role that oxidative damage plays in the process of neurodegeneration and in the increased vulnerability to Al-induced injury in the aged animal. These findings have demonstrated that Al can produce relevant neuropathological and biochemical changes in experimental animals, albeit in an animal system quite different from that in Alzheimer's disease.

Another relevant finding in Al-induced neurodegeneration has been the observation of amyloid precursor protein and Aβ immunopositivity in neurons following Al treatment in rabbits (Huang *et al.*, 1997) and in rats (Shigematsu and McGeer, 1992). Neurofibrillary tangles in Alzheimer's disease also exhibit this Aβ staining, but the most prevalent pattern is the presence of Aβ in the neuritic plaques. It is logical to hypothesize that in Alzheimer's disease, increased Aβ first appears intracellularly, followed by extracellular

deposition. Plassman and Breitner (1996) have described an Al-induced secondary structural transition in the non-Aβ component of Alzheimer's disease amyloid (NACP), also known as α-synuclein, which generated an approximately 33% α-helix, thus rendering the α-synuclein resistant to proteases. Based on this finding it was suggested that Al may influence α-synuclein turnover and induce aggregation via structural modifications, thus leading to Aβ deposition. Paik *et al.* (1999) have investigated the self-oligomerization of α-synuclein by various metal ions including Al. Copper and zinc induced oligomerization with copper being most effective. Aluminum was also studied but had less of an effect. However, this work totally ignored the fact that all metals studied were prepared at a concentration of 0.5 mM in 20 mM MES, pH 6.5 which would allow for a 0.5 mM concentration of both Cu^{2+} and Zn^{2+} but, as was discussed earlier, the concentration of Al^{3+} would be many times lower due to the formation of insoluble Al salts.

Deregulation of Al and iron homeostasis may play a role in Alzheimer's disease and in Al neurotoxicity. A study by Bouras *et al.* (1997) described the presence of high concentrations of Al and iron in the hippocampus and inferior temporal cortex in both Alzheimer's disease and in dementia pugilistica. The predominant findings were those of the coaccumulation of Al and iron, both in neurofibrillary tangles and in the nuclei of tangle-bearing neurons. Also, concentrations of Al and iron in nuclei of tangle-free neurons and neuropil were similar in Alzheimer's disease and in dementia pugilistica. These studies suggest the existence of an association between the deposition of Al and iron with neurofibrillary tangle formation, and support the possibility of a global dysregulation of Al and iron transport in Alzheimer's disease and dementia pugilistica. It has been hypothesized that the deregulation of Al and iron homeostasis permits the existence of colocalization of these two metals, and contributes to the accumulation of metabolic errors, leading to neuronal disorders, including the formation of neurofibrillary tangles and Aβ deposition (Berthon, 1996). Cells in the rat brain possess a specific high-affinity receptor for transferrin that is independent of the metal being transported. This system is postulated to be the route whereby iron in the general circulation reaches the brain (Roskams and Connor, 1990). Transferrin is thought to be mainly an iron transporter protein, based on the relatively high abundance of iron in the circulation and the high affinity of transferrin for iron (Martin *et al.*, 1987). However, as mentioned earlier, transferrin also has a high affinity for Al, although not to the same extent as iron (Martin *et al.*, 1987), and this property should provide an avenue for the transportation and intracellular deposition of Al. Evidence for this pathway is provided in a report that aluminum accumulation was accompanied by an increase in iron uptake in primary cultures of the rat cerebral cortex (Oshiro *et al.*, 1998).

Shin *et al.* (1995) have shown that Al selectively binds to the paired helical filament-tau, induces aggregation, and retards the in vivo proteolysis of this protein aggregate. Their data suggest that Al may serve as a cofactor in the formation of neurofibrillary tangles by its interaction with tau. Madhav *et al.* (1996) using circular dichroism revealed a five-fold increase in the observed ellipticity of the tau-Al assembly which they attributed to Al-induced aggregation of the protein. More recently Tarbox and Goux (1999) have studied Al interactions with tau protein using circular dichroism spectroscopy, and have shown that Al induces marked conformational changes independent of tau phosphorylation.

All of the above studies indicate that Al contributes to the formation of neurofibrillary lesions in neurons, and thus may play a role in the pathology of Alzheimer's disease and other human neurodegenerative disorders. The biochemical similarities of the Al animal model to the intraneuronal lesions of Alzheimer's disease make this system valuable for studying perturbations of the neuronal cytoskeleton, particularly in view of the lack of optimal animal models. In the context of the present review, the animal studies cited do not prove that Al is a risk factor, but convincingly demonstrate that this metal can produce significant neurofibrillary pathology and biochemical changes that are important features of neurodegenerative disorders.

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Aluminium and Alzheimer's Disease
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CHAPTER 11

Aluminium and Iron: Implications for Alzheimer's Disease

Patrick C. D'Haese * and Marc E. De Broe

Department of Nephrology/Hypertension, University Hospital Antwerp, Wilrijkstraat 10, Antwerp, B-2650 Belgium

* Corresponding author, email: dhaese@uia.ua.ac.be

Summary

There is no doubt that aluminium may cause neurotoxic effects. In dialysis patients the element's role in the development of the so-called 'aluminium-related dialysis dementia is now well recognized. The role of the element in the aetiopathogenesis of Alzheimer's disease however, remains controversial.

In contrast to aluminium, iron is essential for neurological function because of its role in oxidative metabolism and because it is a co-factor in the synthesis of neurotransmitters and myelin. Nevertheless, abnormal iron accumulation in diseased brain areas, including Alzheimer's disease, has been reported. There is strong evidence for iron-mediated oxidative damage as a primary contributor to cell death in these disorders.

Since aluminium and iron display a number of similar physicochemical characteristics, interactions between both elements in the development of neurotoxic effects may reasonably be suggested.

Historical Perspective

Aluminium, Iron and Alzheimer's Disease

Aluminium

Alzheimer's disease is a progressive neurodegenerative disease, clinically characterized by a gradual loss of cognitive functions and histopathologically by the presence of neurofibrillary tangles and senile, or neuric plaques. During the last two decades an overwhelming amount of literature data have been reported in connection with the disease. A 1980–2000 Med-line search on 'Alzheimer's disease' revealed 16,362 papers being published on this topic during this time period. Nevertheless, there is still a lack of well-accepted mechanisms on the causes of the sporadic form of this most devastating highly prevalent neurological disorder. The two major hypotheses implicate either the peptide Aβ or microtubule-associated protein tau as central to the development of the

disease although the genetic linkage of sporadic Alzheimer's disease to apolipoprotein E4 is generating much interest (Savory et al., 1996). Alzheimer's disease is an heterogeneous disorder and none of the avenues of investigation reported so far, has led to a well-substantiated mechanism for the pathogenesis of the disease. Therefore other hypotheses and factors including environmental ones, need to be considered and discussed, particularly when supporting data are available. The hypothesis of aluminium as an environmental risk factor falls within this category and there has been a growing, suggestive body of information supporting the view that the element may contribute to, or accelerate, or play a causal role in the pathogenesis of Alzheimer's disease (Savory et al., 1996; Candy et al., 1994).

Out of the various studies, those linking aluminium with the pathogenesis of Alzheimer's disease by induction of an Alzheimer-like pathology in the brain tissue of experimental animals by the direct injection of aluminium salts or the co-localization of aluminium — mainly as the alumino-silicate complex — with neurofibrillary tangles or neuritic plaques, the two major histopathological hallmarks of Alzheimer's disease in human brain tissue are most convincing (Klatzo et al., 1965; Perl & Brody, 1980; Candy et al., 1986). Nevertheless the issue on the pathogenic role of aluminium in the development of Alzheimer's disease remains highly controversial. So, electron microscopic as well as biochemical examination of the diseased nerve cells from both humans with Alzheimer's disease and animals treated with aluminium salts revealed basic structural and biochemical differences between the neurofibrillary changes induced in animals injected with aluminium salts and those found in Alzheimer's diseased patients (Wisniewski and Soifer, 1979).

With regard to the controversy that still exist on increased brain aluminium levels in patients with Alzheimer's disease, authors failing to confirm these findings, ascribed the increased aluminium levels to age-dependent differences or stated that aluminium may become involved and accumulate locally during the progress of the disease (Eichhorn, 1993; Markesbery et al., 1981). With respect to the latter it appears reasonable that after some time during the progress of the disease metal transport is affected, so that more aluminium can get to the brain. In this respect it is worth mentioning that a number of investigators have noted changes in the blood–brain barrier. Moreover, in line with this hypothesis is the notion that aside from aluminium other metals (i.e. lead, mercury, copper, iron, ...) (Eichhorn, 1993; Deibel et al., 1996; Rao et al., 1999) been implicated. Furthermore, when interpreting literature data on brain aluminium accumulation the problems and approaches to the measurement of aluminium should be recognized. Particularly analytical data generated before 1985 should be interpreted with caution. Whilst co-localisation of the element with neurofibrillary tangles or neuritic plaques have been demonstrated by several workers others have been unable to confirm the co-existence of aluminium with the pathological lesions of Alzheimer's disease (Chafi et al., 1991; Landsberg et al., 1992; McDonald et al., 1994).

There have been several studies that indicate a relationship between Alzheimer's disease frequency and aluminium in drinking water (Martyn et al., 1989; Neri and Hewitt, 1991; Jacqmin-Gadda et al., 1996). Most evidence from epidemiological studies indicating an association between increased aluminium exposure and the development of Alzheimer's disease has been provided by Martyn en al. (1989) reporting a significant

increase in the risk (up to 50%) for Alzheimer's disease in districts where the mean water aluminium concentration was greater than 111 μg/L as compared to regions where it was less than 10 μg/L. Supportive data for these findings have also been published by others (Neri and Hewitt, 1991; Jacqmin-Gadda et al., 1996).

Epidemiological studies on the role of aluminium in the development of Alzheimer's disease demonstrate an association but fail to establish cause and effect. Moreover they are difficult to substantiate since the disease may be underreported and the diagnosis may sometimes be wrong.

Adding to the list of studies linking aluminium neurotoxicity to Alzheimer's disease is the use of DFO chelation to treat affected patients. Most of this work in humans has been carried out by Crapper McLachlan et al. (1991) while Savory et al. (1994) applied the chelator to an animal model. Desferrioxamine is a trivalent ion-selective chelating agent with a high affinity for ferric iron (Fe^{3+}) and aluminium (Al^{3+}) and has been used extensively to diagnose and treat aluminium-intoxicated dialysis patients (D'Haese et al., 1995; Barata et al., 1996). In his studies Crapper McLachlan et al. (1991), after a 2-years observation period demonstrated that the rate of decline of the no-treatment group was twice as rapid as the DFO-treated group. In their critical overview on the controversy of the role of aluminium in Alzheimer's disease, Savory et al. (1996) correctly state that the lack of a true control group is an important drawback of this study. Indeed, the controls for ethical reasons, were given oral placebos rather than intramuscular injections as was the route of administration of DFO. Moreover, DFO is also a strong chelating agent for iron, which is critically important in oxidative stress-induced neuronal damage and explains the element's assumed involvement in the development of Alzheimer's disease (see below).

By which mechanism aluminium gets access into the cells of the central nervous system is not yet fully understood. This is mainly due to the lack of a suitable isotope of aluminium (aluminium-26) until recently. Moreover the use of aluminium-26 is extremely expensive as an Accelerated Mass Spectrometer is required. Hence, the knowledge generated with this technique is highly limited also. So, gallium-67 has been used as a tracer for aluminium and was found to have a very low blood–brain barrier permeability consistent with transferrin-mediated transport which appeared to be unidirectional (Pullen, 1990). Moreover, the element's distribution and hence, that of aluminium assumed from this, appeared to be regional with a pattern similar to that of transferrin receptors labelled with ^{125}I-Fe-transferrin, suggesting that the density of transferrin receptors in a particular brain region governs the element's uptake (Pullen, 1990). In line with this mechanism for the entry of aluminium into the cells of the central nervous system are the data reported by Morris et al. (1989) and Roskams & Connor, 1990). These latter investigators reported that aluminium is able to gain access to the central nervous system under normal physiological conditions via the same high-affinity receptor–ligand system that has been postulated for ferric iron delivery to neurons and glial cells; i.e. the transferrin–transferrin receptor system. As iron, aluminium also exerts a high affinity for transferrin and is known to be transported by this protein (Van Landeghem et al., 1994). With the transferrin-mediated uptake of aluminium the protein is supposed to recycle to the serum after delivering the metal to the brain. Aside from transferrin-mediated endocytosis, citrate has also been proposed an important ligand for aluminium and, although still controversial, it has been suggested that entry of the element into the brain is markedly enhanced when complexed

with citrate (Slanina et al., 1984). Others have contested this statement (Quartley et al., 1993). Citrate complexation of aluminium has also been reported a mechanism responsible for the element's efflux from the brain into the blood. This would occur via the monocarboxylic acid transporter located on the cerebral capillaries of the blood–brain barrier (Ackley & Yokel, 1997). These authors further suggested that this transporter may protect the brain from aluminium while defects in the transporter might increase the element's neurotoxic effects. In line with this mechanism are the findings of speciation studies of aluminium in CSF by Van Landeghem (1997) reporting that in contrast to serum, aluminium in CSF appears not bound to transferrin. This was explained by taking into account the molar concentration of citrate in CSF which is 900-fold higher than that of transferrin in contrast to serum where the molar ratio is only 1 to 4. *In vitro* findings have indeed indicated that when the citrate concentration rises relative to the transferrin concentration citrate is able to remove aluminium from its binding protein (Van Landeghem et al., 1997).

Iron

In contrast to aluminium, iron is very well known to play an essential role in biological systems. So, iron management and the timely delivery of appropriate amounts of the element, is crucial to normal brain development and function. Hence, mismanagement of cellular iron can result not only in decreased metabolic activity but also in an increased vulnerability to oxidative damage (Connor & Menzies, 1995). It is therefore not surprising that iron by its ability to generate oxygen free radicals and hence lipid peroxidation has been involved in the pathologies of Parkinson's and Alzheimer's disease (Kala et al., 1996).

The brain requirement for iron is relatively high, consistent with its high energy needs. Nevertheless the entry of iron in the brain is not completely understood, but most reasonably involve the same proteins, and possibly mechanisms that are known to exist elsewhere in the body (Pinero & Connor, 2000). The regulatory control of the blood barrier and the blood–cerebrospinal fluid-barrier prevents the brain from having direct access to iron in the plasma pool. Iron is supposed to be transferred to the brain by plasma transferrin through an interaction between circulatory transferrin and transferrin receptors in the brain microvasculature (Morris et al., 1992) followed by the element's cellular uptake through endocytosis. Transport of iron on transferrin-like molecules (e.g. lactoferrin) at the blood brain barrier may also take place as well as transport through the ventricular system; i.e. the blood–cerebrospinal fluid barrier (Pinero & Connor, 2000). The mechanism of iron efflux from the brain is poorly understood, but studies have shown that with the calculated iron influx, there is also a high efflux (Moos & Morgan, 1998).

Several studies have reported increased total iron levels in Alzheimer's disease as measured by atomic absorption spectrometry (Connor et al., 1992). Others reported on increased levels of iron storage protein, ferritin, in brains of Alzheimer's diseased patients, especially in cells such as microglia that are associated with senile amyloid plaques (Grundke-Ibqal et al., 1990). In combination with increased ferritin levels Fischer et al. (1997) reported transferrin levels to be decreased pointing to an imbalanced iron homeostasis and supporting the oxygen radical hypothesis in Alzheimer brain; in other

words that higher transferrin levels in brain and/or serum may protect against the development of Alzheimer's disease. With regard to the elemental compartmentalization in brain and in line with the above have increased iron levels been found in neuritic plaques as well as in neurofibrillary tangles. Using nuclear microscopy, McDonald et al. (1994) reported iron levels to be significantly increased in both plaque cores and in the background tissue and this in the absence of any difference of aluminium or silicon levels at these ultrastructural sites.

The brain requires a ready supply of iron for normal neurological function, but free iron is toxic (Conner & Menzies, 1995; Connor et al., 1995). Iron promotes conversion of hydrogen peroxide to hydroxyl radicals and, thus may contribute to oxidant stress. These radicals oxidatively modify proteins and make them more susceptible to proteolysis, damage DNA, and peroxidize lipids (Joshi et al., 1994). Although the origin of the free radicals responsible for the elevated peroxidation in Alzheimer's cortex has not been identified, one potential source is the formation of hydroxyl radicals from endogenous hydrogen peroxide in the presence of iron molecules that are not bound to enzymes or storage proteins (Kala et al., 1996). The presence of low molecular weight loosely bound iron molecules has been reported in a variety of tissues and cells (Gutteridge et al., 1991). This pool of iron, which seems to play a crucial role in the transfer of iron from ferritin storage sites to those enzymes needing iron for their biosynthetic activities, may also provide iron molecules for participation in the Fenton reaction leading to the *in vivo* formation of oxygen free radical formation (Kala et al., 1996). Aside from such a mechanism has it been proposed that the tau protein associated with the neurofibrillary tangles may contain iron binding sites and that the binding of iron disrupts the binding of tau to microtubulin molecules; a suggestion which is supported by the fact that administration of an iron-specific chelator, i.e. desferrioxamine seems to have a substantial therapeutic effect in patients with Alzheimer's (Crapper McLachlan et al., 1993). Some evidence has been presented also that iron may effect the processing of amyloid precursor protein to β-amyloid protein. Indeed, iron chelation has been shown to reduce the production of soluble amyloid precursor protein whilst increased iron levels augment it (Bodovitz et al., 1995). Recently, the iron-binding protein p97 has been ascribed a role in the development of Alzheimer's disease. p97 (melanotransferrin) belongs to the group of iron-binding proteins that include serum transferrin, lactoferrin and ovotransferrin and co-localizes with the transferrin receptor at the capillary endothelium of human brain and is specifically expressed on reactive microglial cells associated with amyloid plaques in post mortem brain tissue from Alzheimer's patients; this in contrast with what is seen in microglia not associated with Alzheimer's and those found in brain from patients with other neuropathologies (Kennard et al., 1996).

Informed Opinion

Aluminium and Iron and Alzheimer's Disease

Aluminium and iron exhibit a number of similar physicochemical similarities such as ionic radius, charge density, chelation by some particular drugs and transferrin, etc. This

indirectly implies that these elements may affect each other with regard to their toxicity. It has been postulated that the toxicity of aluminium greatly depends on the degree to which the element is bound to transferrin (Van Landeghem et al., 1997, 1997a & 1998; Smans et al., 2000), a complex providing a form of the metal that can readily enter cells and hence determines the element's selective tissue distribution and toxicity. It has previously been shown that the transferrin binding of aluminium greatly depends on the iron status with which it competes for binding to the latter protein and gets displaced from its binding sites (Van Landeghem et al., 1997a). It has been demonstrated by Van Landeghem et al. (1997a) that iron by occupying specific transferrin binding sites not only limits the number of available binding sites for aluminium but the element also affects the affinity between transferrin and aluminium. In this context one should also interpret the findings by Farrar et al. (1990) suggesting that the transferrin of patients with Alzheimer's disease has an abnormally low affinity for aluminium secondary to a functional defect in the protein and resulting in a greater proportion of non-transferrin bound low-molecular weight aluminium complexes in their plasma, which in turn could move the element readily into the brain exerting its neurotoxic effects. Surprisingly however, these patients also had a significantly higher transferrin–iron saturation as compared to the control groups. Hence, the effect of iron on the transferrin binding of aluminium rather than a so-called defective aluminium transferrin binding, specific for patients with Alzheimer's disease as suggested by Farrar et al. (1990) might be the mechanism responsible for their observations. To which extent the repeatedly reported reduced transferrin levels in Alzheimer's patients (Molashi et al., 1996; Fisher et al., 1997) may contribute to a reduced protein binding of aluminium, possibly causing a higher bioavailability of the element, is not yet clear.

Isolation of ferritin from the cerebral cortex of normal patients and cases of Alzheimer's disease revealed that the protein contained less than 9 atoms of aluminium per ferritin molecule and that there was no difference between both populations (Candy et al., 1994). This made the authors suggest that aluminium is excluded from ferritin binding *in vivo*, most probably due to the composition of the iron/aluminium pool available for uptake rather than the intrinsic properties of the ferritin molecules. This might in turn have important implications for the potential intracellular toxicity of aluminium, since it implies that cells are unable to detoxify aluminium by the same mechanism as that available for iron (Candy et al., 1994).

With regard to possible temporarily relationship between both elements in their accumulation in the brain of Alzheimer's patients, Rao et al. (1999) showed that iron accumulates in the brains of moderately as well as severely affected people while aluminium is mainly deposited in severely affected brain which made the author suggest that iron accumulates early in Alzheimer's disease pathology while aluminium is deposited in a later phase of the disease. This latter notion however, also supports the hypothesis that increased aluminium levels are a consequence rather than being the cause of Alzheimer's disease.

Whilst an association between the deposition of aluminium and iron and neurofibrillary tangle formation resulting from a possible global dysregulation in the transport of both elements has been postulated by Bouras et al. (1997) others reported on a different pattern between aluminium and iron for their deposition in the Alzheimer's brain. So,

Morris et al. (1989) noticed a discrepancy between the localisation of iron and that of transferrin receptors while the distribution of aluminium and transferrin receptors is similar to the pathological changes seen in Alzheimer's disease, with the cortical and limbic regions together with certain subcortical cell nuclei showing the greatest involvement in Alzheimer's disease (Morris et al., 1989).

Recently, aluminium has also been attributed a synergetic role with iron in its neurotoxic effects. As mentioned above an association between oxidative stress and Alzheimer's disease which possibly results from the pro-oxidant properties of β-amyloid present in the senile plaques due to interactions between amyloid and iron has been suggested. Yang et al. (1999) presented some evidence that both aluminium and β-amyloid can potentiate free radical formation by stabilizing iron in its more damaging ferrous form which can promote the Fenton reaction and hence stimulate free radical formation. In line with a possible cooperative action between aluminium and iron in the development of Alzheimer's disease are the findings by Abreo et al. (1999) showing that aluminium enhances iron uptake and expression of neurofibrillary tangle protein in neuroblastoma cells. To which extent the cellular uptake aluminium at the level of the brain also disrupts the cellular iron metabolism at this site is not clear. Previous studies by Abreo et al. (1990) in Friend erythroleukemia cells showed that whilst the uptake of aluminium did not effect the transferrin-receptor mediated endocytosis of iron there was a decreased iron uptake by ferritin despite the increased cellular uptake. They postulated that in the presence of aluminium, iron might probably be allocated in an inaccessible cellular compartment by which the iron binding protein senses inadequate iron. As a consequence the iron-binding protein binds to the iron-responsive elements of the transferrin receptor mRNA and the ferritin mRNA by which degradation of the transferrin receptor mRNA and translation of the ferritin mRNA is blocked, thus resulting in an increased synthesis of the transferrin receptor and decreased synthesis of ferritin. The increase in transferrin receptors may then result in an increased uptake of iron (and perhaps aluminium) to increase. So, aluminium might indirectly affect the toxic effects of iron by inducing an increased iron uptake which in conjunction with a decreased iron entry into ferritin might result in iron being displaced into compartments in which the latter element could participate in peroxidative reactions underlying the element's deleterious neurotoxic effect. An alternative mechanism has been described based on the fact that in Alzheimer's diseased brains a more stable iron regulatory protein–iron responsive element (IRP-IRE) complex, possibly resulting from increased RNase activity has been found (Pinero et al,. 2000a). Such a complex could increase the stability of the transferrin receptor mRNA and inhibit ferritin synthesis, which would also explain the increase, in iron accumulation without an increase in ferritin in Alzheimer's patients (Connor et al., 1992a).

It remains an intriguing question why in the brain aluminium is so toxic at very low concentrations. Indeed, as noted in aluminium-overloaded patients the element seems not to exert any toxic effect when present at high concentrations in e.g. the liver (up to 50 μg/g) whilst in brain serious toxic effects already occur at concentrations that are at least one order of magnitude lower. Although, little is known on the mechanism by which aluminium accumulates in brain cells one can reasonably assume that the element's direct toxicity greatly depends on its subcellular localisation, which in turn is determined by its speciation. In serum the element circulates 80–90% bound to transferrin; the

remaining fraction occurring as a low molecular mass compound most probably as citrate or silicate. As mentioned above it is not yet clear by which pathway aluminium passes the blood–brain barrier. Quartley et al. (1993) reported that after oral administration of aluminium citrate brain aluminium levels did not change while very high concentrations were found in plasma (539 μg/L) and all other tissues under study. These findings are highly suggestive for aluminium citrate not to be able to pass the blood brain barrier. On the other hand has a transferrin receptor mediated uptake similar to that of iron been demonstrated. This finding seems to be corroborated indirectly by data of Van Landeghem et al. (1997) after studying the speciation of both aluminium and iron in cerebrospinal fluid (CSF). They found that the serum metal/CSF metal ratio's of aluminium and iron (both occurring highly protein bound in serum) were comparable (± 20) as opposed to the ratio noted for e.g. silicon (± 1.5) which in serum is known to occur non-protein bound (D'Haese et al., 1995). With the transferrin mediated uptake of either iron or aluminium the protein is supposed to recycle to the serum after delivering the metals to the brain. Subsequently iron is bound by transferrin produced in the brain. Aluminium however, as demonstrated by speciation studies in CSF seems not to be bound by transferrin and hence circulates as a loosely bound compound in the brain which at least in part may explain its high neurologic toxicity at extreme low levels. Although aluminium is a trivalent cation unable to undergo redox reactions it has repeatedly been shown in *in vivo* as well as *in vitro* studies that the element may induce, potentiate or enhance iron-induced oxidative events (Xie et al., 1996; Toda and Yase, 1998; Bondy et al., 1998) which is in line with the element's occurrence as a non-protein bound element in the brain. Although further evidence is still needed stimulation of iron-supported lipid peroxidation through binding to the membrane and promotion of changes in the arrangement of membrane lipids including packing of fatty acids facilitating the propagation of lipid peroxidation has been promoted as a possible underlying mechanism responsible for these findings (Oteiza, 1994) which in turn indicate that the effect of aluminium on iron-induced oxidative stress might contribute to the neurotoxic effects of both these elements.

Whereas in serum iron is transported nearly 100% bound to transferrin, speciation studies by Van Landeghem et al. (1997) indicated iron in CSF to appear only partly bound to transferrin; a finding suggested by others also using other methodologies. The occurrence of the element as both a protein bound and non-protein bound compound in brain allows us to suggest that once across the blood brain barrier, the element will either be transported by transferrin (synthesized by choroid plexus or by oligodendrocytes) allowing its uptake by different cell types presenting the transferrin receptor, or further delivered from the CSF to the rest of the brain via a non-transferrin-mediated mechanism. One can reasonably assume that the cellular uptake of iron as either the iron-transferrin complex or a low molecular compound will also influence the element's toxic effect. This has previously been established for aluminium also. So, has it been shown in parathyroid gland cells that both aluminium citrate and aluminium transferrin are taken up to the same degree but that toxic effects, as determined by a reduced PTH secretion, only occurred after addition of the element as the transferrin complex (Smans et al., 2000). *In vitro* findings by Abreo et al. (1999) indicate that, after addition of aluminium–transferrin or aluminium citrate, the element's uptake by neuroblastoma cells occurs by transferrin-dependent as well as transferrin-independent mechanisms which both inhibit

cell growth and go along with an increased staining of neurofibrillary tangles. To which extent these findings can be extrapolated to the *in vivo* situation deserves further investigations.

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Aluminium and Alzheimer's Disease
The Science that Describes the Link
C. Exley (Editor)

CHAPTER 12

Aluminum and the Blood–Brain Barrier

Robert A. Yokel *

College of Pharmacy and Graduate Center for Toxicology, University of Kentucky Medical Center, Lexington, KY 40536-0082, USA

* Corresponding author, email: ryokel1@pop.uky.edu

Abbreviations: Aβ – amyloid beta protein; AD – Alzheimer's disease; Al – aluminum; Al-citrate – aluminum-citrate; BBB – blood–brain barrier; BBr – brain/blood ratio; CSF – cerebrospinal fluid; ECF – extracellular fluid; i.a. – intraarterial; i.c.v. – intracerebroventricular; i.p. – intraperitoneal; i.v. – intravenous; MCT – monocarboxylate transporter; TfR-ME – transferrin-receptor mediated endocytosis; 4-TMA-AP – 4-trimethylammonium antipyrine

Summary

Aluminum may contribute to Alzheimer's disease by increasing brain aluminum, however elevated brain aluminum has not been consistently found in Alzheimer's disease. Brain aluminum entry from the nasal cavity is probably not significant. Most brain aluminum entry is probably through the blood–brain barrier by transferrin-receptor mediated endocytosis and transporter-mediated processes. There appears to be transporter-mediated brain aluminum efflux. Some aluminum persists in the brain for a very long time, suggesting elevated brain aluminum might result in later life from dietary aluminum. Blood–brain barrier permeability does not appear to be compromised in Alzheimer's disease or produced by aluminum under conditions relevant to the human. Studies assessing the ability of aluminum to alter blood–brain barrier function do not convincingly relate to the human.

Historical Perspective

Aluminum Is a Neurotoxicant

The ability of Al to produce neurotoxicity in animals has been documented (Döllken 1898). There have been many studies of the association between Al in drinking water and dementing illnesses in the human (Yokel 2000). There have also been many studies of Al concentration in bulk brain, neurofibrillary tangles and senile plaques of Alzheimer disease (AD) victims compared to controls (Yokel 2000). An assumption of the latter studies is that elevated brain Al may suggest a role of Al in the etiology of AD. The results have been inconsistent, and have been interpreted to both support and refute a role

for Al in Alzheimer's disease (Yokel 2000). However, elevated brain Al can contribute to iatrogenic encephalopathies in the human. These include the dialysis encephalopathy (dementia) syndrome which is due, at least in part, to Al-contaminated dialysate (Alfrey *et al.*, 1980; Flendrig *et al.*, 1976; McDermott *et al.*, 1978) and mortality in uremic neonates given Al-containing milk formulas and nutrition solutions (Bishop *et al.*, 1989; Freundlich *et al.*, 1985). Therefore, there is concern about conditions that lead to elevated brain Al. One factor contributing to elevated brain Al appears to be age, based on studies of 35 and 9 subjects, respectively (Markesbery *et al.*, 1984; McDermott *et al.*, 1979) although a study of only 4 subjects failed to find an association (Jacobs *et al.*, 1989). Suggestions of an age-related elevation of brain Al and the recognition that Al is a neurotoxicant lead to the suggestion that: "If the estimations regarding the normal rate of uptake for Al^{3+} in the brain are correct, then the toxic level should be reached after 100–150 years if no elimination from the brain occurs" (Ganrot 1986). This chapter will address the distribution of aluminum into and out of the brain, its accumulation in the brain, potential mechanisms of its transport across the blood–brain barrier (BBB), and its potential effects on BBB function, in relation to AD.

The Roles of ^{27}Al and ^{26}Al in the Study of Aluminum Toxicokinetics

Prior to the application of accelerator mass spectrometry (AMS) to quantitate ^{26}Al in biological samples (Flarend & Elmore, 1998), the study of Al toxicokinetics utilized two approaches. The stable isotope of Al, ^{27}Al, was utilized and quantitated by electrothermal atomic absorption spectroscopy. However, due to its ubiquitous presence, supra-physiological doses or concentrations were usually utilized to reliably detect it above inherent ^{27}Al. Based on its chemical similarities to Al, ^{67}Ga was used by two research groups as a model of Al. However, ^{67}Ga does not mimic the toxicokinetics of Al (Allen & Yokel, 1992; Priest et al., 1995). The exquisite sensitivity for ^{26}Al quantitation obtained with AMS (which provides detection of ~1,000,000 molecules) enables the study of Al toxicokinetics at physiologically relevant Al concentrations.

Aluminum Can Enter the Brain after Oral Exposure

Oral administration of ^{26}Al resulted in higher brain ^{26}Al concentrations than seen in control rats (Fink *et al.*, 1994; Walton *et al.*, 1995; Drueke *et al.*, 1997; Jouhanneau *et al.*, 1997). This is not surprising because Al distributes throughout the mammal.

It Has Been Suggested that Aluminum Can Enter the Brain Directly from the Nasal Cavity

The potential routes for Al to enter the brain are from environmental airborne sources that enter the nasal cavity and from blood. From the nasal cavity, Al might directly enter the brain through the olfactory neuron, which runs from the roof of the nasal cavity

to the olfactory bulb. Inorganic Cd, Hg, Mn and Ni can enter the olfactory bulb from the nose. Manganese is further able to distribute across synapses in the olfactory bulb to connecting neurons and therefore continue to distribute to many or all brain regions (Tjalve & Henriksson, 1999). There is evidence that Al can enter the brain through the olfactory neuron (Perl & Good, 1987; Divine *et al.*, 1999).

Blood to Brain Transfer through the Blood–Brain Barrier Provides the Greatest Opportunity for Brain Aluminum Uptake

From blood, Al might enter the brain through the BBB. It could also enter the brain through the choroid plexuses into the CSF housed in the four ventricles within the brain and in the subarachnoid space surrounding the brain. The BBB buffers the brain against sudden changes in blood composition. This isolates the brain from factors that might adversely effect brain chemistry, so that the brain becomes a pharmacological sanctuary. Nevertheless, the primary site of drug and chemical distribution between blood and brain is the BBB (Pardridge 1997). This might be predicted from the comparative anatomy of the BBB and the choroid plexus. The anatomical basis of the BBB is the tight junctions between the endothelial cells surrounding the parenchymal microvessels that perfuse the brain (to see a diagram visit http://www.sfn.org/briefings/blood-brain.html). Also contributing to the BBB are the absence of fenestrations and the low transcytotic activity in the endothelial cells, the pericytes in the basement membrane around the endothelial cells, and the astrocytic foot processes that cover 99% of the surface of endothelial cells. The BBB surface area is $\sim$12 m^2, $\sim$1000-fold greater than the surface area of the choroid plexuses (Pardridge 1998). The choroid plexuses are capillary networks surrounded by epithelial cells that have tight junctions with some properties that are similar to BBB endothelial cells. Like BBB endothelial cells, the choroid plexuses have facilitated diffusion and active transport properties. Substances distributing across the BBB enter brain extracellular fluid (ECF) from which they could enter brain cells. Substances distributing across the choroid plexuses enter the CSF within the 4 ventricles of the brain from which they might distribute into brain ECF and then into brain cells. However, the bulk flow of fluid through brain parenchyma toward the ventricles (Davson *et al.*, 1987) would impede diffusion of substances from the cerebral ventricles into brain tissue. The endothelial cells comprising the BBB have an intimate relationship with brain tissue, providing the opportunity for rapid distribution between blood and brain. The greatest distance between a brain cell and a microvessel comprising the BBB appears to be $\sim$30–50 μm (Arnold Scheibel, personal communication). This extensive network of microvessels provides substance delivery to and removal from the brain. In contrast, some human brain regions are >1 mm from the nearest CSF compartment (ventricle or subarachnoid space). This may present an insurmountable distance between CSF and some brain cells for exchange of substances because the maximal penetration of substances through brain parenchyma has been claimed to be <1 mm (Pardridge 1998). Therefore most blood–brain exchange is through the BBB.

The Primary Route of Brain Entry of Small Molecular Weight Aluminum Species Is through the Blood–Brain Barrier

The relative contributions of the BBB and the choroid plexuses to brain Al entry were assessed by placing microdialysis probes in the frontal cortex, lateral ventricle and jugular vein of the rat. Microdialysis enables repeated sampling of unbound substances that are in the ECF (Benveniste & Huttemeier, 1990; Hammarlund-Udenaes *et al.*, 1997). Aluminum was given i.v. as Al-citrate, the predominant small molecular weight Al species in plasma (Table 1), to achieve Al concentrations that exceeded the metal-binding capacity of transferrin, therefore favoring persistence of Al as the citrate. The Al concentration in the dialysate flowing out of the microdialysis probe implanted in the cortex peaked in the first 5 minute sample, and was higher than in dialysate exiting the probe in the lateral ventricle (Allen & Yokel, 1992). The rapid appearance of Al in the frontal cortex was attributed to brain Al entry through the BBB and not the choroid plexuses, based on the following. The Al concentration was lower in the lateral ventricle than in the frontal cortex, suggesting Al in the cortex did not derive from Al in the lateral ventricle. The bulk flow of brain ECF from brain parenchyma to lateral ventricles would be expected to impede the movement of substances from CSF to brain ECF (Davson *et al.*, 1987). Some regions of the frontal cortex are 1.5 mm from the lateral ventricle and 1 mm from subarachnoid space in the rat, suggesting Al could not have diffused from CSF to the frontal cortex within 5 minutes. Although Al rapidly enters both brain ECF and CSF from the blood, it appears that Al primarily enters the brain through the BBB. Two studies from the laboratory of Sri Melethil also showed that Al, as the chloride and sulfate, can enter CSF from blood in significant concentrations within 30 minutes (Peng *et al.*, 1992; Xi et al., 1992).

A Small Fraction of Aluminum that Enters Systemic Circulation Distributes to the Brain

An i.v. injection of Al-citrate produced a peak Al concentration that averaged 2.8 μM in dialysate of brain ECF in the first 5 minute microdialysis sample (Allen & Yokel, 1992). These results suggest a brain ECF Al concentration of ~87 μM. This estimate is based on the following two observations. Relative recovery of Al from ECF by microdialysis is

Table 1. The main binding ligands for Al *in vivo*, their effective equilibrium constants with Al, their concentrations in plasma and brain extracellular fluid (which are assumed from values in cerebrospinal fluid), and the percentage of Al predicted to be associated with that ligand in plasma and brain extracellular fluid. (Provided by Wes Harris)

Ligand	Effective equilibrium constant with Al (log)	Plasma		Brain extracellular fluid	
		Concentration (μmol/l)	% of Al species	Concentration (μmol/l)	% of Al species
Transferrin	13.7, 12.6	30	91	<0.25	4
Citrate	11.6	99	7–8	180	90
Hydroxide	6.5	0.4	<1	0.4	5
Phosphate	9.3	1100	<1	490	1

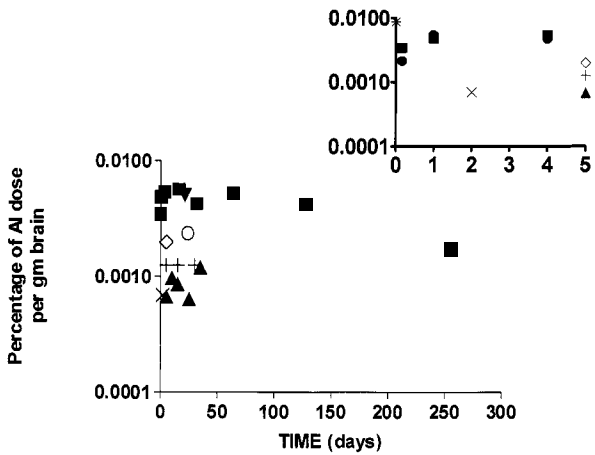

Fig. 1. The percentage of Al per gm brain of rats after systemic (solid symbols) or oral dosing (open symbols). The insert shows the data for days 0–5 only. The percentage after oral dosing has been multiplied by 330 to model oral Al bioavailability of 0.3%. ∗, After i.v. ^{27}Al-citrate (Allen and Yokel 1992). ■, After i.v. ^{26}Al-Tf (Yokel *et al.*, 2000). •, After i.v. ^{26}Al-citrate (Yokel et al, unpublished results). ▼, After i.v. $^{26}AlCl_3$ (Walker *et al.*, 1994). ▲, After i.p. ^{26}Al in acetate-HCl (Kobayashi *et al.*, 1990). ◇, After p.o. ^{26}Al in water (Fink *et al.*, 1994). ○, After p.o. ^{26}Al in water (Walton *et al.*, 1995). +, After p.o. ^{26}Al in HCl, pH 1.6–2 (Jouhanneau *et al.*, 1997). ×, After p.o. ^{26}Al in HCl, pH 1.6–2 (Drueke *et al.*, 1997).

3.25%, obtained from dialysis of blood *in vivo* compared to serum ultrafiltrate (Yokel *et al.*, 1991). Comparable antipyrine concentrations were obtained in dialysate from blood and brain (Yokel *et al.*, 1992), suggesting comparable relative recovery by microdialysis of small molecules from brain and blood ECF. Assuming, within this 5-minute period, that the Al was confined to brain ECF, which represents 15% of brain volume, then brain Al concentration was ~87 μM × 0.15, or ~13 nmoles/g brain. Each gram of brain therefore contained ~0.009% of the 0.5 mmol/kg bolus dose of Al given to these 300 g rats. More recent studies have measured brain ^{26}Al after its oral or systemic (i.v. or i.p.) administration to rats. After systemic administration, each gram of brain had ~0.0008 to 0.005% of the injected dose (Fig. 1). When the brain ^{26}Al concentrations obtained in rats that received oral ^{26}Al are multiplied by 330 to correct for an assumed oral Al bioavailability of 0.3% (Yokel *et al.*, 2001), there was a reasonably similar percentage of the administered ^{26}Al dose/gram brain (Fig. 1). These results demonstrate brain entry of a fairly constant fraction of Al, presumably from blood through the BBB.

Aluminum Crosses the Blood–brain Barrier by One or More Mechanisms other than Diffusion

Brain entry through the BBB might be achieved by very limited diffusion through the pericellular pathway, by diffusion of small (generally <700 Da) lipophilic substances through the endothelial cell membranes, or by transporter-mediated processes (Laterra *et al.*, 1999). The rate of appearance of Al in the brain after i.v. injection of Al-citrate

cannot be explained by its diffusion through the BBB. Diffusion through the BBB positively correlates with the substance's lipophilicity (Levin, 1980; Laterra *et al.*, 1999). The lipophilicity (octanol/aqueous partition coefficient) of Al-citrate (0.0019; Yokel & Kostenbauder, 1987) predicts a BBB permeability coefficient of $\sim 10^{-7}$ cm sec^{-1}, based on the relationship between hydrophilicity and molecular weight (Levin 1980). However, the rate of Al-citrate diffusion through an artificial membrane (phosphatidylcholine/phosphatidic acid liposomes) suggested a permeability coefficient of 2×10^{-11} cm sec^{-1} and a flux rate of 4×10^{-16} mol cm^{-2} sec^{-1} (at ~1 mM Al-citrate; Akeson & Munns, 1989). This rate is considerably slower than predicted from its hydrophilicity and molecular weight (Levin 1980). This deviation from the predicted rate was attributed to hydrogen bonding and hydration of the bound metal (Akeson & Munns, 1989). Therefore, the appearance of Al in the brain was concluded to be more rapid than could be explained by diffusion (Akeson & Munns, 1989). The authors suggested that brain uptake of Al-citrate may be transporter-mediated, citing the energy dependent, protonmotive transport of the divalent cation–citrate complex by bacteria (Bergsma & Konings, 1983).

Estimates of the transfer rate of Al from blood to brain appear in two non-peer-reviewed reports. The rates are expressed as K_{in}, the influx rate constant, which is a measure of clearance from blood to brain. For diffusion K_{in} is equivalent to the permeability $\times$ surface area product. Jagarlamudi & Melethil (1995) reported a K_{in} of 1.34×10^{-2} ml g^{-1} sec^{-1} into cortex from a bicarbonate-containing perfusate, based on a 1 minute *in situ* brain perfusion followed by a 45 second washout. This could be an underestimate if there was significant efflux of Al from the brain during the washout. In contrast, Radunovic & Bradbury (1994) reported a K_{in} of 2.6×10^{-7} ml g^{-1} sec^{-1} into the cerebral hemispheres after a 50 hour perfusion of Al-citrate. This estimate is based on quite variable data and could significantly underestimate K_{in} due to brain Al efflux during the 50 hours.

Results we obtained suggest the presence of a non-diffusive mechanism enabling brain uptake of Al-citrate (Allen & Yokel, 1992), as follows. The flux rate of Al-citrate through a membrane by diffusion was estimated to be 4×10^{-16} mol cm^{-2} sec^{-1} (Akeson & Munns, 1989). The surface area of brain capillaries was estimated to be 240 cm^2 per g brain (Ohno *et al.*, 1978). Approximately 15% of brain volume is ECF. The unbound Al concentration in plasma after an i.v. Al injection was estimated to be ~1 mM based on an Al concentration in blood dialysate of ~30 μM (Allen & Yokel, 1992) and a relative recovery of Al from plasma ultrafiltrate of 3.25% (Yokel *et al.*, 1991). Therefore if 4×10^{-16} mol of Al could diffuse through 1 cm^2 of membrane in 1 second, $\sim 3 \times 10^{-11}$ mole of Al could diffuse through 240 cm^2 of capillary endothelial cells into 1 gram of brain in 5 minutes. If this was initially distributed within the 15% of the brain that is ECF, the ECF Al concentration would be $\sim 2 \times 10^{-7}$ M. This would generate a brain/blood ratio (BBr) of 0.0002, based on 2×10^{-7} M Al in brain ECF/1×10^{-3} M Al in blood ECF. As the BBr that would result from diffusion in 5 minutes is much lower than the BBr achieved, which was ~0.15, Al-citrate influx into the brain must be transporter mediated.

To further assess the presence of transporter-mediated Al transport across the BBB, brain and blood ECF Al concentrations were determined using microdialysis during steady state Al concentrations, over an eight-fold range of Al-citrate infusion rates (Allen *et al.*, 1995). Dialysate Al concentrations rose from 7.5 to 41 μM as the Al infusion rate

was increased from 0.1875 to 1.5 mmol Al/kg/hr. Based on 3.25% relative recovery of Al by microdialysis from plasma, this corresponds to plasma Al concentrations of 230–1200 μM. The BBr remained constant at ~0.15. At equilibrium the following equation applies:

$$Cl_{in} \times C_{bl.u} = Cl_{out} \times C_{br.u} \quad (1)$$

where Cl_{in} is the diffusion clearance into the brain (influx), $C_{bl.u}$ is the unbound concentration in blood ECF, Cl_{out} is the diffusion clearance out of the brain (efflux), and $C_{br.u}$ is the unbound concentration in brain ECF. The lower concentration of unbound Al in brain than blood ECF ($C_{br.u} < C_{bl.u}$), e.g., a BBr < 1, suggests $Cl_{out} > Cl_{in}$, therefore the presence of a process other than diffusion mediating distribution of Al across the BBB, e.g., transporter-mediated transport.

There Are Many Transporters at the Blood–Brain Barrier

Transporter mediated facilitated diffusion and active transport enable brain entry of essential nutrients at rates that greatly exceed the rates that are possible by diffusion through the BBB. There are many transporters at the BBB. Table 2 lists some of the families and a few specific transporters that have been identified at the BBB. Evidence for expression has been obtained with a few of these transporters however most have only been functionally described. Realizing that not all transporters have yet been identified and that the expression of many known transporters has not been determined in the brain, much less at the BBB, it is not possible to compile a complete list of BBB transporters. There are specific transporters for several essential metals, such as copper, iron and manganese (Romero *et al.*, 1996). For example, iron, combined with transferrin, combines with a receptor on the vascular endothelial luminal surface, which is endocytosed in a vesicle to enable distribution across the endothelial cell membrane, by transferrin-receptor mediated endocytosis (TfR-ME). The mechanism of iron distribution from endothelial cell cytoplasm to brain ECF is less well understood. Toxic metals may enter the brain as substrates for essential transporters or as organic complexes that are recognized by transporters for metabolic precursors (Romero *et al.*, 1996), e.g., they may hitchhike through the BBB. For example, the methylmercury-L-cysteine complex is structurally similar to L-methionine and is transported into the brain by the large neutral amino acid transporter (Aschner & Clarkson, 1988; Kerper *et al.*, 1992). Because Al is not an essential element, one would not expect there to be a transporter at the BBB whose function is brain Al influx. Brain Al entry may be mediated by a transporter whose role is other than Al transport.

It Was Suggested that Aluminum Enters the Brain as an Aluminum–Glutamate Complex

Studies with whole blood, incubated with 250 μM Al and up to 95,000 μM L-glutamate, suggested L-glutamate enhancement of Al uptake into erythrocytes (Deloncle *et al.*, 1990). Rats given daily L-glutamate injections for two weeks before a single Al

Table 2. Some of the transporters at the BBB. Transporter, symbol (from LocusLink), distribution (L = luminal, A = antiluminal), type (AT = active transport, FD = facilitated diffusion, RM = receptor mediated, SAT = secondary active transport), typical substrates and blood-to-brain transport rates. From LocusLink (http://www.ncbi.nlm.nih.gov/LocusLink), Laterra *et al.* (1999) and other sources

Transporter	Symbol	L	A	Type	Substrates	Transport rate (nmol/g/min)
hexose (GLUT-1)	SLC2A1	x	x	FD	glucose	700
monocarboxylate	SLC16A1	x	x	FD	lactate, pyruvate	60
anion exchange (band 3)	SLC4				lactate, Cl^-/HCO_3^-, metal–anion complexes	
large neutral amino acid (L-system)		x	x	FD	L-DOPA, phenylalanine	12
small neutral amino acid (A system)	SLC5		x	SAT	alanine, serine, cysteine	
neutral amino acid (ASC system)	SLC7		x		D-serine	
basic amino acid		x	x		lysine	3
acidic amino acid (X^-)			x		glutamate, aspartate	0.2
N-system		x		FD	glutamine and glutamate	
excitatory amino acid (EAAT1-3)	SLC1		x		glutamate	
amine (cation, y^+)	SLC7	x	x	FD	choline	0.2
β-amino acid		x	x		taurine	
low affinity GABA	SLC6?	x				
high affinity GABA	SLC6?		x			
saturated fatty acids					octanoate	
urea	SLC14			FD		
purine		x	x		adenine	0.006
nucleoside	SLC28/29?	x	x		adenosine	0.004
Na/K-ATPase	ATP1		x	AT	potassium	
Ca-ATPase	ATP2	x	x	AT	calcium	
sodium		x		AT	sodium	200
potassium	SCL12		x	AT		12
chloride	SLC12?					140
divalent metal (DMT1, DCT1, Nramp2)	SLC11				Fe^{+2}, Zn, Mn, Cu, Cd, Ca, Ni and Pb	
P-glycoprotein (P-gp) multidrug resistance transporter (MDR)	ABC Subfamily B	x		AT	organic cations with high lipophilicity: vincristine	
multidrug resistance-associated protein (MRP1, 4, 5 and 6)	ABC Subfamily C			AT	organic anions and glutathione, glucuronate and sulfate conjugates	
organic anion transporting polypeptides (oatp)	SLC21		x		negatively charged amphipathic (amphiphilic) organics	
peptide transporter	SLC15?				TRH, α-MSH, IL-1	
transferrin		x	x	RM	transferrin	0.003
many transporters for vitamins and co-factors						
several transporters for hormones						

injection had higher brain Al than rats given daily saline injections before the Al injection (Deloncle *et al.*, 1990). The authors suggest formation of an Al-L-glutamate complex that crosses membranes. Deloncle & Guillard (1990) cited an Al-L-glutamate stability constant of 15.04 for log k_1 of the 1 : 1 Al : L-glutamate complex, to support their conclusion. However, a more accurate stability constant is 7.69 (Martin 1992). Considering this stability constant and the plasma and CSF glutamate concentrations, 58 and 26 μM, respectively, compared to the values for citrate (Table 1) shows why Martin concluded that glutamate is not competitive with most other ligands for Al in physiological systems (Martin 1992).

Aluminum May Be Transported into the Brain by Transferrin Receptor Mediated Endocytosis and by Another, More Rapid, Process

The chemical species of a metal in blood plasma (ECF) suggest the species of the metal available for transport (Romero *et al.*, 1996). The predominant Al species in blood ECF is Al-transferrin (Table 1). The density of transferrin receptors in regions of the forebrain of chronic hemodialysis patients correlated with Al concentration, suggesting that Al might enter the brain via TfR-ME (Morris *et al.*, 1989). Al-transferrin was shown to bind to rat brain synaptosomes with a comparable B_{max} and slightly lower K_D than Fe-transferrin. Al-transferrin and Fe-transferrin competitively displaced each other from their binding site(s). These results suggested that Al might enter the CNS via TfR-ME (Roskams & Connor, 1990).

We gave ^{26}Al-transferrin by i.v. injection to rats. Four hours later, 0.003% of the dose was found/g brain (Fig. 1) (Yokel *et al.*, 2000). At 4 hours 0.08% of the dose was in each ml of blood. If we make the following assumptions: 1) the distribution of the Al-Tf was originally confined to the vascular compartment, 2) Al-Tf was cleared over the four hours by a first order process and 3) the K_{in} of Al-Tf is comparable to the Fe-Tf value ($\sim 3 \times 10^{-3}$ ml blood/g brain/hr; Bradbury 1997), then we conclude that TfR-ME could account for the observed brain influx of ^{26}Al. These results would be compatible with a role for TfR-ME in brain Al influx.

In contrast, the rapid brain entry of Al we observed after i.v. Al-citrate injection cannot be explained by TfR-ME. If we make the following assumptions: 1) transferrin saturation by Al, 2) comparable K_{in} values for Al and Fe by TfR-ME, and 3) the confinement of Al to brain ECF, then the maximum brain ECF Al concentration that could be achieved by TfR-ME in 20 minutes would be ~0.15 μM. This is very much lower than the brain ECF Al concentration achieved in microdialysis studies (18–245 μM; Allen *et al.*, 1995), which was estimated from dialysate Al concentrations and 3.25% relative recovery of Al by microdialysis probes. A marker of BBB integrity (4-trimethylammonium antipyrine, 4-TMA-AP, discussed below) was administered in this study (Allen *et al.*, 1995). No significant disruption of the barrier to this small charged molecule was observed. The results suggest a mechanism for brain Al uptake other than TfR-ME.

These observations are consistent with the effects of transferrin on brain Al entry in rats, determined using the *in-situ* brain perfusion technique (Jagarlamudi & Melethil, 1995). Al was introduced in the perfusate, a pH 7.4 bicarbonate solution, as 'free Al' and

with transferrin in relative Al : transferrin concentrations that would result in up to 20 or 40% of the Al bound to transferrin. The chemical species of the Al that was added as 'free Al' was not reported. Transferrin reduced brain Al uptake, suggesting 'free Al' was able to enter the brain by a more rapid mechanism than by TfR-ME. Brain uptake of Al, infused as Al-citrate, was not influenced by a transferrin receptor antibody in mice nor was brain Al uptake in hypotransferrinemic mice different from controls (Radunovic *et al.*, 1997). These results are consistent with a mechanism of brain Al uptake that is not mediated by TfR-ME.

The more rapid brain uptake of non-transferrin bound Al than Al-transferrin is consistent with results obtained with Fe and Mn. Brain Fe uptake was 80–95-fold greater in hypotransferrinemic than control mice (Ueda *et al.*, 1993). The authors suggested this might be due to transport of Fe-citrate across the cerebral endothelium (BBB) that is reduced when transferrin binds Fe. Similarly, brain entry of Mn, introduced as the ion, was more rapid than could be explained by diffusion, and was impeded by transferrin, suggesting an uptake mechanism other than TfR-ME (Rabin *et al.*, 1993).

In conclusion, there appear to be at least two mechanisms by which Al, Mn and Fe may transfer from blood to brain. They are TfR-ME and a more rapid, transporter-mediated, process that probably transfers Al-citrate.

We Hypothesized that the Monocarboxylate Transporter Mediates Aluminum-Citrate Distribution across the Blood–Brain Barrier

Al forms coordination bonds with the two terminal carboxylates and the hydroxyl group of citrate, leaving the central carboxylate group an unbound anion at physiological pH (Gregor & Powell, 1986). Consideration of this chemical species in light of the expression of the monocarboxylate transporter (MCT) at both the luminal and antiluminal (abluminal) surfaces of the BBB (Gerhart *et al.*, 1997) and the high maximal transfer rate of this transporter at the BBB, 60 nmol/gm brain/min (Table 2), suggested to us that it might mediate Al transfer across the BBB. The estimated Al concentrations we achieved in plasma by Al-citrate infusion during microdialysis studies (0.2–1.2 mM; Allen & Yokel, 1992) are compatible with the K_m of the MCT (1.8 mM) (Pardridge 1988). At the V_{max} of the MCT, 60 nmol/gm brain/min, it could transport 2 μmoles of Al, if Al-citrate serves as a substrate, into 1 ml of brain ECF in 5 minutes, producing 2 mM Al in brain ECF. This rate is more than sufficient to explain the appearance of 2.8 μM Al in dialysate of brain (estimated to reflect 0.08 mM Al in brain ECF) 5 minutes after the i.v. Al injection utilized in brain microdialysis studies (Allen & Yokel, 1992). Therefore, MCT transport could explain the rapid appearance of Al in brain ECF after i.v. injection of Al-citrate.

There Is a Mechanism Mediating Rapid Aluminum Efflux from the Brain

Intravenous injection of Al citrate resulted in a peak rat brain ECF Al concentration within 5 minutes, determined by microdialysis (Allen & Yokel, 1992), suggesting rapid brain entry of Al. Yet injection and infusion of Al to maintain constant blood and brain

ECF concentrations did not result in the same Al concentration in these two compartments on the two sides of the BBB (Allen *et al.*, 1995). Brain ECF Al concentration was 10–15% of that seen in blood. Pharmacokinetic modeling suggested that Al uptake into neurons and glial cells could not account for the lower brain than blood ECF Al concentration, suggesting Al efflux from the brain across the BBB (Ackley & Yokel, 1997). This process cannot be diffusion because the same blood and brain ECF Al concentration should be seen at steady state. Because brain ECF Al was lower than blood ECF Al concentration, the rate of efflux of Al from the brain must exceed its influx rate, according to equation (1), above.

A Putative Mechanism of Brain Aluminum Efflux

Many, but not all, BBB transporters are expressed at the antiluminal membrane (Table 2). An important role for some of these transporters is to protect the brain from accumulation of neurotoxicants, by actively effluxing them from brain to blood. Substances that are effluxed from the brain include AZT (by the nucleoside transporter); cyclosporin, vinca alkaloids, etoposide and taxol (by P-gp and MRP); iodine and thiocyanate; theophylline; atenolol; acetaminophen; fluconazole; baclofen; valproate; methotrexate; β-lactam antibiotics; quinolones and fluoquinolones. These results indicate efflux of a wide variety of substances by many mechanisms. Studies with metabolic inhibitors suggested that lead is transported from brain to blood by Ca-ATPase (Bradbury & Deane, 1993), presumably revealing an efflux mechanism for this neurotoxicant.

Consideration of the Al species in the brain suggests that it is unlikely that TfR-ME significantly effluxes Al from the brain. Due to the very low presumed transferrin concentration in brain ECF, Al-transferrin is not a significant Al species in brain ECF (Table 1). In fact, brain ECF transferrin may be saturated with Fe, leaving no available metal binding sites (Bradbury 1997), reducing brain ECF Al-transferrin to zero. Finally, if TfR-ME does not have a sufficient rate to mediate brain Al influx, above, it cannot mediate a more rapid brain Al efflux. Consideration of the Al species in the brain suggests that the most likely candidate for Al efflux is Al-citrate (Table 1). We hypothesized that the MCT might be mediating Al efflux from the brain, as Al-citrate, based on the following. Al-citrate is the predominant Al species in brain ECF. The MCT is present at both the luminal and antiluminal membrane of the endothelial cells comprising the BBB. The V_{max} of MCT is more than sufficient to mediate brain Al efflux, if Al-citrate serves as a substrate, above. MCT exhibits greater efflux than influx efficiency at the BBB (Oldendorf *et al.*, 1979). Therefore, we tested the role of the MCT to efflux Al from the brain in a series of studies using microdialysis of both frontal cortices as well as blood in the rat receiving an infusion of Al-citrate. After obtaining a steady state Al BBr, which averaged 0.24, the dialysate perfusing one frontal cortex microdialysis probe was changed to a dialysate containing a pharmacological probe selected to inhibit the MCT (Ackley & Yokel, 1997; 1998). This delivery method should produce the highest concentrations of the pharmacological probes in brain ECF immediately surrounding the microdialysis probe, where the effects of transport across the BBB are also being observed. Cyanide, which inhibits electron transfer along the mitochondrial respiratory chain to block cellular

respiration, and 2,4-dinitrophenol, which uncouples oxidative phosphorylation, were tested. They increased the Al BBr to a value not different from one, suggesting the process effluxing Al from the brain was energy dependent. Addition of pyruvate, an MCT substrate, increased the Al BBr, suggesting it competitively inhibited MCT efflux of brain Al. The MCT is a proton co-transporter, therefore its activity positively correlates with proton concentration. Treatments selected to reduce the proton concentration in brain ECF surrounding the microdialysis probe increased the Al BBr, consistent with inhibition of a proton dependent transporter, such as the MCT. All of these observations were obtained in the presence of 4-TMA-AP, which showed no significant disruption of the BBB, suggesting the treatments were not simply opening the BBB. However, none of these treatments specifically tested the hypothesis that the MCT mediates Al-citrate efflux from the brain.

The Residence Time of Some Aluminum that Enters the Brain Is Very Long

Aluminum persistence in mammalian tissues has been reported. The $t_{1/2}$ of Al in selected peripheral tissues ranged from ~40 to well over 100 days in the rabbit (Yokel & McNamara, 1989) and 8–24 days in the rat (Greger *et al.*, 1994). The human $t_{1/2}$ after retirement from occupational inhalation exposure was estimated to be up to 8 years (Ljunggren *et al.*, 1991). After i.v injection of ^{26}Al to a human, a $t_{1/2}$ of 7 years was estimated (Priest *et al.*, 1995).

Al appears to be retained in the human and rat brain for some time. Human brain Al increases with age (McDermott *et al.*, 1979; Markesbery *et al.*, 1984). This could be due to increased exposure with age, a decreased ability to remove Al from the brain with age, or very slow, or no, elimination of Al from the brain. Patients who had elevated Al intake and who received hemodialysis before successful renal transplantation years prior to death had elevated post-mortem brain Al (McDermott *et al.*, 1978; Reusche *et al.*, 1996), suggesting accumulation of Al during hemodialysis that was slowly or not cleared after establishment of renal function.

Animal studies show Al persistence in the brain. When ^{26}Al was given i.p. to rats no decrease in brain Al was seen 5 to 35 days later (Kobayashi *et al.*, 1990). When ^{26}Al was given i.v. to rats that were euthanatized 0.17 to 256 days later the $t_{1/2}$ of brain Al was ~150 days (Yokel *et al.*, 2000). Therefore at least some of the Al that enters the brain resides there for a very long time.

The Blood–Brain Barrier in Alzheimer's Disease

The BBB in those with AD demonstrates histological evidence of pathology, including thinning, tortuous and deformed microvessels accompanied by wrinkling of the vascular basement membrane (Regelson & Harkins, 1997; Caserta *et al.*, 1998; Kalaria 1999). There is evidence for decreased blood flow through the brain (Regelson & Harkins, 1997), and reduced biochemical activity, including GLUT-1 transporter activity, Na/K-ATPase and perhaps hexokinase, in the BBB of AD subjects (Kalaria 1999). Abnormal processing

of amyloid beta protein (Aβ) may occur throughout the body, including at the BBB, where it may contribute to AD pathology (Caserta *et al.*, 1998; Kalaria 1999). It is less clear whether the BBB is more permeable in AD. Studies that have shown an increase of serum proteins in brain ECF of deceased AD patients and in CSF have been discounted as an unreliable method to demonstrate BBB dysfunction and probably the result of changes in the choroid plexus, respectively (Caserta *et al.*, 1998). Several PET studies, using ^{82}Rb, ^{68}Ga-EDTA and meglumine iothalamate, failed to find evidence for a leaky BBB in AD patients (Caserta *et al.*, 1998), leading these authors to conclude that there is no generalized increase in BBB permeability in living AD subjects.

The Effects of Aluminum on the Blood–Brain Barrier

There have been many suggestions that Al can alter the function of the BBB. However, most of the studies have been conducted utilizing conditions and Al concentrations that make their relevance to the human unlikely, as discussed in Informed Opinion. There are also numerous studies that failed to show Al-induced BBB changes. The original observation of Al-induced changes in the brain that generated the hypothesis that Al may play a role in the etiology of AD was the development of neurological symptoms in rabbits after intracerebral injection of Al phosphate, prepared as Holt's adjuvant (Klatzo et al., 1965). Intravenous injection of fluorescein to rabbits demonstrating Al-induced neurological symptoms did not reveal increased permeability of the BBB; e.g. there was no evidence of fluorescein in the brain 24 hours later. However, if fluorescein had entered the brain through compromised tight junctions it may have diffused back into blood over the 24 hours, considering its 286 minute $t_{1/2}$ in the human, and presumably shorter $t_{1/2}$ in the rabbit. On the other hand, 2 hours after i.p. administration of 100 mg/kg (3.7 mmoles/kg) of Al as $AlCl_3$ or Al lactate to the rat, brain entry of ^{14}C-sucrose increased 2.5–3-fold, whereas the same dose of Al as $Al(OH)_3$ had no effect (Kim *et al.*, 1986). Similar results with this i.p. dose of $AlCl_3$ were reported, and are discussed in more detail in Informed Opinion (Yenumala & Melethil, 1993). A study of the time course of brain ^{14}C-sucrose entry after Al-lactate administration showed an increase at 2 and 4, but not 24 hours. Blood Al concentration was higher 24 hours after Al lactate than 2 hours later (480 versus 320 μg/l) and was higher than 2 hours after $AlCl_3$ (250 μg/l) or $Al(OH)_3$ (20 μg/l), which was not increased above saline treatment (Kim *et al.*, 1986). Another group, using the same procedures as Kim et al did not find that Al lactate increased the permeability of the BBB (Favarato *et al.*, 1992). However, Al maltolate and Al acetylacetonate, 2.2 mg/kg (0.8 mmoles/kg), which are hydrophilic and lipophilic stable Al complexes, respectively, did increase BBB permeability to sucrose 1.2 to 4.5-fold. Stefanovich and Joo gave rats 1 mg/kg (4.5 μmol/kg) of Al-gluconate i.v. once or i.p daily for 14 days (Stefanovich & Joo, 1990). To determine BBB changes they gave Evans blue dye 2 hr prior to termination of the rats. Evans blue binds to albumin. Brain Evans blue-albumin increased 2.2-fold in the single and 3.4-fold the repeatedly injected rats, suggesting some opening of the BBB.

The ability of Al to affect BBB permeability was addressed using cultures of monolayers of bovine brain microvascular endothelial cells (Audus *et al.*, 1988a). $AlCl_3$,

≥0.1 μM, increased fluorescein flux across the cells, revealing increased permeability of the monolayer. A higher Al concentration, 1 μM, *reduced* fluid phase endocytosis, suggesting the enhancement of brain uptake of substances in the presence of Al is not due to Al-enhanced pinocytosis. These results, along with the lack of Al alteration of lipid order in either the core of the cell membrane or near its surface, suggest Al interacts with the surface of these cell membranes (Audus *et al.*, 1988a). Al was seen on the luminal surface of some brain microvascular endothelial cells after Al administration to the rabbit (Wen & Wisniewski, 1985). No function was attributed to the presence of Al at this site. The ultrastructural appearance of the capillary endothelial cells and basement membranes of rabbits that received repeated subcutaneous Al injections but had not experienced prolonged convulsions prior to death was normal (De Boni *et al.*, 1976). One percent (75 mM) $AlCl_3$ did not disrupt a dodecyltrimethylammonium model membrane bilayer (Tracey & Boivin, 1983). Unilamellar vesicles primarily composed of phosphatidylserine were prepared as a model membrane having negatively charged phospholipid molecules (Deleers, 1985). Al was found to compete for Ca binding; 100 μM Al (as the chloride) inhibited Ca binding 50%. A similar concentration of Al produced vesicle fusion (intermixing of the lipids), release of a molecule that was trapped in the vesicles and rigidification of the membranes, suggesting Al perturbation of membrane structure (Deleers *et al.*, 1986).

To assess the ability of Al to alter BBB function in vivo, we synthesized a charged analog of antipyrine, 4-trimethylammonium antipyrine, 4-TMA-AP (Allen *et al.*, 1992). Although antipyrine rapidly crosses the BBB, little 4-TMA-AP crossed the BBB, as determined by microdialysis of brain ECF, except when manipulations were conducted that were expected to impair BBB integrity. 4-TMA-AP was used in studies in which Al-maltolate, four Al-3-hydropyridinones and Al-citrate were i.v. bolus dosed and then infused to produce steady state plasma Al concentrations of ~20 μM for Al-maltolate and up to ~1.5 mM for the other Al species (Allen *et al.*, 1994; Allen *et al.*, 1995; Ackley & Yokel, 1997; 1998). None of these Al administrations significantly increased recovery of 4-TMA-AP in brain dialysate, suggesting they did not increase BBB permeability. No significant increase in recovery of 4-TMA-AP from the brain was produced by several conditions that were studied to attempt to elucidate the mechanism of Al BBB permeation (Ackley & Yokel, 1997; 1998). However α-cyano-4-hydrocinnamate did increase 4-TMA-AP in brain dialysate (Ackley & Yokel, unpublished results) presumably revealing the ability of 4-TMA-AP to demonstrate increased BBB permeability in these experiments.

Banks and Kastin conducted a series of studies to investigate the possibility that Al affects the uptake into, or efflux from, the brain of a number of small peptides as well as non-peptides. To study the effects of Al on brain uptake in rats, 100 mg Al/kg was typically given as $AlCl_3$ i.p. 0.5–2 hours before intra-arterial (i.a.) administration of radiolabelled test substance. The subjects were decapitated 5 seconds after test substance injection. This very large dose of Al was given in 10 ml/kg of an acidic (pH 2.89), hypertonic (1.78 mOsm/l) solution. Banks and Kastin did not measure blood Al concentrations in any of their studies. Others reported a blood Al concentration of 300 μg/l (11μM) after 54 mg/kg (2 mmoles/kg) Al as $AlCl_3$ to mice (Leblondel & Allain, 1980) and 250 μg Al/l (9 μM) in rats using the same procedures as Banks and Kastin (Kim *et al.*, 1986). The time of peak Al effect to increase brain concentration of N-tyr-delta-sleep-inducing

peptide (N-tyr-DSIP) and β-endorphin was 2 hours after i.p. Al injection (Banks & Kastin, 1983). They reported that the effect was Al dose dependent (Banks & Kastin, 1983). Al did not have a significantly different effect among the 6 brain regions studied (Banks & Kastin, 1985b). The apparent brain uptake of some substances was affected by Al pretreatment to a greater extent than uptake of other substances. For example, Al increased the brain uptake of rat, but not human, luteinizing hormone (Banks & Kastin, 1985a). The authors suggested that Al increased the BBB permeability to N-tyr-DSIP and β-endorphin (Banks & Kastin, 1983) and several other substances (Banks & Kastin, 1985a) by potentiating the mechanism(s), which are unknown, for their brain entry. The authors did not believe the increased permeability was due to enhancement of pinocytosis or leakiness, BBB disruption, or an action on the test substances (Banks & Kastin, 1985a). This is supported by the lack of Al-induced increase of pinocytosis by brain microvascular endothelial cells *in vitro* (Audus *et al.*, 1988b) and lack of effect of Al on Aβ uptake, which is largely sequestered by the BBB (Banks *et al.*, 1996). Brain albumin uptake was not found 5 seconds after its i.a. injection, suggesting that Al did not increase permeability through the tight junctions between the brain capillary endothelial cells enough to permit diffusion of this fairly large molecule into the brain (Banks & Kastin, 1985a). However, (Kim *et al.*, 1986), using the identical procedure, found increased sucrose influx into the brain, measured 10 minutes after its i.v. injection, discussed above. Banks & Kastin (1989) found a good correlation between enhanced brain uptake and test substance lipophilicity, suggesting that Al enhanced brain uptake of many substances by effecting their transmembrane diffusion (Banks & Kastin, 1989). They also concluded that Al selectively affected the saturable transport of some substances across the BBB (Banks & Kastin, 1989). On the other hand, Al pretreatment slightly inhibited brain uptake of some substances, e.g., $A\beta_{1-28}$, suggesting to the authors that Al inhibited its diffusion through the BBB (Banks *et al.*, 1991). Jakovljevic *et al.* (1991) used a similar procedure to that employed by Banks and Kastin to study the influence of Al on brain uptake. They gave 100 μg Al/kg to mice 2 hours before s.c. injection of quinidine, a cation at physiological pH, and acetylsalicylic acid (aspirin), an anion at physiological pH. They terminated the mice 15–210 minutes later. The authors interpreted their results to suggest that Al significantly inhibited brain entry of quinidine and did not effect brain entry of aspirin. The i.p. dose of Al administered by Jakovljevic *et al.*, was 3 orders of magnitude less than generally administered by Banks and Kastin. However, calculation of BBr values from their data suggests quinidine readily entered the brain in the absence of Al, but Al reduced the BBr, suggesting Al either inhibited quinidine brain entry or accelerated brain efflux. The BBr values for aspirin were greater in the presence than the absence of Al, suggesting either Al facilitated aspirin brain entry or inhibited brain efflux. In the absence of steady state conditions, it is difficult to ascertain what effect of Al might have caused a change in the BBr. This study was not conducted well enough to draw any firm conclusions about the ability of Al to alter quinidine or aspirin, or cation or anion, brain entry.

Banks and Kastin also evaluated the ability of Al to influence permeation of substances across the BBB from brain to blood. For these studies they generally pretreated mice with 100 mg Al/kg (as $AlCl_3$) 1 hour before intracerebroventricular (i.c.v.) injection of the radiolabelled test substance, followed by decapitation 10 minutes later. The peak effect occurred 1 hour after Al injection. They reported an Al-concentration

dependent effect, from 1 μg Al/kg, which decreased brain efflux of a peptide (tyr-MIF-1; tyr-Pro-Leu-Gly-NH_2) by ~12%, to 100 mg Al/kg, which decreased efflux by ~80% (Banks *et al.*, 1988). They found that Al decreased the efflux of several peptides from the brain, interpreting the results as an indication that Al inhibits a peptide transport system as well as other transport systems (Durham *et al.*, 1991).

Some effects of Al on biochemical functions of the BBB have been reported. Thymidine uptake by growing, non-confluent, bovine brain microvascular endothelial cells was half-maximally stimulated by 0.3 μM $AlCl_3$ and 0.5 μM $Al_2(SO_4)_3$, suggesting Al-induced stimulation of DNA synthesis (Audus *et al.*, 1988b). Although they suggest that Al stimulation of DNA synthesis could result in changes in the synthesis of specific transporter proteins or enzymes, they provide no evidence that this occurs, or that it would be detrimental. Cerebrovascular Na/K-ATPase located at the BBB is critical for maintenance of the stable ionic milieu of the CNS. Incubation of cerebral microvessels with 100 μM $Al(NO)_3$ increased Na/K-ATPase activity 55% (Caspers *et al.*, 1990). Neither the mechanism nor the significance of this effect is known. It is not obvious that increasing Na/K-ATPase activity would be detrimental. Similarly, 100 μM Al increased muscarinic receptor density 32% (Grammas & Caspers, 1991). Finally, the same group found that 100 μM Al increased the activity of the small neutral amino acid transporter in cerebral microvessels 95% (Grammas *et al.*, 1992). Although this may render the BBB more responsive to cholinergic agonists it is not obvious that this would be detrimental to BBB function.

In a series of studies Vorbrodt and colleagues also investigated the ability of Al to alter biophysical and biochemical properties of the BBB. Using cultures of microvessels from goat brains, they found that 4 days of exposure to 50 μM Al maltol reduced by 26% the number and increased the distance between surface anionic sites. Based on the small reduction in the number of anionic sites, they note that Al maltol was not able to neutralize surface anionic sites of these cells. They also note that these sites would be easily replaced in vivo by ongoing cell metabolic activity (Vorbrodt & Trowbridge, 1993). Using the same preparation and Al maltol exposure they also found a reduction in the uniformity of distribution and increased scattering of sialic acid residues, which is consistent with their above findings. They found a reduction in Ca-ATPase, particularly in the intercellular clefts, which could compromise the tight junctions because they are Ca dependent (Vorbrodt *et al.*, 1994a). They then assessed the effect of consumption of Al lactate in the drinking water for 6 weeks or 6 months on multiple endpoints. These were brain entry of Evans blue and horseradish peroxidase, blood microvessel activity of alkaline phosphatase and Ca-ATPase, and distribution of anionic sites on the membrane surfaces of the brain microvessels (Vorbrodt *et al.*, 1994b). No changes were seen in any of these endpoints between mice consuming tap water and those consuming water containing 1.76% Al lactate. Intake averaged ~200 mg (7.5 mmole) Al/kg/day.

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If Al plays a role in the etiology or exacerbation of AD it is likely that Al either enters the brain in sufficient amounts to produce toxicity or it alters the function of the BBB. It

is unlikely that the neurotoxicity of Al is a secondary result of peripheral effects. If Al in the brain contributes to AD one might expect to see an elevation of brain Al in AD. Although higher brain Al has been reported in AD subjects than controls in some studies, there are a number of negative reports (reviewed in Yokel, 2000). It is not known whether brain Al is elevated in AD.

There are three routes of distribution that might enable sufficient Al to enter the brain to produce neurotoxicity; from the nasal cavity, through the choroid plexuses and through the BBB. Although it has been suggested that Al can enter the brain directly from the nasal cavity, there has been no quantitation of brain Al entry by this route. A study of brain cocaine uptake directly from the nasal cavity suggested that only a very small fraction was transported through the olfactory bulbs into the brain (Chow *et al.*, 1999). Diffusion through membranes is directly related to lipophilicity and inversely related to molecular weight. Based on the oil/water partition coefficients of ~0.005 for Al (Yokel & Kostenbauder, 1987) and 123 for cocaine (Carmichael & Isreal, 1973) and their molecular weights, diffusion through membranes would be predicted to be ~4 orders of magnitude slower for Al than cocaine. This suggests Al uptake into the brain directly from the nasal cavity would account for an extremely small fraction compared to that absorbed from the nasal cavity into systemic circulation. This assumes that there is not a transporter for Al at the olfactory nerve ending. The insolubility of most non-industrial airborne Al species (aluminosilicates) suggests this is not a significant route of human brain Al uptake. From the nasal cavity, Al might also enter the brain by diffusion into the perineural space surrounding the olfactory neuron. This space is continuous with the cerebrospinal fluid (CSF) compartment that surrounds the brain (Jackson *et al.*, 1979). This has not been assessed, but would likely produce the highest brain Al concentrations near the brain's surface, at least initially, rather than in neurons in deep structures that are affected in AD. Although it seems likely that very little Al would distribute from the nasal cavity to the brain, perhaps sufficient Al could enter the brain during the 50 or more years preceding the onset of AD. Two studies might clarify the potential role for Al absorption from the nasal cavity to contribute to brain Al. One is a confirmation that Al can distribute from the nasal cavity into the olfactory nerve and then across synapses into the brain. The second is an estimate of the percentage of Al present in the nasal cavity that enters the brain.

The most likely route for significant brain Al uptake is through the BBB. Although Al distribution across the choroid plexus into CSF and ultimately into brain parenchyma could contribute to brain Al over decades, the much greater exchange between blood and the brain across the BBB makes this route a more likely candidate. The mechanism of blood-to-brain transfer of small molecular weight Al species, such as Al-citrate, across the BBB has not been identified. There is utility in understanding the mechanism of Al movement across the BBB. If Al permeates the BBB by diffusion the route is most likely *between* the brain microvascular endothelial cells that comprise the BBB, e.g. the paracellular pathway. Presumably anything that increases the permeability of the BBB, evidenced by increased brain entry of marker substances that diffuse across the BBB, would allow greater brain Al entry. However there is little good evidence for increased BBB permeability in AD.

If Al crosses the BBB by a transporter mediated process, changes in transporter activity due to genetic influences, disease states or the concentration of competitive

substrates or transporter modifiers could influence Al movement across the BBB, and brain Al exposure. There is only one published study addressing the possibility of genetic influence on brain Al uptake. They found greater brain Al in only 2 of 5 mouse strains fed Al in their diet when compared to a diet essentially free of Al (Fosmire *et al.*, 1993). The authors suggest that their findings indicate genetic differences in the permeability of the BBB to Al. However, there are other mechanisms that might explain the results, such as differences in Al absorption, the Al species in blood, e.g., the percentage of Al bound to transferrin, and Al retention within the brain.

There is evidence that both TfR-ME and one or more transporter(s) mediate brain Al uptake. The relative importance of these mechanisms has not been addressed. It may be that under conditions when the metal binding sites of transferrin are not saturated, and therefore plasma Al primarily associates with transferrin, that TfR-ME mediates brain Al influx. Because Al cannot be reduced, it has been suggested that TfR-ME would not deliver Al to tissue (Williams 1999) which would rule out this mechanism of Al transport across membranes. The ability of TfR-ME to transport Al into the brain has not been conclusively demonstrated.

Some differences in transferrin have been reported in those with AD compared to controls. There is an increased incidence of a transferrin variant (transferrin C2) in AD (Namekata *et al.*, 1997; van Rensburg *et al.*, 2000) and lower serum and brain transferrin concentrations in those with AD (Fischer *et al.*, 1997). It has been suggested that the C2 variant may increase Fe-, and perhaps Al-induced oxidative injury. However, there does not appear to be a significant difference of Al or Fe binding between C2 and the more common C1 variant (Van Landeghem *et al.*, 1998). Although transferrin is clearly important in the distribution of Al, perhaps also facilitating its entry into the brain, it is open to speculation whether the higher incidence of transferrin C2 in AD might alter Al distribution and thereby influence AD.

There appears to be a mechanism of Al distribution across the BBB other than TfR-ME. The identity of the transporter(s) mediating BBB permeation has not been identified. It would be valuable to identify brain influx and efflux transporters for Al to predict factors, such as metabolic states, drugs and inherited conditions, which might favor uptake of brain Al or disfavor brain Al efflux.

We had suggested that the MCT might mediate efflux of Al from the brain across the BBB. To further assess the role of the MCT as a mediator of Al-citrate transport, studies were conducted with erythrocytes and a murine brain endothelial-derived cell line in the laboratory of Andrew Halestrap, University of Bristol, England. Rat erythrocytes were utilized as a classical preparation in which to study lactate transport, which is mediated by the MCT. Uptake of ^{14}C-lactate was not inhibited by 5- or 10-fold excess Al-citrate. There was no evidence of Al-^{14}C-citrate uptake into erythrocytes. The rat erythrocyte expresses both the MCT (isoform MCT1) and the band 3 anion exchanger (Poole & Halestrap, 1993). These results suggest that Al-citrate does not serve as an effective substrate for either of these transporters (Yokel & Halestrap, unpublished results). These studies were conducted with low mM Al-citrate concentrations, as obtained in plasma in the microdialysis experiments that demonstrated rapid brain Al uptake, discussed above. It appears that neither MCT1 nor the band 3 anion exchanger mediated this brain Al uptake.

To identify the characteristics, and possibly the identity, of the transporter(s) mediating Al-citrate distribution across the BBB, studies were conducted using an immortalized cell line (Yokel & Halestrap, unpublished results). We used b.End5 cells, that were established from brain endothelial cells of Balb/c mice. The uptake of Al-^{14}C-citrate compared to ^{14}C-citrate was determined in cells grown to 50–100% confluence. Uptake of Al-citrate in 1 hour was ~70% greater than citrate, suggesting different mechanisms of uptake. Intracellular Al-citrate achieved ~25% of the medium Al-citrate concentration within 1 hour, whereas diffusion would predict the intracellular Al-citrate concentration would be 1% of the medium at this time, suggesting transporter-mediated uptake. The Al-citrate transporter(s) was not sodium or pH dependent. Decreased Al-citrate uptake in the presence of sodium azide, 2,4-dinitrophenol and rotenone suggested some energy dependence. Ouabain did not inhibit uptake, suggesting Na/K-ATPase was not mediating Al-citrate transport. Uptake was not inhibited by α-ketoglutarate or malonate, suggesting the transporter was not a dicarboxylic acid transporter or α-ketoglutarate exchanger. Uptake was inhibited by numerous compounds, most of which are MCT substrates or inhibitors. Western blot analysis showed expression of MCT1, but not MCT2 or MCT4, by the bEnd.5 cells, consistent with their expression (MCT1) or lack of expression (MCT2 and MCT4) at the rat BBB. However, as results obtained with erythrocytes suggest MCT1 does not mediate Al-citrate uptake, transport appears to be mediated by one or more other transporters. Considering the properties of the transporters that have been identified at the BBB (Table 2), the organic anion transporting polypeptide (oatp) family is a candidate. The inhibition of Al-citrate uptake by 4,4′-diiodothiocyanostilbene-2,2′-disulfonic acid (DIDS) and probenecid, which inhibit organic anion transporters, is consistent with this possibility. However, as DIDS and probenecid inhibit many transporters, these results are not definitive. Sulfobromophthalein and fluorescein, which have been reported to be organic anion transporter inhibitors (Fricker *et al.*, 1999; Ishizuka *et al.*, 1998), concentration-dependently inhibited Al-citrate uptake by b.End5 cells. These results suggest that an organic anion transporter may play a role in Al-citrate distribution across the BBB. The ability of Al-citrate to inhibit uptake of an organic anion transported substrate, PAH, was assessed in oocytes expressing rOAT3. Five mM Al-citrate moderately inhibited PAH uptake, suggesting Al-citrate is a weak inhibitor of PAH transport, and therefore possibly a substrate of an organic anion transporter (B. Feng, K.M. Giacomini & Yokel, unpublished observations). Therefore, a candidate transporter for brain Al influx of small species, presumably Al-citrate, and brain efflux, most likely Al-citrate, is an organic anion transporter, perhaps a member of the oatp family. Further work is necessary to confirm or reject this hypothesis.

Although only a small percentage of circulating Al enters the brain (Fig. 1), the $t_{1/2}$ of ~150 days observed in the rat brain is of concern. This prolonged residence of Al may be consistent with the suggestion that a toxic brain Al concentration should be reached in the human after 100–150 years if no brain elimination occurs (Ganrot, 1986). However, there are two unknowns that impact on this. The human lifespan is about 30 times that of the rat. It is unknown if the translation of this 150 day $t_{1/2}$ to the human should be scaled to our lifespan, resulting in a brain Al half-life of ~12 years. Brain Al concentration resulting from continuous Al intake can be predicted from the amount of Al that enters the brain and the half-life of Al in the brain. For this prediction it was assumed that 0.3%

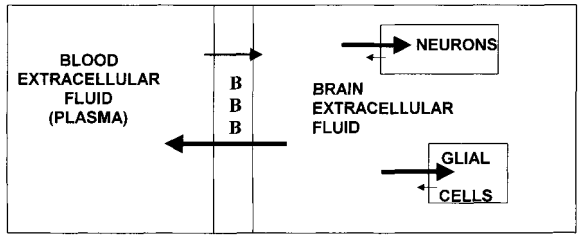

Fig. 2. Compartments of Al distribution between blood extracellular fluid, brain extracellular fluid and brain cells (neurons and glial cells). Distribution of Al across membranes, including the blood–brain barrier (BBB), is shown by arrows. Arrow size indicates the estimated rate of distribution.

of the Al consumed in drinking water was absorbed (Yokel *et al.*, 2001) and 0.03% of the ~5–10 mg Al consumed daily in food (Yokel & McNamara, 2001) was absorbed. Entry of 5×10^{-3} % of the absorbed Al into the brain and a 12 year brain Al half-life were used. The calculation suggests brain Al in the average 60 year-old human should be similar to that reported (Markesbery *et al.*, 1984). Therefore the concentration of brain Al would be primarily a product of dietary Al intake. The second unknown is the location of the Al that is so slowly cleared from the brain. This has been suggested to be cytoplasmic (Reusche *et al.*, 1996) or cytoplasmic and nuclear (King *et al.*, 1994). It is unknown if the Al at this site can cause toxicity, or if it is sequestered in a non-toxic form.

The transport of Al into and out of the brain in light of its persistence in the brain suggest the following hypothesis. Some of the Al released into brain ECF after transport across the BBB is effluxed quite rapidly back into blood. Failing to be transported out of the brain, the Al is taken up by brain cells (neurons and glial cells), in which it persists for a very long time. Fig. 2 illustrates the compartments and distribution of Al involved.

The second site where Al might contribute to AD is the BBB. It appears that there are structural and biochemical, including transporter, abnormalities in AD cerebral microvessels, but no significant compromise of the tight junctions of the BBB. It is unlikely that Al plays a role in the etiology of the structural changes of AD, as no structural changes were seen in Al-treated rabbits (De Boni *et al.*, 1976). The possibility that Al alters transporters has not been rigorously evaluated. Of particular interest is its ability to affect GLUT-1, which has been reported to be decreased in the BBB of Alzheimer disease victims. There is very little data that directly relates to the possibility that Al might adversely effect the biochemical function of the BBB. Increased Aβ appears to accompany AD. There are reports suggesting Al can increase Aβ by a number of mechanisms (reviewed in Yokel, 2000). The ability of Al and Aβ to interact at the BBB to increase damage, perhaps mediated by oxidative injury, that might contribute to AD has not been directly addressed. Another change in the BBB in AD is an apparent reduction of hexokinase activity. A high Al concentration (100 μM; 2700 μg/l) inhibited hexokinase by 66% in brain homogenates (Harrison *et al.*, 1972). Effects of Al on cerebral microvessel Na/K-ATPase, muscarinic receptor density and the small neutral amino acid transporter were reported after exposure to the same Al concentration (Caspers *et al.*, 1990; Grammas & Caspers, 1991; Grammas *et al.*, 1992). This concentration far exceeds that achieved in human blood, and presumably within cerebral microvessel cells, as follows.

Normal plasma Al concentration is generally considered to be <10 μg/l (0.4 μM). Serum Al was found to be slightly elevated in patients with probable Alzheimer's disease when compared to age-matched controls (~13 μg/l vs. 8 μg/l [0.5 versus 0.3 μM]; Zapatero et al., 1995). The studies that have been conducted to assess the ability of Al to alter BBB function have generally used Al concentrations is great excess of these human serum Al concentrations.

Some studies have reported an increase in BBB permeability following Al exposure. For example, the 1 mg/kg (4.5 μmol/kg) i.v. injection of Al-gluconate given by Stefanovich & Joo (1990) increased BBB permeability a few fold (discussed above). If the injected Al was originally confined to blood plasma, its concentration would be ~3600 μg/l (135 μM). Therefore a blood Al concentration >100-fold greater than typically seen in the human would increase BBB permeability only about 2–3-fold. Comparably increased BBB permeability was reported after i.p. $AlCl_3$ injection that produced a blood Al concentration of ~250–300 μg/l (9–11 μM) (Kim *et al.*, 1986). However, if increased BBB permeability correlates with serum Al concentration, very little increased BBB permeability would be anticipated at the lower serum Al concentrations typically seen in humans. The IC_{50} of Al to inhibit Ca binding to phosphatidylserine vesicles (Deleers, 1985) was 100 μM, ~200-fold greater than serum Al concentration of probable Alzheimer's disease sufferers. This suggests it is very unlikely sufficient plasma Al concentrations would be achieved in the general population to produce the effects observed by Deleers (Deleers, 1985; Deleers *et al.*, 1986). Evaluation of studies assessing increased BBB permeability should consider the magnitude of increased permeability (opening) of the tight junctions that would be necessary to permit passage of BBB permeability markers into the brain. Presumably a greater compromise of the tight junctions between brain microvascular endothelial cells is required to permit brain entry of larger molecules. Therefore a greater insult can be implied if albumin (or the Evans blue-albumin complex; molecular weight = 77,000 Da) or horseradish peroxidase (44,000 Da) enter the brain than meglumine iothalamate, ^{68}Ga-EDTA, sucrose, fluorescein, 4-TMA-AP or ^{82}Rb, which have molecular weights of 809, 360, 342, 334, 246 and 82 Da, respectively. The smaller molecules enable a more rigorous assessment of BBB permeability. The reports of no increased BBB permeability to meglumine iothalamate, ^{68}Ga-EDTA, or ^{82}Rb in AD patients or 4-TMA-AP in rats in the presence of high concentrations (200–1200 μM) of Al-citrate suggest there is no great alteration of BBB permeability in AD or resulting from exposure to one of the two predominant Al species in blood.

Banks and Kastin conducted a large number of studies to assess the ability of Al to alter the distribution of substances into or out of the brain. There appear to be shortcomings in the procedures they used. They conducted no studies to determine if the test substances they interpret to have entered the brain actually passed through the brain capillary endothelial cells, or if they simply adhered to the luminal surface of these cells or were sequestered within these cells. Blood was not washed out of the brain after the i.a. injection nor were other procedures conducted to determine if apparent brain uptake is due to significant adsorption of test substances onto the luminal wall of the blood vessel. The test substance within the blood vessels of the brain was not subtracted from that reported to be in the brain. The capillary depletion method should indicate if test

substance was significantly sequestered by the microvascular cells (Triguero *et al.*, 1990). Finally, they provided no evidence that the radioactivity in the brain was still associated with the test substance. It is difficult to conclude if Al administration did increase the exposure of brain cells to the test substances that are reported by Banks and Kastin to be present in the brain in higher concentrations in the presence of Al.

It is difficult to understand how the effect of 1 μg (0.04 μmoles) Al/kg as $AlCl_3$ in physiological saline could be due to the Al as suggested (Banks *et al.*, 1988) if blood Al after i.p. $AlCl_3$ directly correlates with the Al dose. This dose is ~5 orders of magnitude smaller than the 54 and 100 mg Al/kg (2–3.7 mmole Al/kg) doses which produced blood Al concentrations of ~250–300 μg/L (9–11 μM). This suggests 1 μg Al/kg i.p. would increase blood Al 0.003 μg/L (0.0001 μM). Another explanation was provided from the results of a brief study (Yenumala & Melethil, 1993). Rats were given 1 mg Al/kg i.v. or 100 mg Al/kg i.p., as $AlCl_3$. Blood Al concentrations were determined up to 170 minutes later. ^{14}C-sucrose was given 120 minutes after the Al and its distribution into the brain determined. Although blood Al concentrations were ~10-fold higher after i.v. than i.p. Al, the K_{in} of sucrose into the brain increased 2–3-fold after i.p., but only 20–70% after i.v. Al. The authors comment that Al-induced changes in the BBB are not directly related to blood Al concentrations. They noted adverse changes in the liver after i.p. Al administration (Sri Melethil, personal communication). Perhaps the changes in BBB permeability after i.p. $AlCl_3$ administration are the result of disruption of the liver rather than Al.

Administration of 1 mg/kg (0.037 mmole/kg), Al as $AlCl_3$, i.v., reduced the brain efflux of tyr-MIF-1 given i.c.v. 15 minutes later by ~50% (Banks *et al.*, 1988). Assuming the Al was initially confined to blood plasma after the i.v. injection and was then cleared with a $t_{1/2}$ of 1 hour, plasma Al concentration should have been ~15,000 μg Al/l (550 μM). At physiological pH, the Al from the $AlCl_3$ would be expected to rapidly become Al hydroxides. It is unclear what Al species or their concentrations may have been present in the blood after the i.p. and i.v. $AlCl_3$ injections administered by Banks and Kastin and others. With all of these uncertainties it is difficult to translate the findings of Banks and Kastin to the human.

The daily oral consumption of 188–214 mg (7–8 mmole) Al/kg/day from the drinking water by mice (Vorbrodt *et al.*, 1994b), exceeded daily water and food Al intake by the human by ~135,000- and 2000-fold. Al lactate is very soluble. The Al should have remained in solution in the drinking water. If consumption of this amount of Al for ~25% of the mouse's life span had no effect on the BBB it is difficult to believe the human BBB would be influenced by daily intake of 0.001–0.05% as much Al.

Therefore, it appears there is little convincing evidence that Al alters the BBB at Al concentrations that might be present in the blood of the typical human or one who develops AD.

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Aluminium and Alzheimer's Disease
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CHAPTER 13

Aluminium Toxicity in Erythropoiesis. Mechanisms Related to Cellular Dysfunction in Alzheimer's Disease

Alcira Nesse * and Graciela Garbossa

Departamento de Química Biológica, Facultad de Ciencias Exactas y Naturales, Universidad de Buenos Aires, Ciudad Universitaria, (1428) Ciudad Autónoma de Buenos Aires, Argentina

* Corresponding author, email: anesse@qb.fcen.uba.ar

Abbreviations: AD – Alzheimer's disease; Al – aluminium; $Al[acac]_3$ – aluminium acetylacetonate; ATP – adenosine triphosphate; BFU-E – burst-forming units-erythroid; CFU-E – colony-forming units-erythroid; Fe – iron; Hb – haemoglobin; IRE – iron responsive element; IRP-1 – iron regulatory protein-1; IRP-2 – iron regulatory protein-2; mRNA – messenger ribonucleic acid; RBC – red blood cell; Tf – transferrin; TfR – transferrin receptor

Summary

The mechanisms that could account for the aluminium-induced damage on erythropoiesis are thoroughly discussed. The reported mechanisms support that both the disturbance of erythroid progenitor functions and the alterations of mature erythrocyte morphology are consequences of the presence of Al within the organism. Since much evidence is presented concerning similar anomalies in aluminium loading and Alzheimer's Disease, the final hypothesis might be that they share common mechanisms that produce cellular toxicity. Considering Alzheimer's Disease as a systemic dysfunction instead of an organ specific one, the haematological research could greatly contribute to the understanding of the pathophysiology of the disease.

Historical Perspective

Aluminium as a Toxin for the Erythropoietic System

Erythropoiesis is a multistep process that drives committed erythroid progenitors derived from pluripotent stem cells through progressive events of proliferation and differentiation, and results in the development of mature erythrocytes. The lineage committed cells, burst-forming units-erythroid (BFU-E) and colony-forming units-erythroid (CFU-E), support erythropoiesis under the influence of erythropoietin. This hormone triggers the differentiation to proerythroblasts, the earliest recognisable red cell precursors in

the bone marrow. Throughout their maturation process these cells synthesise haemoglobin (Hb). This pathway needs a co-ordinated production of haem molecules and globin chains. Haem synthesis in erythroblasts is regulated by a complex mechanism closely linked to iron (Fe) availability. Likewise, globin chain expression is dependent upon the availability of previously synthesised haem.

Aluminium (Al) loading — as a contributing factor to aggravate anaemia of end-stage renal disease — was first noticed in the Elliot and McDougall research (1978), where the illness was described as a severe microcytic anaemia in patients suffering from dialysis encephalopathy. This clinical association between Al and anaemia was confirmed by the results of other investigations (O'Hare & Murnaghan, 1982; Wills & Savory, 1983; Touam et al., 1983; Eschbach & Adamson, 1985; Abreo et al., 1989). In spite of some anaemia abatement as a consequence of the introduction of deionised-water in dialysis treatment, Al-related toxicity is still reported in haemodialysed patients (Di Paolo et al., 1997; Montenegro et al., 1998). Modifications of haematological parameters in renal patients provided indirect evidence to ascertain that Al is the aetiological agent of anaemia. However, this connection was eventually confirmed by experimental studies. Al-treated uraemic rats exhibited severe anaemia (Kaiser et al., 1984; Drüeke et al., 1986) and impairment of CFU-E growth (Nesse et al., 1997). The normocytic anaemia, characteristic of the uraemic clinical state, turned into microcytic anaemia even though serum Fe concentration remained unchanged (Drüeke et al., 1986).

Considering the multiple factors affecting erythropoiesis in chronic renal disease, the use of experimental animals without renal disorders better established the linkage between Al and the development of anaemia. Regardless of the Al compound and the administration route employed in different experimental models, anaemia was induced in rabbits, mice and rats (Seibert & Wells, 1929; Kaiser et al., 1984; Drüeke et al., 1986; Garbossa et al., 1996; Garbossa et al., 1998a; Vittori et al., 1999).

After chronic oral Al administration, the *in vitro* culture of bone marrow cells showed a decrease in the CFU-E response to erythropoietin (Garbossa et al., 1996; Nesse et al., 1997; Garbossa et al., 1998a; Vittori et al., 1999). The inhibition of CFU-E development could be the earliest clue of Al-induced erythropoiesis impairment since it became evident after short treatments, and it appeared even before the detection of peripheral signs of anaemia (Garbossa et al., 1996).

Though an inhibitory effect over either the synthesis or the secretion of endogenous erythropoietin could be discarded (Garbossa et al., 1996), the possibility of a functional disruption of erythropoietin interaction with its specific surface receptor could not be ruled out (Mladenovic, 1988; Garbossa et al., 1994).

Bone Al deposits found in Al-overloaded patients and rats (Kaye, 1983; Drüeke et al., 1986; Watrin & Galle, 1986; Berry, 1996; Garbossa et al., 1998b) could be taken as an argument to suggest that the presence of the metal for long periods in the bone matrix is responsible for a local cytotoxic injury of CFU-E growth. Accordingly, a decrease in the number of erythroblasts at all maturation stages was observed in rats implanted with Al wires inside their bone marrow (Zaman et al., 1990).

Contrary to general thought, the above-mentioned studies suggest that not only haemodialysed patients but also other patients without renal failure and even healthy subjects are potential targets of Al toxicity. Regarding this topic, the widespread use of

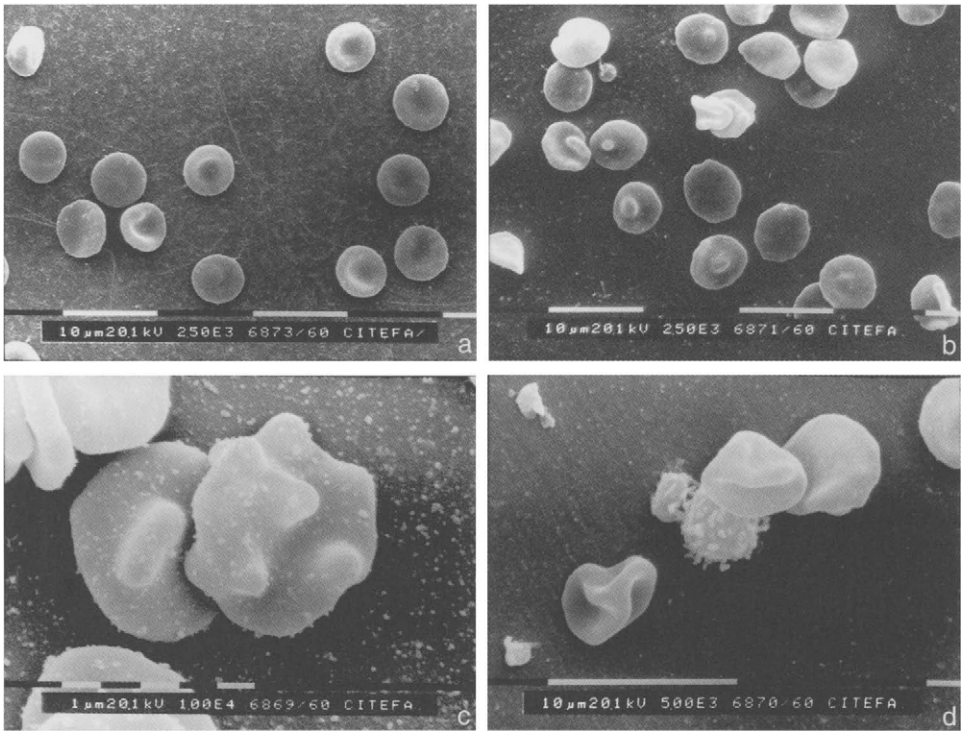

Fig. 1. Scanning electron microscopy of erythrocytes from control (a) and Al-overloaded rats (b–d): (a) normal biconcave erythrocytes (2500×); (b) target cells and leptocytes (2500×); (c) acanthocytes (10,000×); (d) severe loss of the biconcave shapes (5000×).

Al in food, nutritive solutions, cosmetics, pharmaceutical and packaging industries, as well as accidental exposures to the metal, constitute a risk of Al accumulation for human beings.

Another important issue should be considered in reference to Al toxicity on erythropoiesis. The deleterious effect of Al could be restricted not just to the erythroid progenitors. Mature erythrocytes might also be affected.

Al induces changes in the red blood cell (RBC) morphology as it has been established in Al-overloaded rabbits by the presence of anisocytosis (abnormal cell size variations) and poikilocytosis (abnormal cell shape variations) in blood smears (Seibert & Wells, 1929). Similar results were found in rats chronically treated with Al citrate (Fig. 1) (Vittori et al., 1999). The features of the blood films were anisochromia (uneven cellular Hb staining), anisocytosis and poikilocytosis. Moreover, electron microscopy analysis of the RBCs revealed the loss of their typical biconcave shapes, with the appearance of cells with different characteristics, such as thin and plain (leptocytes), spiculated (acanthocytes, echinocytes or crenated cells), displaying just one cavity (stomatocytes) and target cells, strongly suggesting membrane alterations (Vittori et al., 1999).

In vitro studies demonstrated that the hydrolytically stable, highly lipophilic Al acetylacetonate ($Al[acac]_3$) compound also caused a marked membrane destabilisation of

rabbit RBCs, both in terms of increasing osmotic fragility and originating acanthocytes (Zatta et al., 1989).

Not much information is available regarding the effects of Al on human erythrocytes. Short-term *in vitro* assays showed that $Al[acac]_3$ interacted with human erythrocyte membrane, resulting in alteration of its morphology from discoid to both echinocytic and stomatocytic forms (Suwalsky et al., 1999). Likewise, similar alterations were found after *in vitro* aging of human RBC in the presence of other Al compounds (Fig. 2). The incubation of erythrocytes with Al during 14 days at 4°C was effective to induce an increase of the acanthocyte percentage with respect to control erythrocytes aged in the absence of Al (40 versus 8%) (unpublished data). As can be seen in Fig. 2, the stomatocytic shape is the striking characteristic of the erythrocytes treated with Al.

All these atypical cellular morphologies are consistent with their impaired resistance to lysis in hypotonic salt solutions because of the inability of cells to maintain the surface area stable and to control their volumes properly. An increased osmotic resistance seemed to be another feature of the Al-induced anaemia (Seibert & Wells, 1929; Touam et al., 1983; Drüeke et al., 1986; Garbossa et al., 1996; Garbossa et al., 1998a; Vittori et al., 1999) and, together with a mild reticulocytosis (Touam et al., 1983; Vittori et al., 1999) and shortened RBC life span (Garbossa et al., 1998a), suggested that an intensified RBC destruction might be involved as well.

What mechanisms could account for the Al-induced damage of the erythropoietic system?

In order to elucidate the above-mentioned findings we propose two main hypotheses as mechanisms for Al toxicity in the erythropoietic system:

(i) Interference of Al in the uptake and utilisation of Fe
(ii) Interaction of Al with cellular membrane components, affecting not only their structures but also their functions.

Interference of Al in cellular Fe uptake

Al alters erythropoietic cell maturation through a mechanism that depends upon the availability of free binding sites in the transferrin (Tf) molecule, the natural Fe carrier, which is also the main vehicle for Al delivery to the cell (Trapp, 1983; Martin, 1986; Fatemi et al., 1991).

To overcome the *in vitro* CFU-E inhibition due to Al, the requirement is that Tf has to be 100% Fe-saturated (Mladenovic, 1988; Garbossa et al., 1994). Therefore, because of the fact that the normal Fe saturation level of human Tf is between 25 and 45%, even low concentrations of Al in the blood stream would be able to bind to Tf and as a consequence, hazardously affect different tissues.

The question arising is whether the Al–Tf complex could bind to the specific transferrin receptor (TfR) and interfere with the Fe–Tf binding to the same receptor. From interaction assays it became apparent that Fe–Tf and Al–Tf were indistinguishable to the membrane TfR of human reticulocytes (Cochran et al., 1991). The receptor–ligand binding must be interpreted as a dynamic and reversible process. When the equilibrium is achieved, the number of ligand molecules that detach from the receptor equals the number

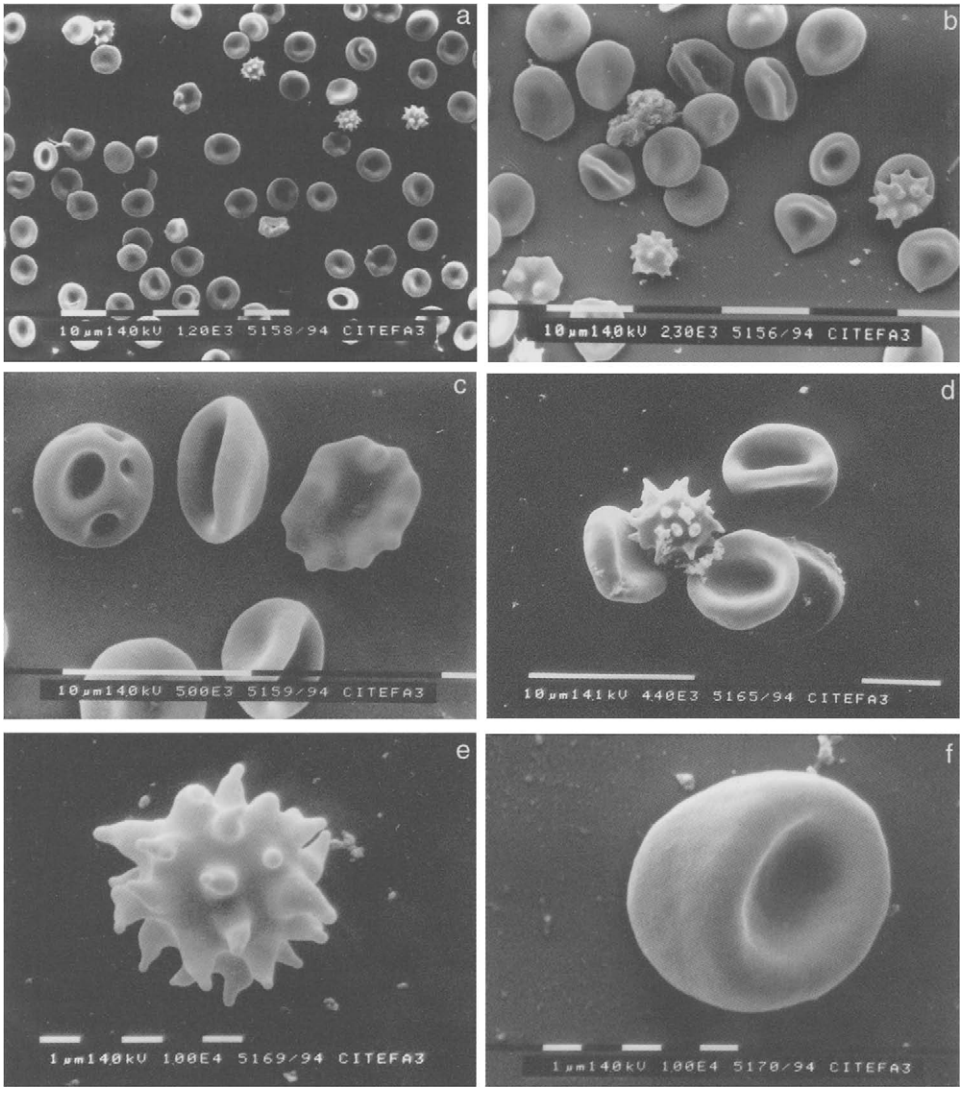

Fig. 2. Scanning electron microscopy of human erythrocytes aged *in vitro* in the absence (a) or presence of Al (b–f): (a) control aged erythrocytes (1200×); (b) leptocytes, acanthocytes and stomatocytes (2300×); (c) details of the loss of the biconcave shapes: cell with membrane invaginations, stomatocyte and crenated cell (5000×); (d) stomatocytes and acanthocyte (4400×); (e) acanthocyte (10,000×); (f) stomatocyte (10,000×).

of molecules bound to the receptor. Whenever a true competition is attained between two ligands, the receptor cannot distinguish between them. This is the situation that probably occurs with the cell surface TfR. The binding of the radioactive tracer ^{125}I–Tf–Fe to the TfR diminished when the competition was established with the physiological ligand Fe–Tf, either as a function of time (Pérez et al., 1999) or as a function of ligand concentration (McGregor et al., 1990). What is surprising is that Al–Tf mimicked the

Fe–Tf binding (Pérez et al., 1999). The Al–Tf complex could not only attach to the TfR but also compete with Fe–Tf for the binding. In fact, calculated values of TfR dissociation constants, $Kd_{Fe\text{-}Tf} = 1.75 \times 10^{-9}$ M and $Kd_{Al\text{-}Tf} = 1.37 \times 10^{-9}$ M, are quite similar (Pérez et al., 1999), indicating that the TfR has the same strong affinity for both complexes. The decline in cellular Fe uptake observed in K562 cells cultured in the presence of Al, recovered control values when Al was taken away from the culture medium (Pérez et al., 2000), thus providing further evidence for a competitive mechanism of binding to the TfR.

It can be argued that binding does not necessarily mean entry. However, an intracellular Al increase was detected in Friend's cells and rabbit reticulocytes (Abreo et al., 1990), and in K562 cells (Unpublished data) incubated with Al–Tf, suggesting that the presence of the complex is essential to gain access to the cell.

The initial competition between Al–Tf and Fe–Tf at the membrane level resulted in an increase in the TfR number (Pérez et al., 1999). This result might account for the higher cellular Fe uptake observed after long term incubation of K562 cells induced to differentiate (Pérez et al., 2000). Nevertheless, the fact that this adaptation allows the cells to acquire the necessary Fe does not assure a normal Fe metabolism.

Disturbance of Fe regulatory intracellular mechanisms

The pathophysiological effects produced by intracellular Al are measured by *in vitro* assays in terms of inhibition of the Hb production. When the erythroleukaemic Friend's virus-infected (Abreo et al., 1990) and K562 (Pérez et al., 1999) cell lines were cultured in an Al containing medium, the Hb synthesis was inhibited.

The finding of significant Fe deposits in bone marrow cells of animals chronically overloaded with Al, concurrently with the depression of CFU-E development as well as the impairment of Hb synthesis (Vittori et al., 1999), supports a mechanism of Al interference with Fe utilisation.

An enlargement of the intracellular Fe pool normally leads to an increase in ferritin synthesis and a decrease in TfR expression, while depletion of the Fe content promotes the opposite biochemical events. Cellular Fe uptake and storage are co-ordinately regulated at post-transcriptional level by the cytoplasmic iron-regulatory proteins 1 and 2 (IRP-1, IRP-2). Under conditions of limited Fe-supply the IRP binding to iron-responsive elements (IREs) blocks ferritin-mRNA translation and protects the TfR-mRNA from further degradation. Unlike IRP-1, which is a stable protein, IRP-2 is immediately degraded as a consequence of an oxidative modification generated by the expansion of the Fe pool, thus resulting in an efficient translation of ferritin-mRNA and rapid catabolism of TfR-mRNA.

Al behaved as an antagonist of the Fe-induced IRE-IRP-2 binding activity and, simultaneously, inhibited the Fe-induced IRP-2 oxidation (Yamanaka et al., 1999). The affinity of Al for the IRP-2 iron binding site(s) must be high enough to establish a real competition with Fe. This mechanism could explain why neither the increase of Fe uptake nor the enlargement of total Fe content induced by Al were sufficient to suppress further Fe cellular incorporation (Abreo et al., 1990; Abreo et al., 1999; Pérez et al., 2000).

Disturbance of membrane structure

Certain RBC membrane disorders involve unusual interactions among membrane components, as well as defects in lipid composition and in the integral and skeletal protein structures. These disorders result in the development of abnormal shapes and altered geometry of RBCs (Mohandas & Morrow, 2000). The RBC shape is determined by the ratio of the surface areas of the two hemileaflets of the lipid bilayer. Shape changes induced in erythrocytes by foreign molecules are due to differential expansion of their two monolayers. Thus, spiculated shapes (echinocytes or acanthocytes) arise when the added compound accumulates in the outer monolayer, whereas cup shapes (stomatocytes) are observed when the compound is inserted in the inner monolayer. The fact that Al induced both acanthocytes and stomatocytes (Suwalsky et al., 1999; Vittori et al., 2000) probably indicates that Al interacts with both the outer and inner moieties of the erythrocyte membrane.

In the RBC membrane the bilayer of amphiphilic lipid molecules is anchored to a network of skeletal proteins through transmembrane proteins. Band 3, the major integral membrane protein, plays an important role in carbon dioxide transport between tissues and the lungs. Apart from its involvement in anion exchange, band 3 forms part of the cytoskeletal complex which controls the mechanical properties of the RBC (Tanner, 1993). This protein interacts with the erythrocyte membrane skeleton that mainly includes spectrin, actin, ankyrin and protein 4.1. The organisation of this protein network of the inner surface in the plasma membrane is responsible for maintaining the shape, stability and deformability of the erythrocyte. An increased membrane protein breakdown induced by Al was found restricted to band 3. After aging human erythrocytes in an Al-rich medium, the immunoblot analysis showed an altered membrane protein pattern which was compatible with band 3 degradation. At the same time, scanning electron microscopy revealed the presence of acanthocytes and stomatocytes (Fig. 2) (unpublished data).

Alterations of the membrane skeleton gave rise to anomalous erythrocyte shapes and, in many cases, haemolytic anaemia (Gallagher & Forget, 1993). Moreover, several band 3 variants have been described associated to acanthocytosis (Kay et al., 1989).

Al and oxidative processes

More evidence has come forward concerning the disruption of the membrane lipid bilayer. Al(III) cannot participate in redox reactions that form free radicals. However, it can produce oxidative injury by facilitating the Fe(II)-mediated oxidation of biological membranes and liposomes (Gutteridge et al., 1985; Xie & Yokel, 1996; Verstraeten et al., 1998). The first step could involve the binding of Al(III) ion to cellular membrane components, as was documented by the detection of measurable quantities of Al in the membrane fraction of human erythrocytes incubated with Al lactate (Panchalingam et al., 1991). Furthermore, Al was found associated to both the inner and the outer monolayers of the human erythrocyte membranes (Suwalsky et al., 1999). In view of its relatively high charge to mass ratio an interesting hypothesis has been proposed: Al(III) may bind to adjacent phosphates of the membrane phospholipid headgroups, and the remaining positive charges of the Al ion could repel each other, inducing the formation of membrane

gaps and exposing fatty acid tails to the Fe(II)-induced free radicals (van Rensburg et al., 1995). The consequent membrane re-arrangement could cause membrane rigidity to increase and fatty acid chain to package (Deleers et al., 1986, Weis & Haug, 1989, Oteiza, 1994). Finally, membrane instability may be responsible for the impairment of biophysical and physiological properties of RBC membranes exposed to Al ion (Xie & Yokel, 1996).

An alternative hypothesis to explain the Fe-mediated lipoperoxidation mechanism is that Al may alter the cellular metabolic activity, producing oxidative stress. A potential target organelle could be the mitochondria. Stable complexes assembled between Al and phosphates or other oxygen donating ligands, could therefore disrupt mitochondrial enzyme activity affecting the electron transport chain. The final effect could be the increment of reactive oxygen species, which can in turn lead to oxidative injury (Campbell et al., 1999).

Al and Fe co-trapping in endocytic vesicles

The mechanism involved in Al facilitation of Fe-mediated lipoperoxidation depends upon phospholipid composition, pH, and Al and Fe concentrations. Acidic conditions inside endocytic vesicles appear to be the appropriate environment to encourage lipoperoxidation, since the peroxidation process was greater at pH 5.5 than at 7.4 (Xie & Yokel, 1996). Furthermore, Al and Fe were found co-trapped in acidic intracellular organelles (Bommer et al., 1983).

Normally, the Fe–Tf–TfR complex is promptly internalised in a clathrin-coated pit, which seals over and fuses to become a multivesicular endosome. An influx of protons into the endosome favours the release of Fe, which must be transported across the endosomal membrane to be available either for cellular use or for storage. Nramp2 (natural resistance-associated macrophage protein 2) has recently been identified as the putative transmembrane protein that transports Fe out of the endosome (Brittenham, 2000). The Tf–TfR complex is recycled back to the cell surface, where apoTf affinity for the TfR is almost lost because of the extracellular pH. The Fe transference from Tf to other ligands occurs in such a way that there is no free Fe to react with oxygen metabolites, but this pathway can be disrupted by the presence of Al.

Taking into account that endosomal acidification seems to be mediated by the ATP-dependent-proton pump, a decay of the activity of this pump due to the presence of Al would prevent the pH to decrease and consequently, impair the dissociation of Tf from its receptor. The final effect would be a failure in the intracellular Fe release. This postulate could be supported by the finding that inside lysosomes, Al(III) compounds reduced the activity of the proton-pump ATPase (Zatta et al., 2000), compromising the functionality of acidic vesicles.

If both ions, Al and Fe, are entrapped inside the same endosome, a normal Fe utilisation could be prevented and finally, Fe accumulation could occur.

Lysosomal trapping has been recognised as an important route for the disposal of trivalent trace elements. Al deposits were detected within lysosomes of liver and spleen macrophages (Bommer et al., 1983), as well as in bone marrow macrophages (Drüeke et al., 1986) from Al-intoxicated dialysis patients. In addition, in Al-overloaded rats

the metal was found in bone marrow cells either in macrophages or in cells of the reticulo-endothelial system. In the former cells Al was localised inside lysosomes as dense inclusions (Drüeke et al., 1986; Berry, 1996), and in the latter ones, inside the organelles having ultrastructural characteristics of lysosomes or phagolysosomes (Watrin & Galle, 1986). The metal was found co-precipitated with Fe (Bommer et al., 1983; Watrin & Galle, 1986) or associated to phosphorous (Drüeke et al., 1986; Watrin & Galle, 1986).

In summary, Al could affect normal Fe availability whether by producing a defective Fe release from Tf due to the insufficient endosome acidification, or by inducing co-trapping of both, Fe and Al, inside the lysosomal compartment. Thus, the inability of Fe to participate in physiological pathways could leave it free to facilitate oxidative processes.

Consequences of Al binding to ATP

ATP hydrolysis is a crucial reaction in the dynamics of the cytoskeleton. Some metabolic events related to cell membrane structure seem to be controlled by cellular redox and energy capacities. The loss of erythrocyte ability to maintain the intracellular glutathione and ATP concentrations triggered membrane proteolytic mechanisms, releasing sialoglycopeptides and modifying the cell surface. These are some characteristics of normal cellular aging that enable the old RBCs to be removed from circulation (Brovelli et al., 1977; Brovelli et al., 1984).

After *in vitro* treatment of human RBCs with Al lactate, the intracellular Al was found bound to ATP, blocking subsequent ATP enzymatic hydrolysis (Panchalingam et al., 1991). The Al attached to ATP forms a very stable complex (Martin, 1986; Panchalingam et al., 1991). Therefore, its presence could produce energy-deprivation, compromising both glycolysis and oxidative phosphorylation, and leading to accelerated aging of RBC.

Relevant Mechanisms for the Aluminium Toxicity in the Erythropoietic System

In view of the above-mentioned findings, we can suggest the following mechanisms in order to explain the toxic action of Al on erythropoiesis.

Once Al bound to Tf reaches the bone marrow, the complex may interact with every cell lineage that carries membrane TfRs. The erythropoietin-induced cells are the most affected because of their high Fe requirement for Hb synthesis.

Through a competitive mechanism, the Al–Tf complex disturbs the free binding of Fe–Tf to its specific membrane TfR, leading to a reduced cellular Fe uptake. At the same time, Al–Tf probably enters into the cell in a similar way as Fe–Tf does. The first cellular modification observed is the increase in surface TfR number, expression that may be triggered either by Fe deprivation or by Al-induced IRP-2 stabilisation. This would be the cell's adaptation in order to obtain the required Fe for its metabolism. If Al continues enhancing IRE binding to IRP-2, by preventing the IRP-2 degradation, Fe would continue entering into the cell, and thus Fe accumulation would occur. Despite this normal or even high availability of intracellular Fe, the Hb synthesis is impaired. Al could also interfere with Fe utilisation, causing its entrapment in an inaccessible compartment.

Apart from all the described events other intracellular mechanisms involving Al-toxicity can be taken into account, such as generation of reactive oxygen species enhanced by Fe-induced oxidative stress, inadequate energy supply conducted by Al–ATP complex formation, impairment of calcium homeostasis, and disturbance of nuclear functions.

If the erythroid progenitors were to be affected by the action of Al on the cellular membrane, the function of surface proteins could be lost or at least depressed. An abnormal performance of the erythropoietin receptor would become a particular cause of ineffective erythropoiesis. Mature erythrocytes do exhibit significant mechanical and morphological changes in conditions of Al-loading. Whether these alterations are produced within the bone marrow during cell maturation or they are acquired within the blood stream during the erythrocyte life span still needs to be clarified. Increased protease activity, Fe-induced lipoperoxidation, decreased ATP availability, altered calcium cellular fluxes or advanced glycation of proteins, could account for the conformational changes in the erythrocyte membrane. These changes could increase cell rigidity, which is reflected in the decrease of osmotic fragility and the shortening of RBC life span. Should the higher *in vitro* osmotic resistance be related to *in vivo* intravascular haemolysis, it would be possible to suggest that the rigidity of cell membrane may be responsible for the increased lysis of the cell along its passage through small vessels because of the reduced deformability of the RBC.

The proposed mechanisms support both, the disturbance of erythroid progenitor functions and the alterations of mature erythrocyte morphology as a consequence of the presence of Al within the organism.

Informed Opinion

Comparative Features of Aluminium Toxicity on Erythropoiesis and Alzheimer's Disease (AD)

The experimental results from various sources have provided good information about reliable mechanisms underlying the toxicity of Al on the erythropoietic system. The main objective sought in this section is to find out whether Al could be considered to be a deleterious factor in AD.

Since there is so much evidence that both, Al-loading and AD present similar metabolic anomalies, the final approach might be that they share common mechanisms that produce cellular toxicity.

There is no information of any clinical study making references about haematological signs of anaemia in AD patients, but this is no proof that the erythropoietic system is unaffected. The erythroid progenitor cells are probably disturbed. However, as they undergo mechanisms of adaptation, only minimal changes in peripheral cells appear. Moreover, these changes are likely to go unnoticed in clinical analysis. This hypothesis could be sustained by the findings reported in two independent investigations that were carried out in Al-overloaded animals (Garbossa et al., 1996) and with erythrocytes from AD patients (Sabolovic et al., 1997). The former demonstrated the inhibition of bone marrow CFU-E growth even before the appearance of peripheral signs of anaemia. The

latter described damaged erythrocyte membranes along with a preponderance of young and recently recruited RBCs. The damage observed in young circulating erythrocytes could be explained as a consequence of Al injury on the whole erythropoietic cell lineage. These findings are in agreement with disturbance of immature cells.

Several reports well describe the Al effect on Fe metabolism in non-erythroid cells. Brain Fe homeostasis appears to rely on the same proteins and mechanisms that are present in extracerebral organs (Koeppen, 1995). As in erythroid progenitor cells, Al was detected in the intracellular medium of brain cells (Roskams & Connor, 1990). To enter these cells, Al ion must cross the plasma membrane being the TfR system the way of entry (Roskams & Connor, 1990). The increase in Fe and Tf uptake was reported to occur in both, erythroid (Abreo et al., 1990; Pérez et al., 1999) and neuroblastoma (Abreo et al., 1999) cell lines grown in Al–Tf containing media. In either case, the Al–Tf complex was responsible for the up-regulation of the TfR expression (Abreo et al., 1990; Abreo et al., 1999).

Interestingly, there is evidence for Fe metabolism disruption in AD (Connor et al., 1992; Smith et al., 1997), and again similar features are shown both in AD and in experimental models of Al toxicity. Considering the importance of IRP–IRE interaction in Fe homeostasis, any mechanism of aberrant Fe utilisation could be ascribed to the disarrangement of this regulatory system. The stabilisation of IRP-2 elicited by Al through the inhibition of the Fe-induced oxidation of this protein resulted in the high TfR biosynthesis and in the suppression of ferritin production (Yamanaka et al., 1999). Both IRP-1 and IRP-2 are expressed in almost all organs and cell lines. The selective association of IRP-2 — but not IRP-1 — to the pathological hallmarks of AD found in the brain tissue (Smith et al., 1998) displayed a resemblance to the Al action on IRP-2. In fact, the increased IRP-2 bound to IREs in AD could explain the paradoxical observation of a high Fe concentration with the concomitant decrease in ferritin synthesis in AD brain tissues (Connor et al., 1992; Loeffler et al., 1995). Therefore, it can be speculated that in both pathological conditions, Al-loading and AD, the Fe-independent induction of the IRE binding activity leads to enlargement of the available cytoplasmic Fe-pool, without an appropriate increase in ferritin content to detoxify Fe. This situation could expose the cells to reactive oxygen species.

There is growing evidence that free radical damage and oxidative stress play a pivotal role in the pathogenesis of AD. Coincidentally, it is interesting to point out that Al is also capable of stabilising Fe(II) ions (Yang et al., 1998). The redox-active Fe was associated with the pathological lesions of AD, the so-called senile plaques and neurofibrillary tangles. This redox activity suggested the possibility for catalytic generation of free radicals at the expense of the consumption of cellular reducing species (Smith et al., 1997). In the same direction, high levels of dietary Al given to adult mice increased the peroxidazibility of brain homogenates (Fraga et al., 1990). A rise in the level of reactive oxygen species was detected after *in vitro* exposure of glioma cells to Al sulphate (Campbell et al., 1999). These oxidising processes could be probably due to a disorganising effect of Al on the membrane in combination with high Fe availability. The subsequent reduction in the glutathione level — the major source of reducing power in the aqueous cell compartment — might further suggest a high pro-oxidant status owing to Al.

Non-erythroid cell membranes appear to be organised in an analogous manner to those of the RBCs, although the former do not need to exhibit the same adaptation to the mechanical, chemical and osmotic forces imposed by the blood stream. Rather, membrane cytoskeletal interactions maintain the distinctive shape of cell surfaces that are crucial for the organisation of tissues. Lipids, integral and skeletal proteins, as well as protein–protein interactions seem to participate in non-erythroid and erythroid cells alike.

There has been much more evidence regarding to membrane alterations in AD peripheral cells than in AD brain cells. Several characteristics, such as membrane fluidity, cell electrophoretic mobility and resistance to lysis in a glycerol-containing medium suggested extensive damage of RBCs in AD (Sabolovic et al., 1997). Platelets from AD patients had a relative excess of their internal membranes with respect to the plasma membrane, which exhibited normal lipidic composition (Cohen et al., 1987). This membrane relative abundance might account for the concomitant increase in the platelet fluidity (Hajimohammadreza et al., 1990; van Rensburg et al., 1992). Similarly, the addition of Al to platelets from healthy subjects caused the cells to become less viscous (van Rensburg et al., 1992). It is well accepted that the fluidity of biological membranes is influenced by factors related to membrane structure. These factors could have a pronounced effect on cell surface properties by modulating the activity of membrane enzymes and other proteins such as ion channels and receptors.

The defective conformation and/or organisation of erythrocyte membrane proteins observed in AD, with band 3, spectrin and glycophorin being the most probable involved (Butterfield & Markesbery, 1980), partially resembled the effects of Al on the erythrocyte ghosts (Vittori et al., 2000). The senescent RBC antigen has been proposed to originate from band 3 degradation of normal erythrocytes to serve as a signal for the removal of aged cells (Kay, 1984). Alzheimer-related changes seem to specifically affect erythrocyte band 3, leading to enhanced protein degradation and accelerated aging of this molecule with the result of premature appearance of senescent cell antigen in the RBC membrane (Bosman et al., 1991). Similar degradation products and altered functions of band 3 were described in brain of AD patients as well, suggesting that the same mechanism(s) could occur in all cells (Kay et al., 1996).

Since not only band 3 but also spectrin and protein 4.1 seem to be affected in AD brains (Sihag et al., 1994; Sihag & Cataldo, 1996; Fernández-Shaw et al., 1997), it can be conjectured that altered expression of membrane proteins could initiate serial events that lead to the disruption of specific membrane domains. Other cells in AD may present similar changes as the brain ones and hence, suffer functional alterations.

Enhanced protease activity may be one of the responsible factors of membrane protein degradation. In this respect, an increase in the activities of apopain and calpain was found in cerebral cortex of rats following a three week Al exposure (Guo-Ross et al., 1998). The serine proteases trypsin and alpha-chymotrypsin were activated *in vitro* by Al action and, at the same time, they gained resistance to protease inhibitors. Taking into account that the Al-activation effect occurred at pH 6.5, it is appropriate to assume that Al-induction of serine proteases takes place in lysosomes of either a neurone or any other cell (Clauberg & Joshi, 1993).

Among others, bone and brain are target organs for Al accumulation in the organism. The multiple Al toxic effects mentioned above could account for the disturbance in the

homeostasis of both erythropoietic and neuronal cells. Nevertheless, a direct action of Al on cell nucleus is also worthy for full attention. In fact, an increased amount of Al was found in a chromatin subcompartment from AD affected brains (Lukiw et al., 1991). Al must be translocated to the nucleus, an action that most probably is carried out by the moiety Al–ATP. Within cellular nuclei, Al was able to modify the structure of chromatin (Walker et al., 1989). Once attached to DNA, Al induced both compaction and precipitation of chromatin (Karlik et al., 1980), thus restraining the access of nucleases to DNA (Walker et al., 1989). In this way, Al could prevent the genes included in the altered chromatin from being expressed. Concerning haematopoietic cells, Al was related to the *in vivo* reduction of the frequency of bone marrow cell divisions and an increment of chromosomal aberrations (Roy et al., 1991). Any biochemical event disturbing the regular mechanism of gene expression might be ascribed to the harmful Al action on DNA. All these mechanisms associated with the activity of Al on chromatin could explain a long-term effect of Al on erythroid progenitors and brain cells, bringing about anaemia and neurological disorders.

In conclusion, the current information encourages the idea that, as in Al toxicity, AD may be related to disturbance of Fe metabolism and alterations in cell membrane. In spite of that, the cellular damage results much more evident in the brain cells, which are highly sensitive to injury. If AD would be considered as a systemic disease instead of an organ specific one, the haematological research could greatly contribute to the understanding of the pathophysiology of the disease.

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Aluminium and Alzheimer's Disease
The Science that Describes the Link
C. Exley (Editor)

CHAPTER 14

Aluminum, Membranes and Alzheimer's Disease

Paolo Zatta [1,*] and Mario Suwalsky [2]

[1] *Centro CNR per lo Studio della Biochimica e della Fisiologia delle Metalloproteine, Università di Padova, Dipartimento di Biologia, Viale G. Colombo 3, 35121 Padova, Italy*
[2] *Faculty of Chemical Sciences, University of Concepción, Casilla 160-C, Concepción, Chile*

* Corresponding author, email: zatta@cribi1.bio.unipd.it

Abbreviations: AChE – acetylcholinesterase; AD – Alzheimer's disease; Al – aluminum; $Al(acac)_3$ – aluminum acetylacetonate; ApoE – apolipoprotein E; APP – amyloid precursor protein; βA4 – amyloid; acac – acetylacetonate; BBB – blood–brain barrier; ADP – adenosine 5′-diphosphate; AMP – adenosine 5′-monophosphate; cAMP – cyclic AMP; ATP – adenosine 5′-triphosphate; ATPase – adenosine 5′-triphosphatase; CNS – central nervous system; DMPC – dimyristoylphosphatidylcholine; DMPE – dimyristoylphosphatidylethanolamine; C/P – cholesterol/phospholipid molar ratio; ER – endoplasmic reticulum; GPC – glycerophosphocholine; GTPase – guanosine 5′-triphosphatase; LDL – low density lipoprotein; MAP – microtubular-associated protein; MAO – monoamino oxidase; PD – Parkinson's disease; SEM – scanning electron microscopy

Summary

Aluminum is able to deeply modify the physico-chemical properties of natural as well as artificial biomembranes. Such modifications,which are strongly correlated to the metal speciation chemical characteristics, can alter relevant cellular, biochemical and physiological parameters (e.g., fluidity, rigidity, transduction of signals, channel permeability and protein activity). In Alzheimer's disease a profound alteration of the cell membrane both in neuronal as well as in non-neuronal cells has been reported. Herein, the most relevant data regarding the interaction between aluminum and cell membranes has been summarized. How the metal ion could eventually have an important role as an aggravating factor in Alzheimer's disease, as well as in other neurodegenerative diseases where the metal ion has been reported to be abnormally accumulated, is also discussed.

Historical Perspective

Membrane and Brain Aging

The *membrane hypothesis* is currently one of the most appealing, among various theories, of aging. Cellular, biochemical and biophysical events occur mainly at the membrane

level, where structural and dynamic properties provide the control mechanisms (Packer et al., 1967; Bowen et al., 1977; Bowen et al., 1979). Cellular membranes are structures that mediate/regulate many cellular functions (e.g., active transport, permeability, signal transduction, etc.). The biophysical structure of the plasma membrane changes with aging and with pathological conditions as the result of damage due to various factors such as oxidative stress, changes of chemical composition, disturbances of homeostasis, neurotransmitter/receptor density reduction, etc. Membrane aging is characterized, among other features, by two main phases: an early one, where the rate of the metabolic pathways is reduced to such a level that the membrane cannot cope with extracellular stressors; consequently, the rate of homeostasis adjustment is reduced concomitantly with a decrease in lipid accumulation and an increase in membrane microviscosity. A second phase is characterized by the aging of membrane proteins together with oxidation, enzyme degradation, cross-linking phenomena, genomic alterations, etc.

Alterations in plasma membranes have been reported by numerous authors in neuronal as well as in non-neuronal cells in Alzheimer's disease (AD) (Zatta & Nicolini, 1995; Bosman et al., 1997). Several studies have emphasized the alteration of some chemical components of the neuronal membrane (Cherayil & Cyrus, 1996; Bowen et al., 1977; DeKosky & Bass, 1982; Pettegrew et al., 1984; Barany et al., 1985; Mason et al., 1996). For instance, a decrement in lipid galactocerebroside, which is a marker for myelin and microsomes, has been reported in senile dementia (Rouser & Yamamoto, 1969; Ansary & Loch, 1975; Pope & Embree, 1976). Wurtman et al. (1990) hypothesized that the abnormal choline utilization, observed in AD, may be involved in the pathogenesis of this disease. These authors demonstrated that in AD brain the very high level of the membrane phosphatidylcholine (PC) breakdown product, glycerophosphocholine (GPC), was twice that of the controls. This increase could reflect accelerated PC breakdown or an impairment in enzymatic processes that allow free choline to be salvaged from GPC. An increased membrane phospholipid degradation in AD, not observed in other neuropathologies, e.g. Parkinson's, Huntington's or Down's syndrome, has also been reported (Nitsch et al., 1992). In this connection it has been demonstrated in our laboratories that Al at μM level strongly activates the enzyme acetylcholinesterase (AChE). This activation is most likely achieved by the interaction between Al^{3+} and γ-peripheral sites of the enzyme with consequent structural changes that could explain at the molecular level the effect produced by Al^{3+} on AChE activity (Zatta et al., 1994).

Aluminum and Membrane Fluidity

Recently, a great deal of attention has been paid to membrane fluidity in relation to neuropathological events. In cellular membranes lipid fluidity is defined as the reciprocal of the structural order of lipid components (e.g., cholesterol and fatty acids). The cholesterol/phospholipid molar ratio (C/P) is one of the most important parameters of the membrane fluidity (Van Blitterswijk et al., 1981) together with the level of unsaturation of phospholipid acyl chains (Shinitzky & Henkart, 1979). Changes in composition and metabolism of fatty acids affect important membrane properties which may contribute to the pathophysiology of aging in that with aging, the level of polyunsaturated fatty acids and

the C/P increase in blood and tissues (Bonetti et al., 1983). A pronounced loss of fatty acids from membranes during senescence, which gives rise to a large increase in C/P, is now well known. This coincides also with increased phospholipase activity and with changes in membrane lipid composition (Calderini et al., 1983).

Modification of membrane fluidity affects cell function and the activity/regulation of membrane-bound enzymes; permeability, membrane fusion, assembly, and lateral diffusion of antigenic components of the cell surface are all influenced by changes in fluidity. The evidence that membrane lipids exert powerful effects and influence the function of membrane-bound enzymes, suggests that subtle displacements in the structure and composition of membrane lipids may prove to be important factors in the etiology of human diseases. Among the many aspects of membrane fluidity to be understood there is the fact that different membranes (e.g., Golgi, mitochondria, endoplasmic reticulum (ER), plasma, etc.) having each its own distinct and peculiar composition, nevertheless succeed in maintaining comparable fluidity (Vessey & Zakim, 1974). In this regard, Mason et al. (1993), found that small angle X-ray diffraction analysis of AD lipid membranes extracted from cortical gray matter revealed a significant structural change with respect to age-matched controls with a reduction of 4 Å in the bilayer width. They also reported a reduction of the C/P of about 35% in AD temporal gyrus as a characteristic of AD. However, a restoration *in vitro* of the correct C/P, gives a virtual restoration of the membrane bilayer width suggesting that the cholesterol deficit played a major role in AD lipid membrane structural perturbation. Membrane dysfunction in connection with structural destabilization could thus contribute to the development of AD independently from other proposed pathogenetic pathways, although concurring synergistically with such pathways. Destabilized membranes may become more susceptible to aberrant enzymatic proteolysis of β-amyloid precursor protein (APP).

Membrane-bound proteins in platelets include the APP from which the β-amyloid deposits may originate. It has been recently demonstrated that activated platelets from AD patients show less APP processing, and secrete fewer soluble fragments than controls (Davies et al., 1997). Biophysical alterations of AD platelet membrane have been reported by several authors (Zubenko et al., 1987; van Rensburg et al., 1992; Von Hungen & Baxter, 1988). Monoamine oxidase (MAO) is an enzyme located on the outer membrane of the mitochondria, which acts on a broad spectrum of phenylethylamines and is involved in the catabolism of dopamine. Its activity increases with aging in the human brain and a greater increase of MAO-B isoform activity has been reported in patients with AD and Parkinson's disease (PD). Platelet MAO-B activity has also been found to increase significantly in demented subjects (Gotz et al., 1998). It is worth noticing that MAO-B is significantly activated by Al(III) in a dose-dependent manner (Zatta et al., 1999). In addition, the neurotoxicological action of Al, studied in single outer membrane channels from rat brain mitochondria, showed that at μM concentration Al^{3+} caused significant changes in the mitochondrial membrane functions (Mirzabekov et al., 1993). It remains to be demonstrated whether the abnormal accumulation of Al in the aging brain and especially in the brain from AD (Edwardson et al., 1992; Zatta, 1993) and Parkinson's disease (PD) patients is directly linked to the abnormal activity of this enzyme.

Aluminum and Sprouting

Synaptic transmission can be blocked by neurotoxic agents; when this occurs, new branches are formed (*sprouting*). Axonal sprouting and synaptic reorganization has been reported after brain injury. In AD cerebral amyloid deposition has neurotrophic effects and this is the main cause of aberrant sprouting, a phenomenon so far underestimated but relevant to the disruption of neuronal connectivity, which may significantly contribute to the clinical manifestations of AD (Scheibel, 1979; DeKosky & Bass, 1982; Phinney et al., 1999). Neuroblastoma cells treated at subtoxic concentrations of Al-lactate show extensive neurite outgrowth and sprouting (Zatta et al., 1992). This kind of neurite morphology is remarkably similar to that described in the aged or in AD brain. Neuritogenesis induced by Al contributes neurites that vary in length and in diameter and sometimes they change their course forming a mesh network. Such growth pattern and extensive sprouting of neurites suggest that Al could be involved in the neuronal remodelling characteristic of AD (Uemura et al., 1993). Several studies have reported the neurotoxic action of Al indicating altered calcium homeostasis (Gandolfi et al., 1998), enhanced cyclic-AMP (cAMP) production, and changes in cytoskeletal protein phosphorylation states and concentration (Jope & Johnson, 1992). In addition, Al inhibits the GTPase activity of the stimulatory G protein, Gs, leading to prolonged activation of Gs receptor stimulation and increased cAMP production (Jope & Johnson, 1992). Al also has a relevant effect on the phosphorylation of microtubule-associated protein 2 (MAP-2) and on the neurofilament NF-H (Zatta, 1995; Shin et al., 1997).

It is now well known that the apolipoprotein E family (Apo E) and its low density lipoprotein (LDL) receptor family regulate the internalization of cholesterol and phospholipids in the brain. Allele $\epsilon 4$ of the ApoE has been found to be strongly linked to both sporadic and familial late-onset AD, raising the possibility that a dysfunction of their lipid transport system associated with compensatory sprouting could be central in AD pathogenesis. ApoE4 in central nervous system (CNS) is particularly relevant to the cholinergic system and to the integrity of phospholipid homeostasis in neurons. Studies of the effect of chronic exposure to Al on the lipid composition in different regions of the monkey brain were carried out. Al administration caused a significant decrease in the total lipid, glycolipid, and phospholipid content of the primate brain. Cholesterol levels and C/P were markedly increased as a consequence of Al administration with a loss of membrane integrity (Sarin et al., 1997).

Aluminum and Blood–Brain Barrier Permeability

The blood–brain barrier (BBB) controls the exchange of material between the blood and the brain maintaining a neuronal homeostatic environmental equilibrium. The BBB is formed by a monolayer of endothelial cells that separate the blood and the fluid medium of the brain; the cells line the brain's venules and arterioles and comprise the capillary bed of the brain (Banks et al., 1995). The microvessels that constitute the BBB are characterized by interendothelial tight junctions (the BBB), pinocytotic vesicles and a high mitochondrial content in endothelial cells. In AD various observations indicate

that there is a reduced mitochondrial content and an increase in pinocytotic vesicles suggesting dysfunction of the BBB (Scheibel, 1984). Current evidences do not exclude the possibility that cerebrovascular functions decline with aging leading to pronounced effects in sporadic and familial AD and to the progression of dementia for reasons yet to be understood (Wada, 1998). Kalaria (1997) suggests that βA4 could be responsible for the cerebral amyloid angyopathy, and for the degeneration of the cerebral microvessels that may deeply affect brain perfusion and BBB functioning. Claudio (1996) suggests that a breach of the BBB is causally involved in the pathogenesis of AD, in that morphological and biochemical abnormalities in the cerebral microvessels seem to be involved in the breakdown of BBB in AD.

Recently, it has been demonstrated that βA4 can cross the BBB and accumulate in the brain circulation. Aluminum alters the structure and the function of βA4 (Fasman, 1996), inhibiting the metalloproteases that process βA4, and changes the permeability of the BBB to peptides of similar size to βA4 (Banks et al., 1996). Thus, aluminum could alter the access of βA4 to the CNS by changing the permeability of the BBB or by affecting enzymatic degradation. Furthermore, it has been demonstrated in our laboratories that Al can profoundly modify the permeability of BBB in rats (Favarato et al., 1992) either in a transient or in a permanent way depending on the metal chemical species used in the experiments. In fact, while a hydrophilic and hydrolytically stable Al species, such as Al-maltolate ($Al(malt)_3$), can alter the BBB transiently, a lipophilic and hydrolytically stable Al species such as Al-acetylacetonate ($Al(acac)_3$) can increase the permeability of the BBB, either permanently or at least for a considerable period of time. Banks & Kastin (1983 and 1989) also found that Al increased the BBB permeability. Vorbrodt et al., (1994) used cultured goat brain microvasculature endothelial cells as a BBB model, and treated them with $Al(malt)_3$; they reported a severe functional alteration which was ascribed to the Al compound. In addition, it has been found that the activity of Ca^{2+}-ATPase is almost suppressed by Al in the interendothelial cleft where junctional complexes are presumably formed (Gandolfi et al., 1998). All these effects can eventually lead to an increase in the BBB permeability due to a breach in the interendothelial junctions and to the neutralization of the negatively charged domains of the endothelial cell surface by Al.

Informed Opinion

Whereas the interaction between Al and biological systems has been the aim of numerous descriptive studies, direct information on the molecular basis of its biological activity is rather scant. The reason for this lack of molecular information is mainly due to the ill-defined chemical speciation of the metal compounds utilized in experimental protocols. For this reason we have used as a chemical model $Al(acac)_3$ (Zatta et al., 1987; Suwalsky et al., 1999a), a chemically well defined, hydrolytically stable and lipophilic compound (Zatta et al., 1997). This complex is effective in producing, at micromolar levels, osmotic fragility of erythrocytes and formation of acanthocytes in a dose dependent fashion (Zatta et al., 1989). Upon administration to rabbits, an aluminum-sensitive animal species, $Al(acac)_3$ was unambiguously shown to be the cause of a myocardiopathy (Corain et al., 1988).

In vivo studies of human erythrocytes showed that $Al(acac)_3$ altered membrane fluidity with an appreciable structural compacting effect on cytoskeletal and transmembrane proteins, as well as a reduction in rotational mobility of cell-surface sialic acid (Zatta et al., 1998). Thus, the erythrocyte can be a useful model for the understanding of the effects of Al on biological cell membranes (Zatta et al., 1998). With this aim, various concentrations of $Al(acac)_3$ and $AlCl_3$ (Suwalsky & Zatta, in press) were incubated with human erythrocytes and membrane molecular models. The latter consisted of multilayers of dimyristoylphosphatidylcholine (DMPC) and dimyristoylphosphatidylethanolamine (DMPE), representative of phospholipid classes located in the outer and inner monolayers of the human erythrocyte membrane, respectively (Devaux & Zachowsky, 1994). These systems had previously been used to determine the interaction and membrane perturbing effects of drugs (Suwalsky et al., 1994; Suwalsky et al., 2000a), pesticides (Suwalsky et al., 1997; Suwalsky et al., 1999b) and other metal ions (Suwalsky et al., 1998; Suwalsky et al., 2000b). Results indicated that 0.1 mM $Al(acac)_3$ and $AlCl_3$ interacted with the human erythrocyte membrane, resulting in gross alterations of its morphology. According to the bilayer couple hypothesis (Sheetz & Singer, 1974), the shape changes induced by foreign molecules in erythrocytes are due to a differential expansion of their two monolayers. Thus, spiculated shapes (echinocytes or acanthocytes) arise when the added compound accumulates in the outer monolayer, whereas cup shapes (stomatocytes) are induced when the compound is inserted in the inner monolayer. The fact that $Al(acac)_3$ produced both echinocytes and stomatocytes (Fig. 1) indicated that the Al complex was inserted in both the outer and inner moieties of the red cell membrane. On the other hand, $AlCl_3$ induced only echinocytosis (Fig. 1), which was an indication that the metal ions were bound only to the outer monolayer of the erythrocyte membrane. Both results were supported by X-ray diffraction experiments performed on DMPC and DMPE bilayers. However, the interaction of $AlCl_3$ with DMPC was stronger than that of $Al(acac)_3$ as the lipid structure was completely destroyed by 1 mM $AlCl_3$, a consequence that was not observed at any concentration of the complex as can be seen in Figs. 2 and 3.

DMPC and DMPE differ only in their terminal amino groups, these being $+N(CH_3)_3$ in the former and $+NH_3$ in the latter. Moreover, both molecular conformations are very similar in their crystalline phases (Suwalsky, 1988) with the hydrocarbon chains mostly parallel and extended, and the polar groups lying perpendicular to them. However, the gradual hydration of DMPC results in water filling the highly polar interbilayer spaces. This phenomenon allows the incorporation of $Al(acac)_3$ and Al^{3+} ions into DMPC bilayers perturbing their structure at concentrations as low as 1 μM. Whereas the Al^{3+} ions could bind to the lipid polar head groups, the acetylacetonate lipophilic moiety would associate with DMPC acyl chain region. On the other hand, DMPE molecules pack tighter than those of DMPC due to their smaller polar groups and higher effective charge, resulting in a very stable bilayer system that is not significantly affected by water (Suwalsky, 1996). However, increasing concentrations of $Al(acac)_3$ and Al^{3+} ions induced molecular perturbations in DMPE bilayers (Figs. 2 and 3).

Additional experiments performed on isolated toad skin showed a significant decrease in the potential difference and short-circuit current after application of $Al(acac)_3$ and $AlCl_3$ (Suwalsky et al., 1999a; Suwalsky & Zatta, in press). These effects can be interpreted as reflecting a slight and transient stimulation followed by prolonged inhibition

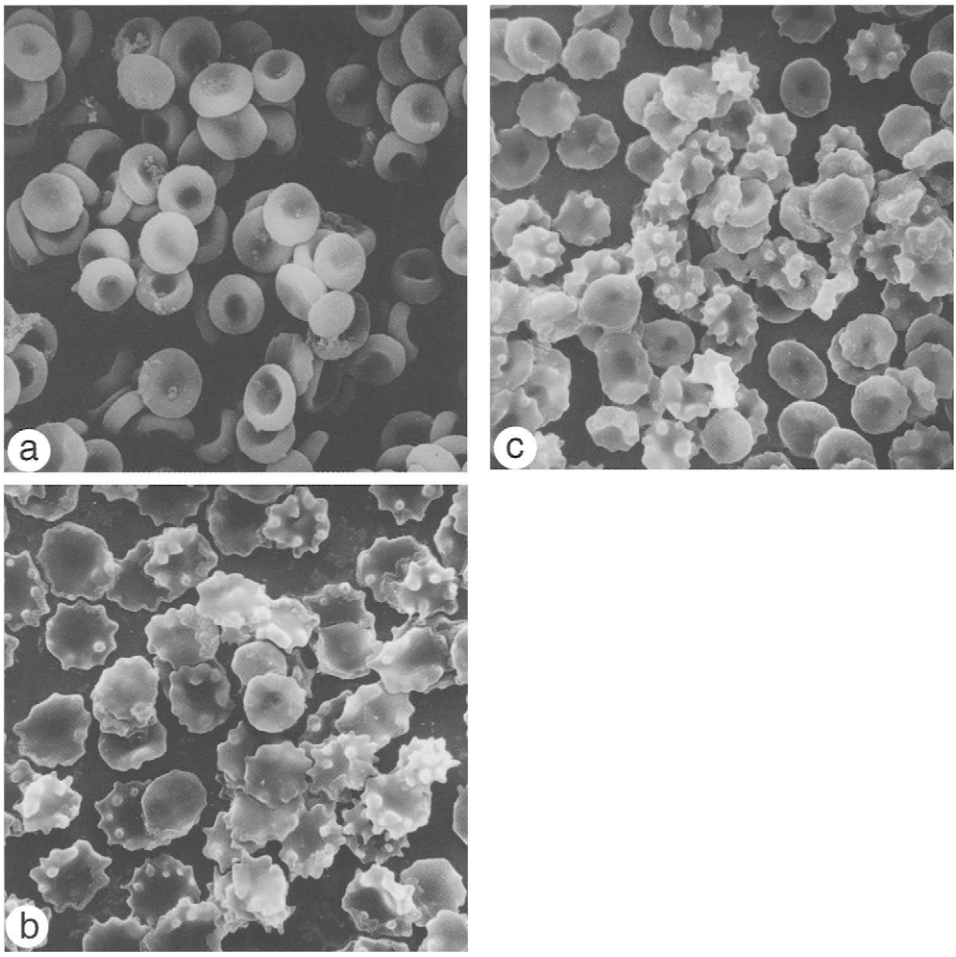

Fig. 1. SEM images of human erythrocytes, 1120×: (A) control; incubated with (B) 1 mM $Al(acac)_3$, and (C) 1 mM $AlCl_3$.

of the active transport of ions by both compounds. However, the effects were far more pronounced with $AlCl_3$ (Table 1). The results are in accordance with a time-dependent, biphasic regulation of ion transport in response to changes in the molecular structure of the membrane lipid bilayer, thus, interfering with channel proteins and/or membrane enzymes or other proteins.

Conclusions

In the last ten years substantial progress has been made to better understand the properties of aluminum with respect to its impact in the biosphere. However, in spite of an enormous number of scientific papers on the chemistry, bioinorganic, biochemistry

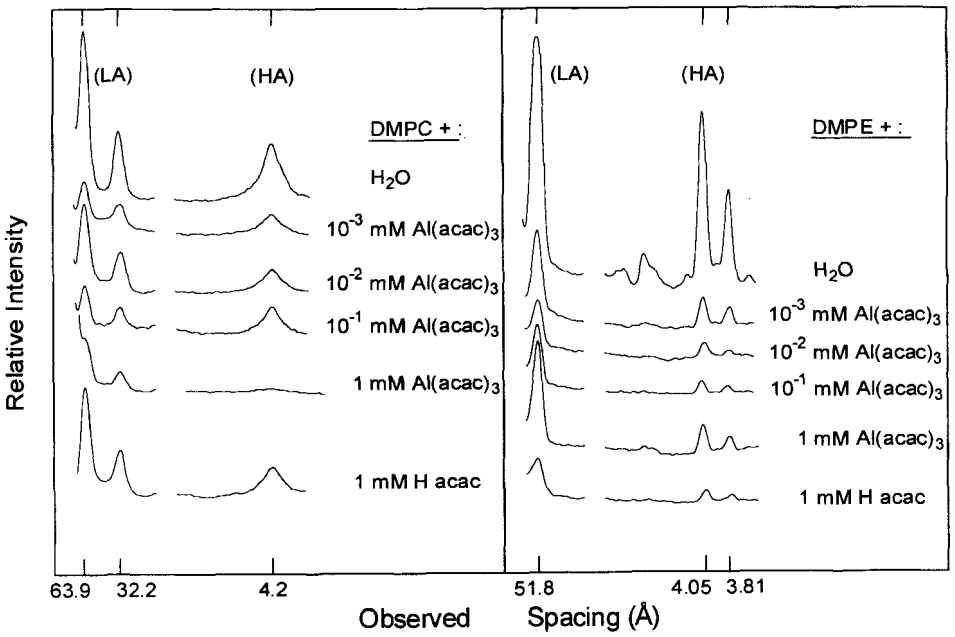

Fig. 2. Microdensitograms from X-ray diffraction diagrams of DMPC and DMPE in water and aqueous solutions of Al(acac)$_3$. LA: low-angle reflections; HA: high-angle reflections.

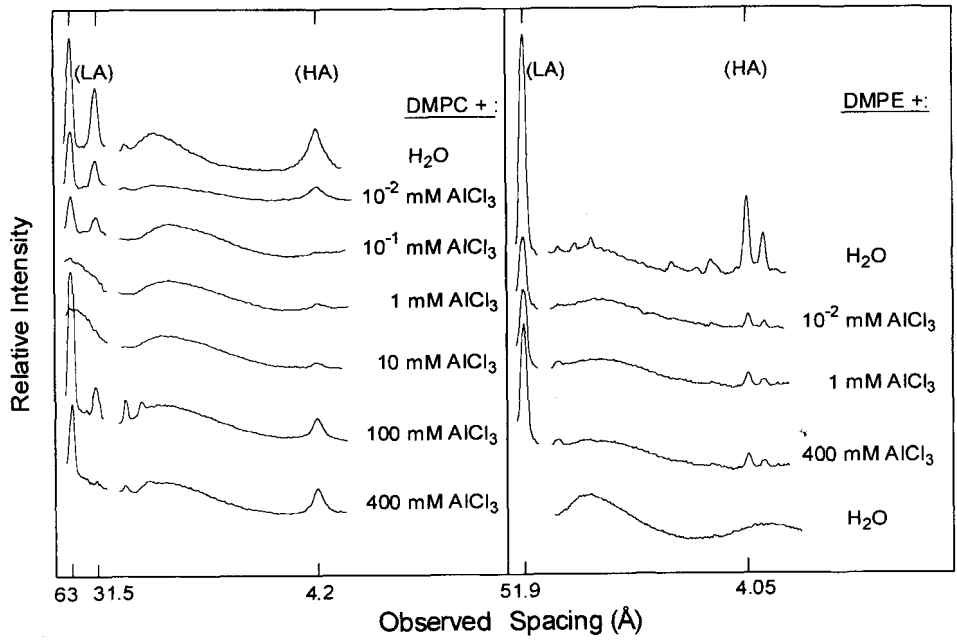

Fig. 3. Microdensitograms from X-ray diffraction diagrams of DMPC and DMPE in water and aqueous solutions of $AlCl_3$. LA: low-angle reflections; HA: high-angle reflections.

Table 1. Effects of aluminum acetylacetonate [Al (acac)$_3$] and of aluminum chloride [$AlCl_3$] applied in the solution bathing the outer surface, on the potential difference (PD) and on the short-circuit current (Isc) of the isolated toad skin (Suwalsky et al., 2000a, 2000b; Suwalsky & Zatta, in press)

Agent	% decrease in PD	% decrease in Isc	*n*
Al (acac)$_3$			5
0.16 mM	1.0 ± 0.9^{NS}	2.1 ± 1.0^{NS}	
0.24 mM	25.0 ± 2.0*	31.7 ± 3.6*	
0.32 mM	43.0 ± 5.0**	53.0 ± 6.6**	
$AlCl_3$			7
0.01 mM	14.0 ± 3.0^{NS}	16.0 ± 4.3^{NS}	
0.03 mM	5.9 ± 2.8^{NS}	7.0 ± 3.0^{NS}	
0.10 mM	16.0 ± 2.0**	32.0 ± 3.0***	

Values are means ± standard error of the means; *n* = number of experiments. In contrast with control values, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$; NS = not significant.

as well as toxicology of aluminum (about 1200/year in the Medline data bank), it is still premature to define the precise molecular mechanisms that could fully explain the influence of this metal ion on the biological targets, including cell membranes. In the context of this review, experimental data confirm that aluminum is able to deeply alter the molecular structure of the lipid bilayer, thus modifying biophysical and physiological properties of the cell membrane such as fluidity, rigidity, transduction of signals, channel permeability and protein activity. To further complicate the scenario, a great number of differences have been found between data obtained from *in vitro* and *in vivo* experimentation. Often, results obtained by *in vivo* models are more dramatically relevant than those obtained using *in vitro* models, suggesting a more holistic action of aluminum toward biological targets.

An improved understanding of the role of the metal speciation appears to be one of the most important issues to be studied in order to comprehend aluminum toxicological properties. This aspect is extremely important if it is considered that aluminum is present everywhere in our everyday life, as in food, beverage and pharmaceutical products. In relation to a possible connection between aluminum and AD, while a direct connection with the etiopathogenesis of this disease is still to be proven, several elements seem to indicate that the metal ion could be somehow involved concurring in the aggravation of some pathological events observed in this devastating syndrome, especially in those individuals more prone to aluminum uptake, on the bases of the following observations.

(i) Aluminum produces a profound alteration of the biophysical status of biomembranes, consequently, it can induce a strong modification of cell membrane anisotropy altering strategic physiological functions of the lipid metabolism as well as of the biochemical and physiological properties of proteins associated with the lipid bilayer.

(ii) The increase of phosphatidylcholine derivatives in the cell membranes of AD (Pettegrew et al., 1984; Wurtman et al., 1990), could facilitate an Al^{3+} overaccumulation in the surface of the membrane. In addition, the hyperphosphorylated tau proteins associated with the cytoskeleton, as observed by several authors in AD, is an elective place for Al^{3+} accumulation as hypothesized by Zatta (1995) and partially proven

(Shin et al., 1995; Shin et al., 1997) with a consequent possible blockage of the axonal flux (Zatta, 1995).

(iii) The interaction between Al and biomembrane produces a significant alteration that could contribute to a reduction of the width of the lipid bilayer as observed in AD by Mason et al. (1993), thus favoring abnormal exposure of the amyloid segment of the APP to a more strategic proteolysis by some putative yet to be discovered secretase.

(iv) Al altering membrane biophysical properties of mitochondria (Mirzabekov et al., 1993) and ER, and inhibiting Ca^{2+}-ATPase activity (Gandolfi et al., 1998) can potentially alter Ca^{2+} homeostasis influencing apoptotic phenomena.

(v) The structural destabilization of the cell membrane by Al could induce a more relevant production of free radicals by transition metals, mainly Fe(II) increasing oxidative phenomena (Zatta et al., 1989; Zatta et al.,1997; van Rensburg et al., 1992) as observed in AD.

(vi) Work on experimental animals has shown that Al, acting as a membrane destabilizer at the BBB, can profoundly modify its permeability in a speciation-dependent way (Favarato et al., 1992).

Further studies are thus necessary to better understand the role of Al in connection with AD, if any. It is a fact that the molecular, biophysical and chemical bases of individual sensitivity to aluminum, and the consequent involvement as a potential risk factor or at least as an aggravating cofactor for AD, as well as for other neurodegenerative diseases, is still an issue to be explored in the near future.

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Aluminium and Alzheimer's Disease
The Science that Describes the Link
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CHAPTER 15

Iron Homeostasis and Aluminium Toxicity

Roberta J. Ward * and Robert R. Crichton

Unité de Biochimie, Université Catholique de Louvain, 1348 Louvain-la-Neuve, Belgium

* Corresponding author, email: ward@bioc.ucl.ac.be

Abbreviations: NTA – nitrilotriacetate; Al-NTA – aluminium nitrilotriacetate; Fe-NTA – iron nitrilotriacetate; IP – intraperitoneal

Summary

The focus of this chapter will be directed at similarities between aluminium and iron, both from a chemical and biochemical view point, and a discussion of whether the accumulation of aluminium in the brain of Alzheimer patients is due to its capacity to use specific iron protein carrier systems. Brain iron is elevated in many brain regions of Alzheimer patients as well as the neurofibrillary tangles and senile plaques, sometimes in association with an increased aluminium content, such that the important question to be addressed is whether the brain iron homeostatic mechanisms are, or could be, perturbed by the presence of aluminium.

Historical Perspective

Aluminium is an element which has no known biological role (Frausta da Silva & Williams, 1991). Its chemistry shows similarities with two other groups of elements, firstly the divalent metals magnesium and calcium and secondly the trivalent metal ions, chromium and iron (Williams, 1999). Although iron and aluminium have similar chemical properties (Table 1), the inability of aluminium to change between two valency states will be an important discriminator within various biological systems where an oxidation-reduction step is required.

Aluminium accumulation in the brain was initially assayed in two groups of patients: firstly post mortem tissue of Alzheimer patients often associated with neurofibrillary tangles and amyloid plaques (Wisniewski & Wen, 1992) and secondly in renal dialysis patients, where high circulating levels of aluminium, caused by blood exchange with dialysates containing large amounts of aluminium, resulted in an increased flux of aluminium across the blood brain barrier, BBB (Morris et al., 1989). Experimentally it was shown that IP injection of rats with various aluminium compounds also resulted in accumulation of aluminium in the brain (Florence et al., 1995). This led to substantial

Table 1. Comparison between iron and aluminium chemistry within biological media

	Fe^{3+}	Al^{3+}
Valency in biological systems	$^{2+}$ or $^{3+}$	$^{3+}$
Ionic radius	0.58–0.65	0.48–0.54
Preferred ligands	O_2^-, OH, N-carboxylic phenolate, imidazole phosphate	O_2^-, OH, N-carboxylic, phosphate
Hydrolysis/polymerisation	Yes	Yes
Solubility of oxyhydroxide polymers	Insoluble	Insoluble
Geometry of co-ordination	Octahedral and others	Octahedral
Transport in the serum	transferrin	transferrin
Ability to cross blood brain barrier	Yes	Yes
Biological importance	Yes	Not identified, toxic
Capacity to traverse GI tract	Yes	Poor
Storage	Ferritin and haemosiderin	Not known

interest in the possibility of an etiological link between aluminium and neurodegeneration — notably Alzheimer's disease, and a focus on how aluminium could cross the blood brain barrier. Since there is considerable evidence that there is disruption of iron homeostasis in Alzheimer's disease (Good et al., 1992) the hypothesis that aluminium might exerts its toxic effect by interfering with iron transport and iron homeostasis had been advanced.

Iron and Aluminium Uptake and Transfer Across the GI Tract

Approximately 12–18 mg Fe/day is ingested by normal subjects, mainly as Fe^{3+}, of which 1 mg is absorbed. Two of the uptake systems for iron which have recently been identified require the reduction of Fe^{3+} to Fe^{2+} (Fig. 1):

(i) a duodenal ferric reductase will reduce Fe^{3+} to Fe^{2+} prior to its entry into the enterocyte via the divalent cation transporter DCT1 (Nramp2) (Andrews, 1999). Within the enterocyte, the iron can either be stored within mucosal ferritin or traverse to the basolateral membrane where it is transported across the basolateral membrane by IREG1, a transmembrane iron transporter protein (McKie et al., 2000). The membrane-bound copper oxidase hephaestin promotes oxidation to Fe^{3+} and facilitates its incorporation into circulating apotransferrin (Vulpe et al., 1999);

(ii) a second uptake and transport mechanism, predominantly for haem, involves a putative haem receptor on the apical side of the enterocyte membrane, a reductive step by haem oxygenase (Boni et al., 1993) (which releases Fe^{2+}, porphobilinogen and CO): thereafter Fe^{2+} can be incorporated into the scheme described above.

The daily intake of aluminium is reputed to be 3 mg/day, although the GI tract appears to rigorously exclude aluminium (because of its chemical nature), 0.06–0.1% of the ingested aluminium in the form of Al^{26} being absorbed.(Moore et al., 2000) with almost 100% of the ingested dose recovered in the faeces (Priest et al., 1998). Alzheimer patients may have a significantly increased absorption of aluminium. In some investigations, of young and elderly patients by either an aluminium absorption test (Taylor et al., 1992) or

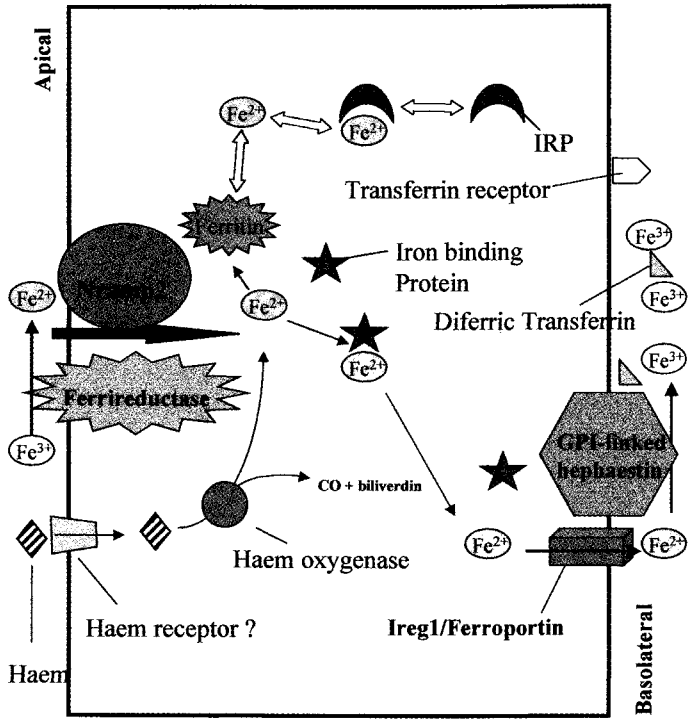

Fig. 1. Regulation of iron uptake in the enterocyte.

with Al^{26} (Moore et al., 2000), respectively, a higher absorption of the ingested dose by comparison to age matched controls was identified.

Two possible systems may be appropriate for aluminium uptake and transport:

(i) a mobilferrin-intergrin pathway (Conrad et al., 1999) which is reputed to transport Fe^{3+} directly from the gastrointestinal tract, and requires no oxidation-reduction step
(ii) the passage of Al^{3+} between the gap junctions of the enterocytes (Hopfer, 1991), possibly as aluminium citrate, thereby bypassing the apical and basolateral membranes of the enterocyte.

Distribution of Iron and Aluminium in the Blood

Iron is present in the serum at concentration between 3 and 5 μg/ml in normal subjects and is predominantly bound to transferrin, >90%, with little associated with albumin or a low molecular weight constituents in normal subjects (Crichton, 1991). Despite the difficulties of assaying aluminium in the blood, significantly elevated serum aluminium levels in Alzheimer patients have been assayed (Basun et al., 1991), e.g. 0.66 μmol/l by comparison to controls, 0.21 μmol/l in some studies (Roberts et al., 1998) although this has not been substantiated in other studies (Pailler et al., 1995). Much higher levels of

aluminium are assayed in the serum of susceptible renal dialysis patients, 5 μM (Fatemi et al., 1991).

Aluminium can bind to transferrin; many in vitro studies have shown that between 60 and 90% of the administered aluminium dose is associated with transferrin (Fatemi et al., 1991; Soldado Cabezuelo et al., 1997). Defective binding of iron and aluminium to a transferrin variant C2, TfC2, in Alzheimer's disease was suggested by Van Landeghem et al. (1998) although these authors were unable to show any significant differences between TfC2 and other TF variants with respect to the binding of iron and aluminium. The non-transferrin bound fraction of aluminium was associated with both albumin and a low molecular weight species (Fatemi et al., 1991) possibly a phosphate, silicate (Hodgkins et al., 1993) or more likely a citrate complex (Van Ginkel et al., 1990). Fractionation of a plasma sample from a haemodialysis patient (with a high plasma aluminium concentration of 5μM) found that 60% of aluminium was bound to transferrin, 34% to albumin with the remainder associated with citrate (Fatemi et al., 1991).

Melanotransferrin or p97 is elevated in the serum of Alzheimer patients compared to controls (Kennard et al., 1996) although the significance of this finding is undefined. Melanontransferrin mRNA is expressed in normal tissues with the highest levels in the salivary glands (Richardson, 2000). It was first described as a marker antigen for human melanoma cells, and has a striking structural similarity to human serum transferrin and lactoferrin. It is predominantly expressed at the cell surface as a glycosylphosphatidyinositol anchored protein, has only one iron binding site and may have some involvement in cellular iron uptake since it co-localises with the transferrin receptor in the brain capillary endothelium (Food et al., 1994).

Entry of Iron and Aluminium into Cells via Transferrin–Transferrin Receptor Pathway

Iron and aluminium, bound to transferrin, will have access to cells via the transferrin –transferrin receptor pathway. The stability constants for iron and aluminium are similar; at pH 7.4 the stability constant, log K for the two binding sites on transferrin are 12.9 and 12.3 for aluminium and 20.7 and 19.4 for iron (Aisen et al., 1978). The metal:transferrin –transferrin receptor complex invaginates into a clathrin-coated pit, where the vesicle is pinched off from the membrane. After budding is complete, the coat proteins are removed, resulting in the formation of smooth-surfaced vesicles, which can then fuse with the target membranes of the endosomes. Fusion of the two membranes delivers the vesicle contents into the interior of the endosome, while the vesicle membrane is added to the endosomal membrane. The interior of the endosomal compartment is maintained at a pH of approximately 5.5 by the action of an ATP-dependent proton pump in the endosomal membrane which pumps protons into the endosomal lumen from the cytosol. If, as seems likely, iron is released from the transferrin–transferrin receptor as Fe^{3+} at the acidic pH of around 5.5 within this compartment (Bali et al., 1991, Sipe & Murphy, 1991) aluminium could be released from transferrin at this stage. It is becoming clear now that Fe^{3+} is subsequently reduced by a ferrireductase within the endosome and is then transported out of the endosome by the divalent cation carrier Nramp2, or DCT1, a transmembrane iron transporter that functions with concomitant transfer of H^+ (Andrews,

1999). Apotransferrin bound to the transferrin receptor then return to the cell surface, where the apotransferrin is released for reutilisation, completing a highly efficient cycle. Exactly how aluminium could exit the endosome remains unknown.

Subcellular studies of aluminium distribution in various cell lines and animal models, after aluminium loading, e.g. bone marrow cells (Watrin & Galle, 1986), Friend erythroid leukaemic cells (Abreo et al., 1994) or of rat liver loaded with cold (van der Voet et al., 1992) or radioactive aluminium Al^{26} (Ward, Day, King and Crichton, unpublished data) have all shown that the majority of aluminium accumulates within the mitochondrial fraction which may be responsible for a decreased state 3/4 respiration in brain mitochondria (Swegert et al., 1999). Similarly, increasing the iron load of mitochondria will result in significantly reduced State 3 oxidation (Ward et al., 2000). Fibroblasts of Alzheimer patients do exhibit altered mitochondrial function with reduced calcium uptake, while exposure of such cells to iron increases calcium uptake as well as their sensitivity to reactive oxygen species (Kumar et al., 1994) thereby indicating that iron and perhaps aluminium might exert mitochondrial toxicity by interfering with calcium homeostasis.

It is clear that much lower concentrations of aluminium are detectable in the cytosolic fraction after aluminium loading. This may be related to the inability of aluminium to exit the endosome. The speciation of aluminium within the cytosolic fraction after equilibrium gel filtration chromatography showed that the aluminium was not present in the form of a citrate complex or associated with transferrin (van der Voet et al., 1992). However the fact that iron chelators, such as desferrioxamine, and the hydroxypyridones, are able to chelate aluminium from the brains of experimentally aluminium loaded rats (Florence et al., 1995) might confirm the presence of the trivalent aluminium ion within a low molecular weight pool from which this metal ion is readily chelated (Crichton & Ward, 1993).

Non-Transferrin Uptake Pathways for Iron and Aluminium into Cells

Hypotransferriaemic mice have severe anaemia, indicating that the transferrin–transferrin receptor cycle is essential for iron uptake by erythroid cells. Despite the lack of transferrin, it is known that hypotransferriaemic mice can still accumulate iron thereby indicating that other non-receptor mediated uptake systems for iron and presumably aluminium must exist. Takeda et al. (1998) showed that there was non-transferrin mediated iron uptake in glial cell cultures from hypotransferrinemic mice. However recent investigation of the transferrin independent uptake pathway by Oshiro et al. (2000) showed a reduction of transferrin receptor expression after incubation of primary cultures of neuronal and glial cells with either iron or aluminium nitrilotriacetate, the low molecular weight aluminium complex inducing the largest decrease. Both of these low molecular weight compounds would be expected to enter into the cytosolic pool such that the iron would be sensed by the iron homeostatic mechanism and transferrin receptor expression would be down-regulated.

Mechanism of Cellular Iron Homeostasis in Cells

Iron homeostasis in mammalian cells is regulated by balancing iron uptake with intracellular storage and utilisation. This is largely achieved at the level of protein synthesis (translation of mRNA into protein) rather than at the level of transcription (mRNA synthesis). This makes it much easier to control levels of protein expression by changing the rate of specific mRNA synthesis by the use of inducers and repressors. Regulatory sequences in mRNA's are generally located in the non-coding, or untranslated regions (UTRs) of the mRNA, situated at the 5′- and 3′-extremities of the coding part of the mRNA sequence: those in the former are usually associated with the initiation of translation, in other words ribosome binding, whereas those at the 3′-UTR are associated with mRNA stability and degradation, ie. mRNA turnover.

Stem loop structures designated as IREs (Iron Regulatory Elements) are found in the 5′- and 3′-untranslated regions of ferritin and transferrin receptor mRNAs, respectively (Fig. 2). Two closely related cytosolic IRE-binding proteins (now known as Iron Regulatory Proteins — IRP's), designated IRP-1 and IRP-2 have been identified in many mammalian cell types (Fig. 3). They act as iron sensors, essentially by existing in two different conformations. When iron is in short supply, the apo- IRP's can bind to

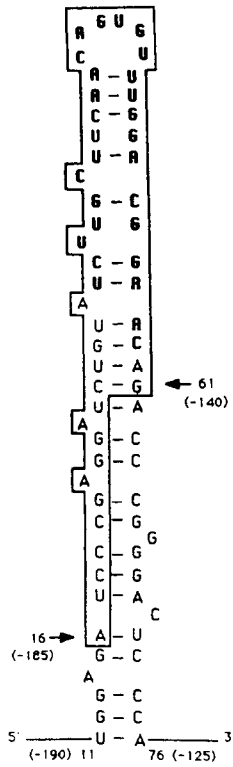

Fig. 2. Nucleotide structure of stem loop.

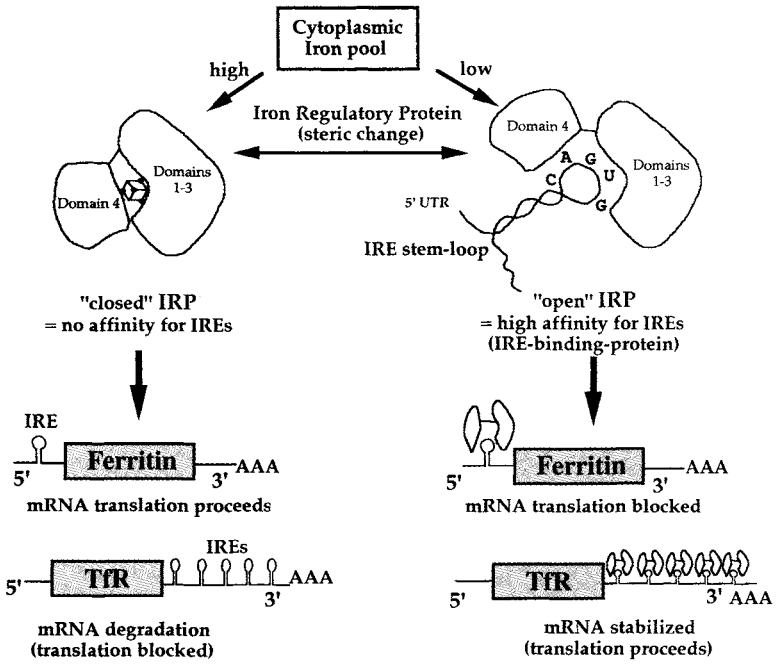

Fig. 3. Iron-dependent co-ordinated control of ferritin and transferrin receptor expression at the translational level.

the IRE's (Fig. 3) with high affinity. When the iron supply to cells is increased, IRP-1 incorporates an iron sulphur cluster, converting it to a form which is unable to bind to IREs, whereas IRP-2 is rapidly degraded. Both IRP-1 and IRP-2 have high affinities for wild-type IREs (Henderson et al., 1993; Guo et al., 1994; Samaniego et al., 1994), and are strongly induced in iron-deficient cells while their IRE-binding capacity is rapidly lost after iron administration. IRP-1 is expressed in all vertebrate tissues (Henderson et al., 1993; Patino & Walden, 1992) with measured levels of between 50,000 to 100,000 molecules per cell (Müllner et al., 1989; Haile et al., 1989). Alterations in IRP-1 activity occur without significant changes in the total amount of IRP-1. It is proposed that the apo-IRP-1 (without its iron–sulphur cluster) is able to bind to RNA with high affinity, whereas the holo-IRP-1 (with the 4FE-4S cluster) is unable to bind RNA, but has aconitase activity. IRP-1 is presumed to form a four-domain protein with a deep cleft between domains 1 and domain 4 connected by a flexible hinge linker. Solution studies imply amino acid residues 121–130 and the region close to Cys437 as being directly in contact with IRE (Paraskeva & Hentze, 1996). IRP-1 containing the 4Fe–4S cluster is inactive in IRE-binding, whereas, in iron deficient cells, the apoprotein form accumulates and is active in binding to IREs. The interconversion of these two forms constitutes the iron sensor which in iron replete cells allows ferritin synthesis to take place while in iron deficient cells transferrin receptor synthesis is increased. Therefore in iron deficiency anaemia, there would be upregulation of transferrin receptor translation and any transferrin–iron or transferrin–aluminium bound would have greater access to the cell.

IRP-2 is less abundant than IRP-1 in most cells, and tissue distribution studies have indicated greatest expression in intestine and brain (Henderson et al., 1993; Samaniego et al., 1994). Human IRP-2 is 57% identical and 79% similar to human IRP-1, but is slightly larger with a molecular weight of 105kD due to the presence of a 73 residue inclusion as compared to IRP-1. No aconitase activity has been found for IRP-2, although it has the equivalent cysteine residues that coordinate the iron sulphur cluster in IRP-1. Whereas the apo-protein binds to IREs like IRP-1, in contrast to IRP-1, IRP-2 is degraded in iron-replete cells.

The 5′UTR's of both H and L chain ferritin mRNAs contain putative stem-loops (Aziz & Munro, 1987) in which there is a highly conserved sequence of 28 nucleotides (Fig. 2). These sequences are known as iron regulatory elements (IREs) which have extensive homology with the single IRE found in both the 5′UTR of erythroid cell δ-aminolaevulinate synthase and mitochondrial aconitase, as well as with those found in the 3′UTR of transferrin receptor mRNA. The location of the IRE in the 5′UTR of ferritin mRNA suggested that it functioned to control initiation of translation and ribosomal binding. The position of the single-copy 5′-UTR IRE's in ferritin, erythroid cell δ-aminolaevulinate synthase and mitochondrial aconitase mRNA's is absolutely crucial for their activity as translational regulators.

An IRE mRNA stability element has only been found to date in the transferrin receptor mRNA. There are five IRE's in the 3′UTR of TfR mRNA, which are highly conserved. They all share the same structural features with other IRE's sufficient for IRP recognition. When IRPs bind to the IREs in transferrin receptor mRNA, they stabilise the mRNA against nuclease degradation, and permit synthesis of the transferrin receptor. In contrast, in the absence of IRP binding, the transferrin receptor mRNA is degraded, preventing its synthesis. Concomitantly the translational blockage of the ferritin mRNA is removed, and synthesis of the storage protein can proceed.

It was therefore important to know whether aluminium could interfere with iron homeostasis namely by altering IRP-1 and IRP-2 levels in brains of Alzheimer patients. Smith et al. (1998) showed that IRP-1 levels in Alzheimer brains were similar to that of control brains although aluminium levels were not assayed. In contrast IRP-2 showed important differences, and was associated with intraneuronal lesions, including neurofibrillary tangles, senile plaque neurites and neuropil threads (Smith et al., 1998). Although IRP2 has no Fe–S cluster it does exhibit several redox sensitive cysteine residues; (Bouton, 1996). In vitro studies of murine erytholeukemia cell lines indicated that the presence of aluminium in such cells antagonised the iron induced decrease in the IRE binding activity of IRP-2, the aluminium stabilising the IRP-2 preventing its degradation such that the IRE binding of IRP-2 was enhanced (Yamanaka et al., 1999). This would result in upregulation of transferrin receptors with an increased flux of metals associated with transferrin, namely iron and aluminium.

Many studies have investigated the effects of increased aluminium accumulation upon iron homeostasis in experimental animal models as well as cell lines. The brains of experimental animals which have been loaded with aluminium intraperitoneally show elevation of both aluminium and iron after three months of loading with aluminium gluconate (Florence et al., 1995) (Table 2). Aluminium loaded cultured Friend erythroleukemia cells show increased iron uptake by transferrin receptor-mediated endocytosis (Albeo et

Table 2. Aluminium and iron content of specific brain regions after IP aluminium gluconate loading (2 mg Al, 3 times a week) for eight weeks

Tissue	Aluminium (ng Al/mg protein)		Iron (ng Fe/mg protein)	
	control rat	aluminium loaded rat	control rat	aluminium loaded rat
Liver	0.2 ± 0.06	8.89 ± 2.41**	112 ± 26	2365 ± 511**
Cortex				
Frontal	0.35 ± 0.05	1.42 ± 0.18**	6.5 ± 3.4	11.54 ± 1.11*
Temporal	0.25 ± 0.05	2.23 ± 1.02**	3.7 ± 1.3	24.35 ± 2.13**
Parietal	0.23 ± 0.05	1.53 ± 0.23**	2.2 ± 0.07	4.32 ± 0.87*
Hippocampus	0.53 ± 0.13	2.25 ± 0.36*	4.11 ± 1.07	23.3 ± 7.0**

al., 1994); the increase in iron uptake was not paralleled by increased iron exit. Ferritin synthesis was depressed which may be due to the fact that the elevated iron was localised within the mitochondria such that the cell was unable to sense changes in cellular iron homeostasis as the iron sensors IRP1 and IRP2 are present in the cytosol.

Incubation of a murine nuroblastoma cell line with either aluminium transferrin or citrate (Abreo et al., 1999) resulted in an increased aluminium uptake, as well as increased rates of uptake of iron and transferrin In contrast, Oshiro et al. (1998) showed that aluminium loading of primary cultures of foetal rat cerebral cortical cells increased transferrin independent iron uptake, but decreased the transferrin receptor mRNA level and increased aconitase activity of the IRP 1 protein.

The Ability of Aluminium and Iron to Permeate the Blood–Brain Barrier (BBB)

The flux of these two metals across the BBB either under normal circumstances or in patients with neurological lesions remains unknown. The BBB appears to form a very efficient barrier under normal circumstances to exclude iron passage across BBB. Gallium[67] has been used to investigate BBB permeability (gallium has stability constants for transferrin similar to iron, log K for the two binding sites of 20.3 and 19.3) and enters rat brain via the BBB and localises to transferrin receptors in the hippocampus, amygdala and cerebral cortex (Pullen et al., 1990). Other studies where an aluminium glutamate complex was used also showed that in this form aluminium was able to pass the BBB and deposit aluminium in the brain cortex (Deloncle et al., 1990).

Iron Homeostasis and Aluminium Toxicity in the Brain

In normal circumstances iron is predominantly sequestered to the oligodentrocytes, possibly by transferrin as this protein is also evident within these cells. Transferrin receptors are expressed on blood vessels, large neurons in the cortex, striatum and hippocampus as well as oligodentrocytes and astrocytes. Connor et al. (1992) investigated the distribution of transferrin and ferritin in Alzheimer diseased brain tissues by

immunohistochemical and histochemical staining; these two iron binding proteins were predominantly in oligodendrocytes in a similar fashion to that of normal brain tissue although transferrin was also distributed around the senile plaques and found in the astrocytes of the cerebral cortical white matter as well as the microglia. Transferrin is detectable immunocytochemically in senile plaques of Alzheimer's disease brains, and when the Tf allele frequency was assayed, unexpectedly the C2 allele frequency in AD patients homozygous for the ApoE epsilon 4 allele is markedly increased two-fold (Namekata et al., 1997). Morris et al. (1994) investigated the distribution of transferrin receptors in the normal and AD hippocampal brain using [3H]-transferrin ([3H]-Tf) binding and identified significant reductions in [3H]-Tf binding in the hippocampal CA1, CA2 and CA4 pyramidal cell layers and in areas where senile plaques and neurofibrillary tangles occurred. Such results indicated that another iron uptake system apart from that of the transferrin mediated system might be significant in the pathogenesis of Alzheimer's disease.

Recently receptors for ferritin have been identified in white matter of the brain (Hulet et al., 1999), although there are no reports of their distribution in brain of normal subjects or Alzheimer patients. Early studies of brain ferritin isolated from orally aluminium supplemented rats, and from Alzheimer patients (Fleming & Joshi, 1987) or after in vitro incubation of ferritin with aluminium citrate (Cochran & Chawtur, 1988) found significant amounts of aluminium associated with ferritin. However later studies (Dedman et al., 1992a) of isolated ferritin from the brains of either renal dialysis patients (high circulating aluminium levels), Alzheimer patients, or in vitro after its incubation with aluminium salts (Dedman et al., 1992b) showed that there was less than 10 aluminium atoms per molecule of ferritin.

The role that melanotransferrin may play in iron uptake in Alzheimer patient's brain could be an important factor in the iron loading; it has been detected in a subset of reactive microglia associated with senile plaques (Jefferies et al., 1996), although further studies are needed.

Lactotransferrin has a similar structure to transferrin, and is considered to be an antioxidant possibly functioning by scavenging excess iron. Lactotransferrin expression was up-regulated in both neurons and glia in affected AD tissue (Kawamata et al., 1993; Leveugle et al., 1994) and was very strongly associated with amyloid deposits and extracellular neurofibrillary tangles. It was also identified in a minority of intracellular neurofibrillary tangles, neuropil threads, and degenerative neurites. It is possible that affected neurons in Alzheimer's disease either take up or synthesise lactotransferrin at an abnormally elevated rate. This excessive accumulation of lactotransferrin may lead to cytotoxic effects resulting in the formation of intracellular lesions and neuronal death.

Does the Elevated Brain Iron Content Cause Redox Changes?

The presence of increased amounts of iron in the brain may indicate that redox changes have occurred, which would alter the activities of certain cytoprotective enzymes and antioxidants. However our studies (Zhang, 1995) showed that, despite such increases of iron in various brain regions of experimentally aluminium loaded rats, this was not accompanied by changes in the activities of catalase, glutathione reductase and

glutathione. Only superoxide dismutase activity in the frontal cortex was significantly enhanced ($P<0.05$) after two months of aluminium gluconate loading. In contrast, other studies of experimental aluminium overload in animals, showed decreases in glutathione reductase (Katyal et al., 1997), superoxide dismutase, catalase and glutathione peroxidase in the brain (Julka & Gill, 1996) although the iron content was not assayed. However only a very short period of aluminium overloading was used such that it may have been insufficient to aluminium load the brain. Another study where the cytoprotective enzymes were assayed in the frontal cortex of AD patients reported no changes in SOD and glutathione peroxidase activities (Hayn et al., 1996), as well as no changes in malondialdehyde, a marker of lipid peroxidation, in frontal, parietal and occipital cortex when compared to controls.

Informed Opinion

It is of considerable interest that of the four mechanisms which have now been proposed for iron uptake and transfer across the GI tract, aluminium uptake by at least two of them would be excluded. Furthermore the suggestion that aluminium uptake and transfer would be enhanced during iron deficiency anaemia (Fernadez-Menendez et al., 1991) is unlikely as it is these two pathways which require an oxidation-reduction step which are unregulated during periods of iron paucity. This would effectively prevent their utilisation by aluminium. Aluminium uptake and transfer by the GI tract of Alzheimer's patients would appear to be enhanced (Moore et al., 2000) although only few patients have been studied. Furthermore it is unknown whether in such patients elevated levels of aluminium are detectable within the blood.

Various studies have attempted to investigate the speciation of aluminium in blood in an attempt, firstly to ascertain the pattern of aluminium binding to plasma proteins and low molecular weight species, and secondly to ascertain whether a transport system for aluminium across the blood brain barrier could be established. Although transferrin has many variants (C1 and C2 accounting for the majority of the population in all races) there is no convincing evidence that transferrin variants have any influence on either iron or aluminium binding or delivery in Alzheimer's disease.

Early studies which detected the presence of aluminium in the neurofibrillary tangles and/or the amyloid plaques began the debate as to how aluminium might be able to traverse the blood brain barrier. Aluminium is certainly able to traverse the BBB, either as a low molecular weight complex (using either gallium (an analogue of aluminium) or Al^{26} (Farrar et al., 1990)) or bound to transferrin, after interaction with transferrin receptors at the blood brain barrier (Roskams & Connor, 1990). Whether the permeability of the BBB to metal ions such as iron and aluminium is altered in patients with Alzheimer's disease remains unclear. It is of interest that untreated genetic haemochromatosis and thalassaemia major patients, with a high transferrin saturation in their plasma, i.e. 100% saturation such that a non transferrin bound iron (NTBI), is detectable, show no reported increased incidences of either Parkinsons disease or other neurological lesions. This might indicate that iron bound either to transferrin or NTBI is unable to permeate the BBB.

In renal dialysis patients with increased circulating levels of aluminium, there is increased deposition of aluminium in the brain, particularly in neurons with high densities of transferrin receptors in the cortex and hippocampus (Edwardson et al., 1992). In addition approximately 30% of such patients show beta-amyloid deposits. However neurofibrillar tangles are not observed in such patients indicating that aluminium may not directly be involved in their formation. However aluminium accumulation in various cell lines (Albeo et al., 1999) in the brains of experimental animals (Troncoso et al., 1986) as well as patients with Parkinsonism dementia/amyotropic lateral sclerosis of Guam does result in the presence of neurofibrillar protein thus indicating a link with aluminium accumulation. Differences in the chemical composition and ultrastructure of these neurofibrillar tangles between animal models and Alzheimer's disease do exist, paired helical filaments are found in patients while a single straight filament is present in animal models (Wisniewski & Wen, 1992).

However it remains a matter of debate as to the role of aluminium in the formation of the neurological lesions associated with Alzheimer's disease (Chafi et al., 1991). It is clear that there is an increase in the iron content of senile plaques and tangles and pyramidal neurons (Watt, 1996; Makjanic et al., 1998), thereby substantiating the fact that some disruption of iron homeostatic mechanisms does occur in Alzheimer patients.

Although it is established that IRP activity is a critical determinant of the expression of a number of proteins of iron metabolism, many issues are not understood. There is evidence which suggests that there can be selective regulation of IRP1 and IRP2 such that it is of interest to know whether there are specific mRNA targets for one or the other IRP. It is unknown whether there is cross talk between IRP-1 and IRP-2 to modulate iron metabolism.

The role of phosphorylation in the regulation of IRP-1 and IRP-2 remains enigmatic (Eisenstein, 2000) but may be related to the stabilisation of IRP-2 by aluminium in the presence of iron. Aluminium may interfere with calcium and magnesium homeostasis; both of these divalent metals playing important roles in the phosphorylation process.

Although aluminium can enter the cell via the transferrin–transferrin receptor pathway it is unknown whether accumulation of aluminium within the endosome will induce toxicity with the subsequent release of aluminium into the cytosol. The evidence that hypotransferranaemic mice can transport iron into cells indicates that there is another pathway, as yet undefined, which could be utilised by aluminium. A candidate iron transporter protein has been identified as a 160 kDa trimer in brush border plasma membrane of the upper intestine, although such a carrier awaits identification in other tissues (Teichmann & Stremmel, 1990). The transferrin independent pathway for the uptake of iron and other transition metals remains to be elucidated. However when iron and aluminium use this pathway, there is down regulation of transferrin receptors indicating that the cell is sensing the increased flux of these two elements into the cell and the accumulation within the LMW pool. Why aluminium should induce these changes is unknown but might be related to an increase in the transporter protein. However NTA is notoriously toxic to cells such that the use of LMW complexes of iron and aluminium should also be investigated in future studies to confirm the observations of Oshiro et al. (2000).

We would endorse the work of Dedman et al. (1992b) that ferritin is unable to accumulate aluminium. It is known that for iron incorporation into ferritin, an oxidation

reduction step is necessary which would effectively exclude the ability of aluminium to enter ferritin. Our recent studies (Ward, Day,King and Crichton unpublished data) with Al^{26} showed that less than 1% of the injected Al^{26} was associated with isolated liver ferritin. In vitro studies by Dedman et al. (1992) did show that aluminium could be incorporated into ferritin when the iron storage protein was reconstituted in the presence of both iron and aluminium citrate, up to 120 atoms of aluminium/atom. Once the nucleation centre within ferritin is formed, aluminium may indeed enter ferritin. However we believe that many of the in vitro studies of aluminium incorporation into ferritin have not been carried out under physiological conditions. Whether aluminium can interfere with iron uptake into ferritin is unknown.

Changes in iron homeostasis would appear to be a major contributory factor in Alzheimer's disease, although whether this is the primary or secondary effect is unanswered. Despite the initial studies which implicated aluminium as a major factor in the development of senile plaques and neurofibrillary tangles, present results would indicate that the brain regions of Alzheimer patients do not always show significant increases in aluminium content. However if aluminium is able to enter the systemic circulation, it seems to be able to traverse the blood brain barrier and form deposits in the brain.

The presence and synthesis of amyloid beta peptides may be accelerated by the presence of Fe as well as Cu and Zn. In addition AβP looses its helical conformation in the presence of 10, 20 and 50 dm^{-1} aluminium (Exley et al., 1993). The binding of Zn^{++}, Cu^{++} and Fe^{++} ions to Alzheimer's A beta peptide were studied by fluorescence titration and it was shown that the metal binding to peptide affinities were Fe<Cu>Zn (Garzon-Rodriguez et al., 1999). Furthermore the binding of these transition metals to the amyloid beta peptides appears to preserve their catalytic redox activity (Sayre et al., 1999). It has also been suggested in a few papers (Potter, 1992; Blom & Linnemann, 1992; Kalaria et al., 1996) that the amyloid beta protein deposition in Alzheimer brains is associated with a predominantly local acute phase response that causes release of various inflammatory and immune system mediators. Such an inflammatory response could explain why iron accumulates within the neurological lesions of Alzheimer's disease and also why the activities of cytoprotective enzymes and antioxidant content are not significantly altered.

The recent reports of defective copper homeostasis in Alzheimer patients (Torsdottir et al., 1999) (i.e. significantly lower serum levels of caeruloplasmin and ferrioxidase activity than age matched controls) was noteworthy, since caeruloplasmin plays an important role in the egress of iron from cells and its decreased activity might be one of the explanations to explain the accumulation of iron in cells. Erythrocyte SOD activity was advocated to be a marker of the disease state as it diminished with the duration of the disease (Torsdottir et al., 1999) although the relevance of this enzyme, as a marker of copper status (DiSilvestro et al., 1992) remains undefined.

Copper and iron homeostasis are intertwined, although the exact mechanism awaits clarification (Ward et al., 1998). As yet no information of such inter-relationship within specific brain regions has been studied.

Although there is no definitive treatment for Alzheimer's disease, various pharmacological strategies are being actively investigated. Antioxidant therapy should disrupt or prevent the free radical/beta amyloid recirculating cascade and the progressive neu-

rodegeneration. The role of copper in the pathogenesis of Alzheimer's disease remains unknown and further studies of brain copper concentrations as well as the use of copper chelators may identify its role.

The use of non-steroidal anti inflammatory drugs may prevent the inflammatory response, which occurs possibly as a result of the amyloid protein, and therefore diminish the deposition of both iron and copper. An inverse relationship between use of anti-inflammatory drugs and the occurrence of Alzheimer's disease has been found. Removal of both iron and aluminium by iron chelators desferrioxamine and other hydroxypyridones, could be of therapeutic efficacy.

Iron is a major contributor to many neurological diseases, and clarification of its aetiology and pathogenesis is clearly an important area for research within this millennium such that appropriate therapeutic agents can be developed.

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Aluminium and Alzheimer's Disease
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CHAPTER 16

Oxidative and Inflammatory Properties of Aluminum: Possible Relevance in Alzheimer's Disease

Stephen C. Bondy * and Arezoo Campbell

Department of Community and Environmental Medicine, Center for Occupational and Environmental Health, University of California Irvine, Irvine, California 92697-1820, USA

* Corresponding author, email: scbondy@uci.edu

Abbreviations: Al – aluminum; AD – Alzheimer's disease; ROS – reactive oxygen species; NFT – neurofibrillary tangles; HNE – 4-hydroxynonenal; SOD – superoxide dismutase; TNF – tumor necrosis factor; IL – interleukin; ACT – antichymotrypsin; NSAIDs – nonsteroidal anti-inflammatory drugs; iNOS – inducible nitric oxide synthetase; CNS – central nervous system; GFAP – glial fibrillary acidic protein

Summary

Both inflammation and the production of reactive oxygen species may be beneficial short-term response to extracellular stressors, and kill pathogens that have accessed nervous tissue. These factors, however, when active over an extended period following an ineffectual response to a persistent stimulus, can become harmful. Colloidal Al could constitute such irresolvable foci that cannot be cleared by activated microglia. This can lead to chronic inflammatory responses that eventually overwhelm neurons and impair their function.

Historical Perspective

The toxicity of aluminum (Al) has been the subject of much research in the past few decades. Although it has been believed that environmental levels of the metal are generally innocuous to human health, a causal role for Al has been established in dialysis dementia (Alfrey et al., 1976), osteomalacia (Bushinsky et al., 1995) and microcytic anemia without iron deficiency (Touam et al., 1983). Aluminum has also been implicated in Alzheimer's disease (AD) by epidemiological reports and studies describing elevated levels of Al in AD brains although this remains a controversial issue. Other clues come from disorders related to AD. In Down's Syndrome, which leads to early onset of AD (Schupf et al., 1998), aluminum has been reported to be absorbed from the gastrointestinal tract to a greater extent than normal (Moore et al., 1997). The transgenic TS65Dn mouse model for

this disorder which bears a trisomic segment of murine chromosome 16 homologous to human chromosome 22, has a significantly higher brain Al content than the corresponding diploid control (Berg et al., 2000). The exact mechanism of Al toxicity is not known but there is considerable evidence that show the metal's capacity to exacerbate the generation of reactive oxygen species (ROS) despite the fact that Al is a trivalent cation incapable of redox changes.

Correlation with, and Possible Causation of, Alzheimer's Disease with Excess Generation of Free Radicals

Recent studies suggest that oxidative stress may play a role in a wide range of neurological diseases including AD (Bondy, 1998). The frontal cortex of AD patients shows a significantly higher ability to produce ROS compared to control brains (Zhou et al., 1995). Carbonyl modifications are increased in the AD brain, especially in neurofibrillary tangles (NFT), and this oxidative marker may provide a clue for the mechanism by which the cytoskeletal abnormality forms and leads to the pathological lesions (Smith et al., 1996).

A significant increase in lipid peroxidation has been found in the temporal cortex of AD patients when compared to age-matched control brains (Marcus et al., 1998). Levels of 4-hydroxynonenal (HNE) an advanced end product of lipid peroxidation, are elevated in the amygdala, hippocampus and the hippocampal gyrus of AD patients (Markesbery & Lovell, 1997). A parallel increase in the level of free HNE in the ventricular fluid of patients with AD has also been reported (Lovell et al., 1997). When AD brains are treated with antibodies against both 4-hydroxynonenal and neurofibrillary tangles, those neurons lacking tangles also display HNE-pyrrole immunoreactivity (Sayre et al., 1997). This implies that oxidative stress is not merely a consequence of tissue damage but may be a pre-existing state, which may subsequently lead to neuronal damage. Smith et al. (1998) showed that HNE and the antioxidant enzyme heme oxygenase-I, as well as tau-reactive dystrophic neurites, are all located at the periphery of amyloid plaques. HNE is capable of modulating the tau protein by covalently binding to it and this may lead to increased phosphorylation of tau protein (Mattson et al., 1997).

Role of Aluminum on ROS Promotion

Iron is a pro-oxidant metal present in most cell compartments, and aluminum potentiates the capability of Fe to promote oxidative stress and lipid peroxidation in isolated biological preparations (Gutteridge et al., 1985, Oteiza et al., 1993, Ohyashiki et al., 1998, Bondy and Kirstein, 1996). It has been proposed that metals without redox capacity such as aluminum can make fatty acids more available to attack by free radicals, thus facilitating the propagation of lipid peroxidation (Oteiza et al., 1993, Ohyakishi et al., 1998). Aluminum is capable of enhancing iron-based oxidant events in protein-free liposomes with a negative charge on their outer surface suggesting that the electrostatic attraction of cations to the surface negative charge plays a role in metal-induced potentiation of

ROS (Bondy et al., 1998a). Since the potentiation of iron-based ROS production by aluminum can occur in liposomes containing no protein, altered membrane configuration or competition for iron-binding sites on proteins cannot be invoked in order to account for this phenomenon.

Both aluminum and β-amyloid peptides are not intrinsically pro-oxidant but there is a strong resemblance between their ability to stabilize iron in the ferrous form and thus enhance the Fenton reaction (Yang et al., 1999). This may provide a mechanism by which these materials can promote free radical generation by iron and also suggests parallels between the toxicity of amyloid peptides and Al salts.

In vivo studies have demonstrated that aluminum plays a role in ROS generation. Intraperitoneal injection of aluminum gluconate, over a 21-day period, increases the rate of ROS formation in cortical tissue (Bondy et al., 1998b). The brain of rats treated with aluminum lactate for 4 weeks showed an increase in lipid peroxidation and a significant decrease in the antioxidants, superoxide dismutase (SOD), catalase, and glutathione peroxidase (Julka & Gill, 1996). This is paralleled by the finding that SOD and catalase levels were depressed in the temporal cortex of AD patients (Marcus et al., 1998).

Reactive oxygen intermediates have been a threat to organisms ever since the advent of aerobic metabolism. When bacteria are exposed to ROS, they initiate the synthesis of an array of proteins with protective functions (Müller et al., 1997). It is likely that throughout evolution, this response of organisms to reactive oxygen compounds, has led to the ability of cells to utilize these oxidant molecules in promoting a defensive reaction to signals associated with pathogenic events. Exposure of rat glioma cells to aluminum sulfate for 48 h caused an increase in the generation of ROS. However, the salts did not elicit a similar response in rodent neuroblastoma cells (Campbell et al., 1999). This effect was reproduced in human cell lines exposed to different concentrations of aluminum (Campbell and Bondy, 2000). This substantiates the concept that short-term production of reactive oxygen intermediates may not simply be undesired products of oxidizing reactions but may be important stress-induced messenger molecules. Indeed, hydrogen peroxide has been shown to mediate important events in the initiation of innate immunity by controlling the activation of the transcription factor NF-κB, which is involved in inflammation and the immune response (Schmidt et al., 1995).

There is a great evolutionary conservation of components of the innate immune response in organisms that diverged over a billion years ago. The existence of proteins required for defense against infection has been found in *Drosophila*. The NF-κB system in mammals is a homologue of these transcription factors. The protein domains of these activators of the host defense system have been highly conserved in organisms as divergent as plants, *Drosophila*, and mammals (Medzhitov & Janeway, 1998).

NF-κB is activated by an array of different pathogenic conditions. Viral and bacterial products, eukaryotic parasites, inflammatory cytokines, physical and oxidative stress, as well as some drugs such as phorbol esters, all activate NF-κB (Baeuerle & Henkel, 1994). This pathway controls the expression of genes involved in stress and inflammation (Schreck et al., 1992; Beauparlant & Hiscott, 1996). NF- κB is present in the cytoplasm as an inactive complex bound to the inhibitory subunit IκB. In this conformation, the transcription factor is unable to translocate to the nucleus. However, extracellular stress factors can result in the phosphorylation and subsequent release of the inhibitory subunit

(Schreck et al., 1992). Degradation of the IκB depends on phosphorylation of the subunit by a kinase complex that is activated by cytokines. This leads to the ubiquitinylation and proteolytic obliteration of the inhibitory subunit (Stancovski & Baltimore, 1997). Once the dimer is free, it can then enter the nucleus and bind to the promoter region of a variety of genes involved in the stress response and immunity.

The common factor responsible for the release of IκB appears to be the cell's redox status, which is determined by the levels of reactive oxygen intermediates. Very low amounts of hydrogen peroxide, but not superoxide, has been shown to activate NF-κB. In cells, which over express hydrogen peroxide or superoxide dismutase (an enzyme that converts superoxide to hydrogen peroxide), there is an increase in tumor necrosis factor (TNF)-induced NF-κB activation. (Schmidt et al., 1995). Hydrogen peroxide may function as an extracellular messenger since it is relatively stable, uncharged and thus diffusible. It is also readily degraded by catalase and thus is easily detoxified (Müller et al., 1997).

Macrophages and microglial cells produce H_2O_2 at inflammatory sites and several other factors, which activate the transcription factor, also increase ROS production. Factors leading to such activation include UV radiation, LPS, TNF, and IL-1 (Baeuerle & Henkel, 1994). In the case of TNF-induced activation of NF-κB, alteration in mitochondrial electron flow has been shown to underlie the increased production of ROS (Schmidt et al., 1995). The relevance of NF-κB to neurodegeneration is further suggested by a correlation between the amount of activated NF-κB and a key inflammatory enzyme, COX-2, in both aging and AD temporal lobe neocortex (Lukiw & Bazan, 1998).

The Role of Inflammation in Alzheimer's Disease

In the cerebrospinal fluid of AD patients, the level of interleukin-1 (IL-1) type II receptor is elevated (Garlind et al., 1999). IL-1β is a very strong inducer of IL-6 and this stimulation is dependent on transcription and protein synthesis (Cadman et al., 1994). Both cytokines induce the synthesis of acute-phase proteins such as antichymotrypsin (ACT) and TNF, (Dunn, 1991).

Microglia appear to play an active role in neurodegeneration by becoming activated following irresolvable neuroinflammation, and secreting complement proteins, oxygen radicals, cytokines, prostaglandins and adhesion molecules that can be toxic to healthy neurons. This process which may be consequent to a futile attack upon indigestible amyloid aggregates, may ultimately lead to dementia (Bondy and Campbell, 2000). Since the pathological lesions of the AD brain contain activated microglia and astrocytes associated with elevated levels of cytokines, complement proteins and acute phase proteins, it may be that progression of the disease involves chronic neuroinflammation.

The prevalence of AD in rheumatoid arthritis patients, who consume anti-inflammatory drugs over an extended period, is lower than in the normal population (McGeer et al., 1990). Seventeen separate epidemiologic studies all suggest that anti-inflammatory drugs may play a protective role against AD (McGeer et al., 1996). Nonsteroidal anti-inflammatory drugs (NSAIDs) are able to suppress microglial activation (Mackenzie et al., 1998). By this means, NSAIDs may reduce the inflammation associated with senile

plaques and thus slow down the disease process. Ibuprofen, a cycloxygenase-inhibiting NSAID, has been shown to decrease levels of inducible nitric oxide synthetase (iNOS) mRNA in primary glial cell cultures (Stratman et al. 1997) and the glucocorticoid dexamethasone dose-dependently reduces the release of IL-6 from human astrocytoma cell lines (Blom et al., 1997).

Lipopolysaccharide-induced chronic inflammation can cause extensive astrogliosis in the temporal lobe regions of the rat brain. The activation of the astroglial cells is associated with an increase in the production of IL-1β, hippocampal cell loss, and impairment of spatial memory, all of which mirror changes seen in the AD brain (Wegrzyniak et al., 1998).

Aluminum-Induced Inflammation

Several studies have found that Al can cause inflammation in a variety of non-nervous tissues. Low doses of aluminum, present in parenteral nutrition formula, can produce marked portal inflammation correlating with the duration of exposure and the amount of Al accumulated in the liver (Demircan et al., 1998). Rats exposed to oral doses of aluminum chloride and aluminum lactate expressed an increase in plasma α 1 globulins, consequent to inflammation (Cherroret et al., 1995). Alum precipitate, which is composed of a suspension of aluminum hydroxide, is used as an adjuvant in vaccines used to inoculate humans. The effectiveness of the adjuvant is attributed to the irritant effect of alum, which increases macrophage processing of the antigen (Benjamini & Leskowitz, 1991). Aluminum sensitization can develop in some children vaccinated for diphtheria, tetanus and pertussis, using vaccines, which contain aluminum hydroxide as an adjuvant. Al-induced inflammatory nodules are sometimes formed in adults revaccinated for hepatitis B (Cosnes et al., 1990).

There was a correlation between the total concentration of Al deposited with fibrosis and focal lung inflammation in an occupational health study of workers exposed to metals and aluminum. Workers with the mildest histological findings also had the lowest concentration of Al particles analyzed from transbronchial biopsies (Schwarz et al., 1998). A case study of a 72 year old woman with end stage renal failure reported elevated serum Al content as well as amyloid deposits in her joints. The synovial region contained an amorphous material surrounded by chronic inflammatory cells. The mineralization front of her bones stained positive for aluminum and she showed signs of osteomalacia (Isaacs et al., 1992). To determine whether aluminum is responsible for the articular toxicity often found in chronic renal failure patients on hemodialysis, rats were injected in the knee with either Al hydroxide or Al lactate. The aluminum hydroxide remained in the vicinity of the injection site for an extended period and induced an increase in the number of leukocytes. Aluminum lactate caused an increase in the infiltration of inflammatory cells and also caused an increase in the production of eicosanoids (Chary-Valckenaere et al., 1994).

Primary glial cells are more vulnerable than neurons to long term exposure to aluminum chloride. While primary cerebellar neurons, containing only 1% glial cells, did not exhibit susceptibility to aluminum chloride, neuronal–glial mixed cultures consisting of 10% glial cells showed a marked decrease in neuronal viability. This suggests the

activation of glia by Al salts leading to harmful effects on neurons. Aluminum was found to be associated with the cells and the level of the association of aluminum with cells was higher in the mixed cultures than in the primary neurons, suggesting a basis for the responsivity of glial cells to Al (Suárez-Fernández et al., 1999). We have also reported that, following incubation with Al salts, cells of glial origin are associated with aluminum to a greater extent than those of neural origin (Campbell et al., 1999). Finally, Al induced apoptosis only in primary astrocytes and not in primary neuronal cultures (Suárez-Fernández et al., 1999). This supports the concept that neurodegeneration may initially be due to the compromised state of the astroglial cells leading to the secondary loss of viability and function of neuronal cells. Aluminum exposure may activate glial cells and enhance oxidant processes occurring within them, thus indirectly jeopardizing the integrity of neuronal cells.

Informed Opinion

Cytokines in Nervous Tissue

The central nervous system is largely but not completely isolated from surveillance by the systemic immune system by the blood brain barrier (Brightman et al., 1995). However, local immune responses are important in cerebral defense processes. As a result of the presence of pathogens or the occurrence of trauma, a range of interleukins, such as IL-1, IL-6 and IL-8, can be synthesized, by activated microglia and macrophages (Dunn, 1991). Their normal function is to recruit more of these cell types to the pathologic site and thus coordinate the immune response. Their integrated action can effect wound healing, destruction of pathogens and the regulation of tissue regrowth and wound healing. These antigen-nonspecific soluble factors are not stored within cells but are synthesized and secreted as needed. They typically have a short half-life but can damage the CNS when their presence is prolonged. Extended production of these chemotactic factors can lead to cytotoxicity due to the recruitment and activation of cells producing high concentrations of ROS (Dunn, 1991).

Superoxide and nitric oxide are necessary for the destruction of invasive pathogens through cell-mediated killing. This may explain why ROS formation in response to a stressor such as aluminum, is only increased in the immunocompetent cells derived from glia and not in cells of neuronal origin (Campbell et al., 1999; Campbell and Bondy, 2000). The cell generally has enough antioxidant reserve so that it is protected against the harmful effects of reactive oxygen intermediates. Cells also have the capacity to react to oxidative stress by production of increased amounts of antioxidant enzymes. Only when the antioxidant capacity of cells are overwhelmed can these intermediates cause cell damage (Keller et al., 1997; Blanc et al., 1997).

When the cell is functioning normally, the production and detoxification of ROS is tightly controlled. However, chronic exacerbation of levels of ROS can ultimately compromise cell integrity. Thus, controlled free radical formation in conjunction with an acute inflammatory event may serve a benign regulatory function, but if these processes occur chronically, they can eventually be harmful.

The outcome of the cellular immune response at the cell level to foreign antigens is determined by the nature of the substance eliciting the reaction. Soluble materials will be digested by phagocytes and the inflammation will resolve itself. Bacteria can be phagocytosed and also dispersed by proteolytic processes. However, if the agent is indigestible, it can persist and result in a state of chronic inflammation (Dunn, 1991). Since aluminum salts form colloidal species in solution, the aluminum-induced increases in ROS generation observed in intact cells may result from an innate immune response to extracellular aluminum particles.

There is limited direct evidence of Al-induced inflammatory events in the diseased central nervous system. Extended aluminum lactate treatment of rabbits increased glial fibrillary acidic protein (GFAP) concentrations in the frontal cortex (Yokel & O'Callaghan, 1997). Reactive astrocytes, which produce GFAP, are associated with both senile plaques and cerebral microvessels (Cullen et al., 1997) and GFAP is increased in the temporal cortex of AD patients (Panter et al., 1985). The finding that Al salts can increase the expression of activated NF-κB in isolated human glioblastoma cells constitutes further evidence for a primary inflammatory role of glia following aluminum intoxication (Campbell & Bondy, 2000).

The cerebral microvasculature becomes more prone to damage during aging and this may result in the compromise of the blood brain barrier (BBB) (Kemper, 1984). Since

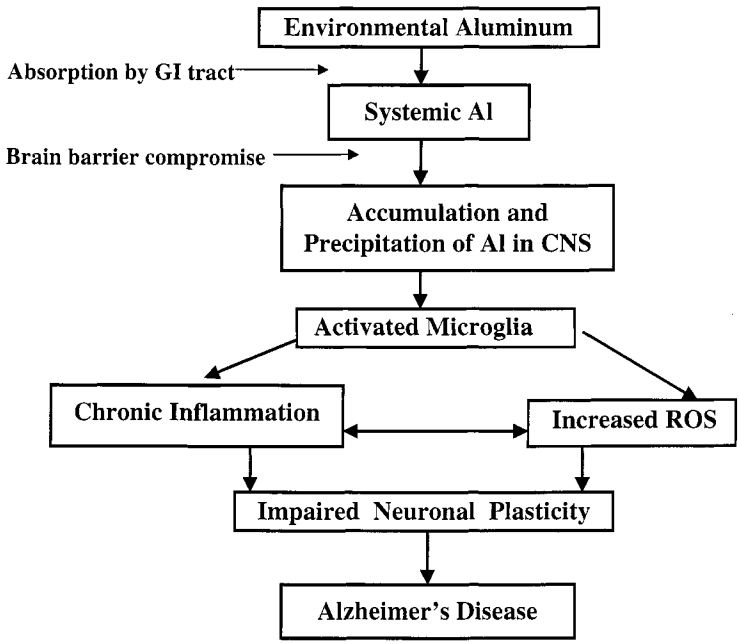

Fig. 1. The potential link of aluminum exposure to Alzheimer's disease. Accumulation of extracellular aluminum in the CNS leads to an innate immune response comprising of increased inflammatory and oxidative events. The persistence of insoluble aluminum particles leads to unresolved microglial inflammation and consequent continuation of the production of harmful reactive oxygen species, which lead to neuronal malfunction.

this barrier is the major mechanism by which the brain keeps out foreign antigens, jeopardizing the BBB could lead to compounds such as aluminum, which are generally confined to the systemic circulation, to enter the brain. Cerebral levels of aluminum have in fact been found to increase with age (Shimizu et al., 1994; McDermott et al. 1979). Once inside the brain, the metal may activate glial cells and cause a chronic inflammatory response, which can then lead to the formation of senile plaques (Müller et al., 1997).

Aluminum has clearly been shown to be capable of inducing both inflammation and excess ROS. While the issue of aluminum as a contributor to AD, remains controversial, much evidence comes from both animal studies and human clinical reports. Furthermore, the mechanistic basis of both the pro-oxidant and glial-activating properties of aluminum is increasingly becoming evident. Immunological failure to disperse xenobiotic inclusions, such as colloidal aluminum particles, within the CNS can lead to chronic inflammatory responses that ultimately involve neurons and impair their function (Fig. 1).

While the relation between Alzheimer's disease and aluminum exposure has yet to be unequivocally demonstrated in clinical and epidemiological settings, a firm mechanistic basis which could subserve such a relation is emerging. The feasibility of this outline of how aluminum may promote extended and interactive oxidant and inflammatory events will be enhanced by new mechanistic and clinical information.

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Aluminium and Alzheimer's Disease
The Science that Describes the Link
C. Exley (Editor)

CHAPTER 17

Glutamatergic Neurotransmission, Aluminium and Alzheimer's Disease

Vicente Felipo *

Laboratory of Neurobiology, Instituto de Investigaciones Citologicas, FVIB, Amadeo de Saboya 4, 46010 Valencia, Spain

* Email: vfelipo@ochoa.fib.es

Abbreviations: AMPA – α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid; APP – amyloid precursor protein; CM – calmodulin; EAAT – excitatory amino acid transporter; GABA – γ-aminobutyric acid; LTP – long-term potentiation; NMDA – N-methyl-D-aspartate; nNOS – neuronal nitric oxide synthase; NO – nitric oxide; sGC – soluble guanylate cyclase

Summary

Glutamate is the main excitatory neurotransmitter in mammals. Glutamatergic neurotransmission modulates many important cerebral processes. Alteration of glutamatergic neurotransmission may lead therefore to neurological alterations and to neurodegeneration and neuronal death. Glutamatergic neurotransmission may be altered at several different steps: the content (expression, synthesis and/or degradation) of the main proteins involved in glutamatergic neurotransmission (e.g. the different types of glutamate receptors or transporters); the regulation of the spatial location of the receptors and transporters; the function of the receptors and transporters, which is modulated in different ways including phosphorylation–dephosphorylation, binding of co-agonists, etc. Alterations in the release or uptake of glutamate may result in altered extracellular glutamate, leading to altered neurotransmission. Also, alterations in any of the steps of the signal transduction pathways associated with the different types of glutamate receptors would also result in impaired glutamatergic neurotransmission. The effects of aluminium and of Alzheimer's disease on different steps of glutamatergic neurotransmission are reviewed.

Historical Perspective

Glutamate is the main excitatory neurotransmitter in mammals and has two main types of receptors: metabotropic receptors associated to G proteins which modulate different enzymes and ion channels (phospholipases C and D, adenylate cyclase, etc.) and ionotropic receptors whose activation leads to the opening of ion channels allowing

the transport of Na^+, K^+ and Ca^{2+}. There are at least 8 different types of metabotropic glutamate receptors (mGluRs) associated with different signal transduction pathways, and the main types of ionotropic receptors are NMDA, kainate and AMPA receptors.

Abnormalities of glutamatergic neurotransmission play a major role in the mechanisms of neurotoxicity of different toxic agents as well as in the origin of neurodegenerative diseases such as amyotrophic lateral sclerosis, Huntington disease and Alzheimer's disease (e.g. Appel, 1993; Martin et al., 2000; Taylor-Robinson et al., 1994; Reynolds & Pearson, 1994). A major role for altered glutamatergic neurotransmission has also been reported for the pathogenesis of hyperammonemia and hepatic encephalopathy. The process of glutamatergic neurotransmission involves several steps which can be altered by neurotoxicants or in the pathological situations mentioned above. The first step is the release of glutamate from the presynaptic neuron. Glutamate in the extracellular space activates glutamate receptors present in the synaptic membrane, leading to activation of the signal transduction pathways associated to these receptors. To avoid continuous activation of glutamate receptors, glutamate is removed from the synaptic cleft by specific glutamate transporters located mainly in astrocytes.

All these steps are tightly modulated under physiological conditions in different ways and alteration of any of the above steps would result in altered glutamatergic neurotransmission. Some of the parameters that can be altered under pathological conditions are:

1. The content (expression, synthesis and/or degradation) of the main proteins involved in glutamatergic neurotransmission (e.g. the different types of glutamate receptors or transporters).
2. The regulation of the spatial location of the receptors and transporters. Only the receptors or transporters present in the membrane can recognize extracellular glutamate. Many of these proteins are associated to form clusters to improve the yield of the neurotransmission process.
3. The function of the receptors and transporters, which is modulated in different ways including phosphorylation–dephosphorylation, binding of co-agonists, etc.
4. Alterations in the release or uptake of glutamate may result in altered extracellular glutamate, leading to altered neurotransmission.

Alterations in any of the steps of the signal transduction pathways associated with the different types of glutamate receptors would also result in impaired glutamatergic neurotransmission.

Therefore there are a large number of possible sites or molecular targets for interference by aluminium of glutamatergic neurotransmission. Some of the studies on the effects of Al on different steps of glutamatergic neurotransmission are summarized below. We first summarize some of the alterations in glutamatergic neurotransmission reported in Alzheimer's disease.

Glutamatergic Neurotransmission in Alzheimer's Disease

There are a large number of studies on the alterations of different steps of glutamatergic neurotransmission in Alzheimer's disease. It is not possible to include an extensive review

of this subject in this chapter. We will provide a general view of the alterations of glutamatergic neurotransmission in Alzheimer's disease and focus the review mainly on the alterations induced by Al on different aspects of glutamatergic neurotransmission and on the possible contribution of these alterations to the pathogenesis of Alzheimer's disease.

Glutamatergic denervation in Alzheimer's disease

The major histopathological features in Alzheimer's disease are the presence of plaques in the cortex and hippocampus and tangles in pyramidal neurons in these areas. A significant proportion of these pyramidal neurons are glutamatergic, thus suggesting that glutamatergic neurotransmission may be affected in Alzheimer's disease. Several studies, using different experimental approaches, have been used to determine if there is a loss of glutamatergic innervation in Alzheimer's disease. Initial studies suggesting a loss of glutamatergic neurons were carried out by measuring the content of free amino acid neurotransmitters in postmortem (or antemortem) brain tissue from patients with Alzheimer's disease. Most of these studies showed a selective significant reduction of glutamate content in cortex of patients (Ellison et al., 1986; Sasaki et al., 1986; Lowe et al., 1990) and suggested a loss of glutamatergic neurons. The decrease in glutamate was more remarkable (83% decrease) in the hippocampal perforant pathway (Hyman et al., 1987) and the authors proposed that this diminution of glutamate is a direct neurochemical correlate of perforant pathway destruction and that disruption of this crucial pathway contributes to the memory dysfunction in Alzheimer's disease. In contrast to these reports, Tarbit et al. (1980) reported no change in free glutamate in hippocampus in Alzheimer's disease and Klunk et al. (1992) found an increase in glutamate content.

Other studies used synaptosomal uptake of [^{3}H]D-aspartate as a marker for glutamate-releasing neurons. Using this technique, a significant loss of glutamatergic innervation in Alzheimer's disease was reported by different groups (Procter et al., 1996; Hardy et al., 1987; Cross et al., 1987; Procter et al., 1988; Cowburn et al., 1988).

A direct visualization of the loss of glutamate-immunoreactive fibers has been reported by Kowall & Beal (1991). Using immunohistochemical techniques, these authors also showed that glutamate-stained neurons contain neurofibrillary tangles. These findings are consistent with the observation that glutamatergic pyramidal neurons involved in corticocortical connections are preferentially vulnerable in Alzheimer's disease.

The loss of glutamatergic pathways reported in the above articles in both the hippocampus and the cerebral cortex may contribute to cognitive dysfunction in Alzheimer's disease.

Glutamate receptors in Alzheimer's disease

Using an autoradiographic assay, Greenamyre et al. (1987) examined the binding of [^{3}H]glutamate to different receptor subtypes in hippocampal sections from patients with Alzheimer's disease and from controls. The binding to NMDA receptors was reduced by 75–87% and the binding to 'quisqualate' receptors by 45–69%. The authors suggest that the marked loss of NMDA and 'quisqualate' receptors in hippocampus makes it

likely that neurotransmission to and through the hippocampus is impaired in Alzheimer's patients and that this may explain, in part, the memory deficits of these patients. The results of this study were later 'reanalysed' by the same group in a subsequent more detailed study (Penney et al., 1990) which confirmed that there is a decrease in the binding to NMDA receptors. A decrease in the binding of ligands to NMDA and 'quisqualate' receptors in the CA1 region of hippocampus from patients with Alzheimer's disease was also found by Jansen et al. (1990). The term 'quisqualate' receptor corresponds to an old nomenclature used to classify glutamate receptors and corresponds to receptors responding to quisqualate, which comprise both metabotropic glutamate receptors and some AMPA receptors.

A large number of more detailed studies, using more specific ligands for specific subtypes of glutamate receptors have been carried out in the last ten years to analyse the possible alterations of glutamate receptors in Alzheimer's disease. It is not possible to mention here all these articles, but we summarize the main findings for each type of glutamate receptors.

AMPA and kainate receptors in Alzheimer's disease

As is the case for most of the aspects covered in this review, there are conflicting reports in the literature about the alterations of AMPA receptors in Alzheimer's disease. However, in this case, it seems that there is a general consensus that there is a selective reduction of AMPA receptors in different brain areas in Alzheimer's disease. Reports supporting this reduction have been published by different groups (e.g. Dewar et al., 1990 and 1991; Armstrong et al., 1994; Yasuda et al., 1995; Ikonomovich et al., 1995; Armstrong & Ikonomovich, 1996; Carlsson et al., 1993; García-Ladona et al., 1994; Thorns et al., 1997). Some groups report that AMPA receptors are reduced in the more vulnerable regions but are not affected or increased in other areas (e.g. Ikonomovich et al., 1995).

A possible mechanism for the reduction of AMPA receptors in Alzheimer's disease has been reported by Chan et al. (1999). These authors showed that caspase activation leads to proteolysis of AMPA receptor subunits and suggested that glutamate receptor activation in Alzheimer's disease may be a trigger for caspase-mediated proteolytic degradation of AMPA receptors.

Only a few studies have been done on the possible alterations of kainate receptors in Alzheimer's disease. Cowburn et al. (1989) reported no change in either the affinity or the number of kainate receptors in any of the regions studied. Dewar et al. (1991) reported a selective significant increase in kainate receptors binding in some layers of the cortex.

NMDA receptors in Alzheimer's disease

As mentioned above, the studies of Greenamyre et al. (1987), Penney et al. (1990) and Jansen et al. (1990) reported considerable reductions in the binding of ligands to NMDA receptors in hippocampus of patients with Alzheimer's disease. Ninomiya et al. (1990) also reported a decrease in [^{3}H]TCP binding in frontal cortex. Other authors reported that binding to NMDA receptors is only reduced in some specific regions of the brain but is

not affected in other areas (e.g. Carlson et al., 1993; Simpson et al., 1988). Geddes et al. (1986) found no change in the binding to NMDA receptors, while Ulas et al. (1994) reported an increase of NMDA-sensitive glutamate binding in striatum of patients with Alzheimer's disease.

The reason for the large discrepancies in the results reported in different studies is not clear, but at least two possible explanations have been proposed. Ulas et al. (1992) studied the binding of [^{3}H]MK-801 to NMDA receptors and found a marked intersubject variability in control and Alzheimer's disease group, which was not related to age, sex or postmortem delay. Some patients showed markedly reduced receptor binding while others showed no changes or even increased binding. This intersubject variability may be one of the reasons for the discrepancies in the literature.

Another possible source of variability is that different ligands and binding techniques have been used in different studies. The main techniques used are the binding of ligands to membranes prepared from samples from patients and controls and the binding to sections of brain tissue and analysis of the binding by autoradiography. DelBel & Slater (1991) found a significant reduction in the binding of [^{3}H]glycine to NMDA receptors in samples from patients with Alzheimer's disease when they used isolated membranes, but not when they used sections and autoradiography.

Other authors propose that the amount of NMDA receptors is not significantly affected in Alzheimer's disease but its modulation by glycine is impaired. Steele et al. (1989) reported that glycine-dependent binding of [^{3}H]MK-801 to NMDA receptors is reduced in the patients while the zinc modulatory site is not affected. Similar results were also reported by Procter et al. (1989) while Palmer & Burns (1994) found no alterations in glycine-stimulated binding of [^{3}H]MK-801 to NMDA receptors.

Glutamate concentration in cerebrospinal fluid in Alzheimer's disease

As mentioned above, Alzheimer's disease is characterized by the degeneration and loss of neurons from many brain regions including the cerebral cortex and hippocampus. Pyramidal neurons are the predominant cell type affected in these areas. Glutamate is the major excitatory neurotransmitter for these pyramidal neurons and has both neuroexcitatory and neurotoxic effects. Increased extracellular glutamate (due to excessive release, reduced uptake or both) may be a mechanism whereby neuronal loss may occur in Alzheimer's disease. As it is not possible to measure extracellular glutamate in brain of patients, an indirect measurement of it can be the determination of glutamate in cerebrospinal fluid. Several groups have reported increased glutamate in cerebrospinal fluid in patients with Alzheimer's disease (Pomara et al., 1992; Csernansky et al., 1996; Jimenez-Jimenez et al., 1998). Basun et al. (1990) found no change in glutamate while Tohgi et al. (1992) and Martinez et al. (1993) reported decreased glutamate in cerebrospinal fluid in Alzheimer's disease. Smith et al. (1985) showed that there is a correlation between CSF glutamate levels and cognitive test scores in patients with Alzheimer's disease.

Glutamate transport in brain in Alzheimer's disease and excitotoxic neurodegeneration

The results of different studies, including those mentioned above, lead to the proposal that glutamate excitotoxicity may play an important role in the pathogenesis of Alzheimer's disease. Excessive activation of glutamate receptors leads to neuronal degeneration and death. This excessive activation may be due to altered function or content of the receptors or to increased extracellular glutamate, which in turn may be due to increased release or decreased uptake by the glutamate transporters. The effects of Alzheimer's disease on glutamate transport and transporters has been studied by several groups. As mentioned in the section on *Glutamatergic denervation in Alzheimer's disease*, initial studies used synaptosomal uptake of [^{3}H]D-aspartate as a marker for glutamate-releasing neurons. Using this technique, a significant decrease in glutamate uptake in Alzheimer's disease was reported by different groups and was interpreted as a loss of glutamatergic innervation (see above). Masliah et al. (1996) confirmed the decrease in uptake of [^{3}H]D-aspartate and found a direct correlation with the levels of synaptophysin immunoreactivity and an inverse correlation with progressive accumulation of brain spectrin degradation products, an indicator of excitatory amino acid-induced neuronal death. These authors proposed that decreased activity of glutamate transporters might be associated with increased excitotoxicity and neurodegeneration.

During the last decade the existence of at least 4 different types of glutamate transporters has been demonstrated. Some authors have studied whether there is a differential alteration of glutamate transporters in Alzheimer's disease. Scott et al. (1995) reported that pharmacologically distinct neuronal transporters occur in various cortical brain regions of Alzheimer's disease patients, as compared to controls. Moreover, the differences were present both in areas spared from damage and in susceptible areas. These alterations would increase the excitotoxic potential of glutamate. Li et al. (1997) showed, by using Western blotting and specific antibodies, that the amount of the glutamate transporter EAAT2 is significantly decreased in Alzheimer's disease, while the content of EAAT1 and EAAT3 are not affected. The decrease in EAAT2 was associated with decreased transport. Beckstrom et al. (1999) reported that Alzheimer's disease brains can have both normal and reduced levels of EAAT1 and EAAT2.

Masliah et al. (1998) studied whether deficient glutamate transport in Alzheimer's disease could be associated with cell damage and caspase activation and found that there was a strong negative correlation between glutamate transport and apoptosis and caspase 3 immunoreactivity, supporting the possibility that excitotoxic injury associated to deficient glutamate transport might lead to cell death via caspase 3 activation.

Two Different Hypothesis for Excitotoxic Neurodegeneration in Alzheimer's Disease

Two different hypothesis have been proposed involving glutamate neurotoxicity in the pathogenesis of Alzheimer's disease. The first one was the 'classical' excitotoxicity mediated by excessive activation of glutamate receptors (e.g. Maragos et al., 1987; Palmer & Gerson, 1990). A different hypothesis has been proposed by Olney et al. (1997) suggesting that the NMDA receptor system is rendered hypoactive in Alzheimer's

disease and that this hypoactivity of NMDA receptors triggers a disinhibition syndrome in which low-grade chronic excitotoxic activity causes widespread neuronal degeneration. Although we can not discuss this matter in detail in this review, it is important to discern which mechanisms are actually working in Alzheimer's disease because this will be important to design possible clinical treatments.

Therapeutic Implications of Glutamatergic Alterations in Alzheimer's Disease

The identification of the molecular mechanisms involved in the origin of Alzheimer's disease would allow to design appropriate clinical treatments. Some clinical treatments have been proposed considering the glutamatergic hypothesis of Alzheimer's disease, including modulation of AMPA receptors or the use of D-cycloserine (see for example: Francis et al., 1993; Madsen et al., 1994; Chessell et al., 1991). Tekin et al. (1998), considering that excitotoxic damage by glutamatergic hyperactivity is involved in neurodegeneration in Alzheimer's disease, used lamotrigine, which inhibits presynaptic glutamate release, in patients with the diagnosis of probable Alzheimer's disease and found that administration of 300 mg/day lamotrigine improved word recognition, naming and depressed mood. We can not review here all the literature about this point but the clarification of the contribution of altered glutamatergic neurotransmission to the pathogenesis of Alzheimer's disease and the identification of the steps affected would allow the design and test of new clinical treatments.

Glutamate, Induction of Paired Helical Filaments, Phosphorylation of Tau Protein, Beta-Amyloid and Other Aspects of Alzheimer's Disease

Alterations in glutamatergic neurotransmission have been correlated with alterations of other parameters characteristic of Alzheimer's disease. It is not possible to summarize here all these studies. We will only point out some of them as a reference. DeBoni & McLachlan (1985) reported that glutamate can induce formation of paired helical filaments in cultured human neurons, suggesting a role of alterations of glutamate in the appearance of these filaments in Alzheimer's disease. Several groups have reported that glutamate modulates phosphorylation of Tau proteins, suggesting a possible contribution of altered glutamatergic neurotransmission to the hyperphosphorylation of Tau in Alzheimer's disease (Sindou et al., 1994; Anderton et al., 1995; Davis et al., 1995; Couratier et al., 1996). A potentiation of glutamate neurotoxicity by beta- amyloid has been reported (e.g. Koh et al., 1990; Mattson et al., 1992). Willoughby et al. (1995) proposed that sustained NMDA receptor activation can regulate alternative splicing of APP. Le et al. (1995) reported that beta-amyloid can lead to apoptotic neuronal death mediated by activation of NMDA receptors. It has been also shown that beta-amyloid inhibits glutamate uptake in cultured astrocytes, thus contributing to increased extracellular glutamate and increased excitotoxicity (Harris et al., 1995; Parpura-Gill et al., 1997). These and other reports suggest that altered glutamatergic neurotransmission may be involved in the origin of some of the characteristic alterations found in Alzheimer's disease.

Aluminium and Glutamatergic Neurotransmission

Aluminium and release and uptake of glutamate

Albrecht et al. (1991) used primary cultures of astrocytes and showed that incubation with 0.5 mM aluminum chloride for 5 min did not induce a release of glutamate, while 5 mM aluminium chloride increased glutamate release to more than 10-fold the basal levels. As discussed by the authors, brain aluminium concentrations in aluminium-related neurological disorders do not usually exceed 0.25 mM (see below), however, they may reach higher values locally.

Provan & Yokel (1992) used rat hippocampal slices preloaded with [^{14}C]-glutamate and studied the effects of Al chloride on the release of labelled glutamate induced by depolarization with K^+. They showed that Al inhibits the calcium-dependent component of the potassium-induced release of glutamate in a concentration dependent manner, with an IC_{50} of 40 μM. The inhibition was nearly complete at 300 μM $AlCl_3$. The assays performed to shed light on the possible mechanism responsible for Al-induced decrease in glutamate release suggested that it is mediated by decreased Ca channel function.

A similar study was reported by Gilbert & Shafer (1996), also using rat hippocampal slices preloaded with [^{14}C]-glutamate and studying the effects of Al (100–1000 μM) on the release of labelled glutamate induced by depolarization with K^+. They found no effect of Al on glutamate release from the slices.

Wong et al. (1981) studied the rate of accumulation of [^{14}C] L-glutamate in purified rat forebrain nerve-ending particles and showed that $AlCl_3$ inhibits the transport of glutamate in a dose-dependent manner. The concentration that gave 50% inhibition (IC_{50}) of glutamate transport was 299 μM.

Sass et al. (1993) studied the effect of Al on [^{14}C]glutamate uptake by astrocytes in culture and found that Al (100 μM) did not affect the uptake of glutamate by astrocytes.

The above studies suggest that Al can influence glutamate release or uptake only at high concentration.

Aluminium concentration in brains of people with aluminium-related neurological disorders and of people with Alzheimer's disease range, according to the literature, between 30 and 250 μM (Lai & Blass, 1984; Crapper et al., 1976). In brains of normal (control) humans the concentration of Al reported is around 7–10 μM (Roider & Drasch, 1999; Crapper et al., 1976). It is therefore possible that the concentration of Al in brain under pathological conditions may be enough to alter glutamate transport and glutamatergic neurotransmission.

Aluminium and glutamate receptors

The effects of aluminium on the content or function of the different types of glutamate receptors has not been extensively studied.

Hubbard et al. (1989) studied the effects of Al on the binding of [^{3}H]MK-801 to NMDA receptors in normal human cerebral cortex membranes. Al showed negligible inhibitory effects in the concentration range where the free ions can be precisely controlled (up to 0.4 nM). Buffers with higher Al concentrations, containing amorphous

$Al(OH)_3$, showed potent inhibition of [^{3}H]MK-801 binding in a concentration dependent manner, with an IC_{50} of 30 μM. The mechanism of the interference by Al of the binding of the agonist to the NMDA receptor is not clear. In principle, Al may interfere with the receptor or with the agonist. If the effect of Al is on the receptor this may be relevant for glutamatergic neurotransmission.

Atterwill et al. (1996) exposed primary neuronal cultures to Al at 1 μM or 7.4 mM. Al induced enhanced neural metabolism at low concentrations in vitro in contrast to depressed metabolism and cellular viability at high concentrations. The NMDA receptor antagonist MK-801 reversed the metabolic rise at low Al levels but not the loss of cell viability at higher Al concentrations. The authors suggest Al, at μM concentrations, 'activate' free-radical mediated metabolic changes, leading to excitotoxic events by way of the NMDA receptor.

One way to determine activation of ionotropic (mainly NMDA) glutamate receptors in intact neurons in culture is to measure the increase in intracellular calcium induced by glutamate. Cucarella et al. (1998) showed that in vitro treatment of primary cultures of cerebellar neurons with 50 μM Al did not affect intracellular calcium levels nor glutamate-induced rise of intracellular calcium in the postsynaptic neurons, suggesting that, under the assay conditions used, acute or chronic treatment with 50 μM Al do not affect significantly activation of glutamate receptors.

Mundy et al. (1997) showed that pretreatment of primary cultures of cerebellar neurons with 300 μM Al for 1 hour, followed by washing of Al resulted in an increase in the accumulation of ^{45}Ca induced by glutamate, which was prevented by MK-801, an antagonist of NMDA receptors. The authors suggest that exposure to Al may reduce the capacity of the neurons to buffer increases of intracellular Ca^{2+} induced by activation of NMDA receptors or, alternatively, Al could potentiate glutamate actions at the receptor level, increasing Ca^{2+} flux through the channel.

The differences between the results reported by Cucarella et al., and by Mundy et al., may be due to the different concentration of Al used (50 vs 300 μM) and/or to the fact that determination of Ca^{2+} was carried out in the presence of Al by Cucarella et al., but in the absence of Al by Mundy et al (see below Interactions between glutamate and Al).

Another way to determine activation of glutamate receptors in brain slices or neurons is to measure glutamate-activated currents. Platt et al. (1994) studied the actions of Al on glutamate-activated currents of acutely isolated hippocampal neurons. NMDA, AMPA and glutamate mediated currents were reduced by 50% in the presence of 50 μM Al. Higher concentrations (100 μM) inhibited all currents completely and irreversibly. Al per se had no influence on resting membrane current or voltage-activated sodium currents. The estimation of the concentration–response relationship of the action of aluminium on NMDA-activated currents revealed a threshold concentration 10 μM. The authors suggest that glutamate receptors are putative sites of action in aluminium neurotoxicity.

Trombley (1998) used primary cultures of olfactory bulb neurons and examined the effects of Al on whole cell currents evoked by glutamate, kainate, NMDA or GABA. In contrast with the study of Platt et al. (1994), Trombley did not found any effect of Al (10–100 μM) on glutamate-, kainate- or NMDA-induced currents, but showed a selective potentiation of non glutamate ($GABA_A$) receptors.

As is the case for the effects of Al on other aspects of glutamatergic neurotransmission,

the above studies report contradictory results. The reasons for these discrepancies are not clear. The experimental systems used and the end point determined are different and further, more detailed studies, are required to clarify under which conditions and by which mechanisms Al may interfere with glutamate receptors.

Aluminium and long-term potentiation

Hippocampal long-term potentiation (LTP) is the most intensively studied model of synaptic plasticity in mammalian brain. LTP is a complex process which is considered the molecular basis for some forms of learning and memory. NMDA receptor-dependent LTP in the hippocampus may be considered the link between glutamatergic neurotransmission and the processes of learning and memory formation.

It has been shown that Al impairs performance on different cognitive tasks in rats, mice and rabbits. An alteration of glutamatergic neurotransmission and of LTP may be responsible for the impairment in cognitive function in animals treated with Al.

The effects of Al on LTP has been studied by different groups. Farnell et al. (1982) injected rabbits with Al (10 μmol) into the lateral ventricle. At different days after injection some animals were killed and hippocampal slices were used to record LTP. After Al treatment, Farnell et al. found a decline in the ability of slices to demonstrate LTP which was detectable 5 days after Al injection and increased with time up to 20 days post-injection.

Platt et al. (1994b) studied the effect of intracerebroventricular injection of Al in rats on LTP determined in the dentate gyrus in the animal in vivo. Acute injection of Al (to reach 10 μM in brain) during baseline recording did not affect the induction or maintenance of LTP. Injection of larger doses of Al (to reach 25 or 100 μM) did not affect significantly the induction of LTP although reduced slightly its maintenance.

In the same work, Platt et al. also studied the effect of daily (chronic) treatment with Al for 5 days (25 μM each day) on LTP in vivo and found that this chronic treatment resulted in a strong inhibition of LTP.

In this study, Platt et al. also assessed the effects on LTP of in vitro exposure of hippocampal slices from normal rats to Al. At 25 μM, Al reduced slightly the magnitude of the potentiation without affecting the maintenance. At 100 μM, Al reduced the magnitude of the potentiation and impaired its maintenance. Gilbert & Shafer (1996) also examined the effects of Al added in vitro on LTP in rat hippocampal slices. In contrast to the report of Platt et al. (1994b), in this study acute exposure to Al (100 μM) did not affect the magnitude or maintenance (measured up to 1 h posttrain) of LTP.

The above results suggest that chronic exposure to Al in vivo impairs LTP. This impairment may contribute to the alterations in learning ability in animals treated with Al.

Aluminium and the glutamate–nitric oxide–cGMP pathway

Binding of agonists to glutamate receptors leads to activation of signal transduction pathways which are integrated and finally result in a biological response. Different signal transduction pathways modulate different cerebral processes. An important pathway associated with activation of glutamate receptors is the glutamate–nitric oxide–cGMP pathway,

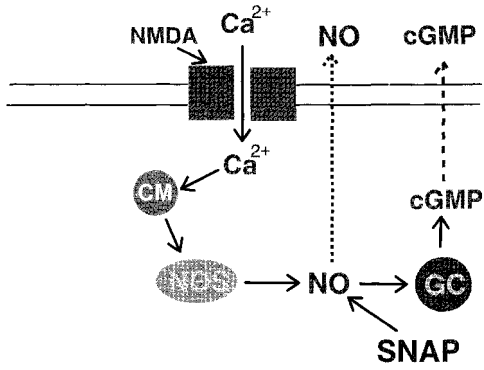

Fig. 1. The glutamate–nitric oxide–cGMP pathway.

which modulates cerebral processes such as communication between neurons and astrocytes and between different neurons, the release of some neurotransmitters in some brain areas, the process of long-term potentiation discussed above, some forms of learning and memory formation, and some circadian rhythms (e.g. the sleep-waking cycle).

Activation of ionotropic glutamate receptors (mainly NMDA) leads to the opening of the ion channel and allows the entry of Ca^{2+} into the post-synaptic neuron, increasing intracellular Ca^{2+} concentration. Ca^{2+} binds to calmodulin (CM) and activates different enzymes including neuronal nitric oxide synthase (nNOS), leading to increased nitric oxide (NO) which, in turn, activates soluble guanylate cyclase, leading to increased cGMP. Part of this cGMP is released to the extracellular space. NO is a gas which can diffuse to the neighbouring cells and activate soluble guanylate cyclase in astrocytes or other neurons close to the neurons in which nNOS was activated (see Fig. 1).

Effects of Al added in vitro to normal neurons in culture

The effects of different treatments with Al on the glutamate–nitric oxide–cGMP pathway have been studied. Cucarella et al. (1998) studied the effects on this pathway of short-term (4 h) or long-term (8–14 days) treatment with Al (50 μM) added in vitro to cultured neurons from normal rats. Short-term exposure to Al did not affect glutamate-induced activation of nNOS and reduced slightly nitric oxide-induced activation of soluble guanylate cyclase, resulting in a slight inhibition of the pathway.

Chronic exposure of the neurons to Al reduced glutamate-induced activation of nNOS by 38% and nitric oxide-induced activation of guanylate cyclase by 33%. This indicates that chronic exposure to Al interferes with two steps of the pathway resulting in a remarkable 77% reduction in the glutamate-induced formation of cGMP.

Effects of prenatal exposure to Al on the glutamate–nitric oxide–cGMP in cultured neurons ex vivo

Cucarella et al. (1998) treated pregnant rats with Al-sulphate (3%) in the drinking water from the first day of pregnancy and prepared primary cultures of cerebellar neurons

from 7–8-days-old pups. They prepared parallel neuronal cultures from control pups and from those prenatally exposed to Al. Both neuronal cultures were prepared and maintained in a similar way, without addition of Al in vitro at any time. The cultures were used for the assays 12–15 days after seeding. In neurons from rats prenatally exposed to Al but not exposed to it during culture, glutamate-induced activation of nNOS was reduced by 85%, nitric oxide-induced activation of guanylate cyclase was not affected and the whole pathway (glutamate-induced formation of cGMP) was reduced by 81%. These results indicate that prenatal exposure to Al induces a strong and long-lasting impairment of glutamate-induced activation of nNOS which is maintained 15 days after withdrawal of Al. It is not clear at this moment whether the maintenance of the effect is due to the permanency of Al within the neurons or to a developmental effect.

Llansola et al. (1999) studied the effects of prenatal exposure to Al on the content of the proteins involved in the glutamate–nitric oxide–cGMP pathway: calmodulin, neuronal nitric oxide synthase and soluble guanylate cyclase. For these studies, prenatal exposure to Al was carried out as in the work of Cucarella et al. (1998). Some rats of each litter were used to prepare primary cultures of cerebellar neurons and sister rats from the same litters were sacrificed at 17–22 days and the cerebella were also used to analyse the content of the proteins. The content of the proteins of the glutamate–nitric oxide–cGMP pathway were analysed in cultured neurons 10–12 days after seeding. Al was not added to the culture medium at any time, the analysis was carried out therefore 10–12 days after withdrawal of the source of Al. Prenatal exposure to Al reduced the content in cultured neurons of nNOS by 52% and of guanylate cyclase by 33% and increased the content of calmodulin by 61%. The content of MAP-kinase, used as a reference protein unrelated to the pathway was not affected.

Llansola et al. (1999) also analysed the effect of prenatal exposure to Al on the content of these proteins in the whole cerebellum, containing neurons and other cell types (astrocytes, etc.). Prenatal exposure to Al reduced the content of nNOS and soluble guanylate cyclase in whole cerebellum by 38% and 40%, respectively, and increased the content of calmodulin by 107%. The effects found in cultured neurons were therefore very similar to those found in the whole cerebellum.

Llansola et al. (1999) also reported that prenatal exposure to Al prevented, in primary cultures of cerebellar neurons, glutamate-induced proteolysis of the microtubule-associated protein MAP-2, disaggregation of microtubules, and neuronal death, indicating an impairment of other NMDA receptor-associated signal transduction pathways.

Effects of chronic exposure to Al in the drinking water on the glutamate–nitric oxide–cGMP pathway in rat brain in vivo

Hermenegildo et al. (1999) used in vivo brain microdialysis in freely moving rats to study the effects of chronic administration of Al-sulphate (2.5%, corresponding to 0.2% Al) in the drinking water (for 3–5 weeks) on the glutamate–nitric oxide–cGMP in the cerebellum of the rat in vivo. The concentration of Al reached in the cerebellum was 96 μM, as measured by inductively coupled plasma mass spectrometry (ICP–MS). As shown in Fig. 1, part of the cGMP formed following activation of the glutamate–nitric oxide–cGMP pathway is released to the extracellular space. Under appropriate conditions,

determination of extracellular cGMP in the extracellular space is a good measure of the activation of the pathway. To assess the function of the pathway, Hermenegildo et al. (1999) administered NMDA through the microdialysis probe to activate the pathway and measured cGMP in the extracellular space. The increase in cGMP induced by NMDA was decreased by ≈50% in rats chronically treated with Al., indicating that some step of the glutamate–nitric oxide–cGMP is impaired in the cerebellum of these rats in vivo. Acute administration of Al did not affect the pathway.

To assess whether nitric oxide-induced activation of guanylate cyclase is altered in the rats treated with Al, Hermenegildo et al. (1999) administered, through the dialysis probe, the nitric-oxide-generating agent SNAP, which produces NO which activates guanylate cyclase (Fig. 1). Activation of guanylate cyclase by NO was increased (240% of controls) in rats treated with Al. Hermengildo et al. (1999) also showed that basal extracellular cGMP and basal activity of guanylate cyclase (assayed ex vivo) are significantly reduced in cerebellum of rats chronically exposed to Al. These results indicate that chronic exposure of rats to Al in the drinking water impairs the glutamate–nitric oxide–cGMP at different steps, reducing glutamate-induced formation of NO and basal activity of guanylate cyclase and increasing NO-induced activation of guanylate cyclase.

The above results clearly show that Al impairs the glutamate–nitric oxide–cGMP pathway. As mentioned above, this pathway modulates important cerebral processes such as communication between neurons and astrocytes, LTP, some forms of learning and memory, etc. As discussed in this chapter, all these processes are also altered by Al. May be the alterations in the glutamate–nitric oxide–cGMP pathway are responsible for the impairment of some of these processes and for some of the neurological alterations induced by Al in humans.

Soluble guanylate cyclase is also altered in temporal cortex of patients with Alzheimer's disease

Bonkale et al. (1995) compared the kinetic profiles of the particulate and soluble forms of guanylate cyclase in the superior temporal cortex from a series of control and Alzheimer's disease subjects. Particulate guanylate cyclase was not significantly different between groups. The activity of soluble guanylate cyclase under basal conditions and in the presence of the activator nitric oxide was reduced by ≈50% in superior temporal cortex of patients with Alzheimer's disease. It is not clear whether Al may play a role in the reduction of soluble guanylate cyclase activity, but comparing the results reported by Bonkale et al. (1995) and by Hermenegildo et al. (1999), the reduction in the basal activity of soluble guanylate cyclase is similar in rats chronically exposed to Al (reaching 96 μM Al in the cerebellum) and in the patients with Alzheimer's disease. As mentioned above, Al in brains of people with Alzheimer's disease range between 30 and 250 μM, therefore, the Al present in these brains may be enough to induce the alterations in soluble guanylate cyclase.

Aluminium and Interaction Between Astrocytes and Neurons

Under adequate conditions, astrocytes protect neurons from glutamate excitotoxicity. Sass et al. (1993) whether prior exposure of astrocytes to Al (in the form of aluminum citrate) interfered with the ability of astrocytes to protect neurons from glutamate excitotoxicity. They used cultures of neurons and astrocytes grown on separate coverslips, subjected either the neurons or the astrocytes to specific treatments and recombined the cells into the same dish by moving coverslips from dish to dish. They confirmed that astrocytes could protect neurons from glutamate-induced death when glutamate (100 μM) is added to the culture medium. They also found that prior treatment of astrocytes with 100 μM Al impairs the ability of astrocytes to prevent neuronal death. No differences were observed in the ability of control and aluminum-treated astrocytes to take up glutamate. The authors suggest that Al may cause astrocytes to: (a) secrete a factor that makes neurons more susceptible to glutamate-induced toxicity; (b) secrete a neurotoxic factor in the presence of glutamate; or (c) reduce secretion of a factor that protects neurons from glutamate excitotoxicity.

Such an interference by Al of the interaction between astrocytes and neurons may also contribute to alterations of glutamatergic neurotransmission.

Suarez-Fernandez et al. (1999) have recently shown that the interaction between astrocytes and neurons play an essential role in the mediation of the neurotoxic effects of Al. These authors used primary cultures of astrocytes and showed that long-term treatment with Al induces apoptosis of astrocytes. When they used primary cultures of neurons containing only 1% of astrocytes, Al did not induce neurotoxicity. However, when the neuronal cultures contained around 10% of astrocytes, Al induced neurotoxicity. These results indicate that astrocytes play a role in the mediation of the toxic effects of Al on the neurons.

Direct Interaction of Al with Glutamate. Aluminium, Ammonia and Alzheimer's Disease

Deloncle et al. (1990) treated a group of rats with daily subcutaneous injections of glutamate for two weeks, while the controls were injected with sodium chloride. Then both groups were injected i.v. with Al. The concentration of Al reached in the brain cortex was significantly increased (about 3-fold) in the rats treated with glutamate vs controls. Deloncle et al. (1990) proposed that Al, which in vivo yield stable complexes with aspartic and glutamic acids, can be transferred through the blood–brain barrier in the form of glutamate complex and be deposited in the brain.

In a subsequent paper, Deloncle & Guillard (1990) proposed that Al forms stable complexes with glutamate within the brain thus interfering with both the function of glutamate as a neurotransmitter and its metabolism. Ammonia is a normal product of degradation of proteins and other compounds, but at high concentrations is toxic, leading to functional disturbances of the central nervous system, which can result in encephalopathy of different grades. To avoid the toxic effects of ammonia in brain it is detoxified by glutamine synthetase which uses glutamate, ammonia and ATP to form glutamine. Deloncle & Guillard (1990) proposed that glutamate bound to Al can not be used by glutamine synthetase and therefore ammonia can not be detoxified, resulting in increased ammonia

levels in brain which would be responsible for the neuronal death, affecting each and every neurotransmitter system and leading to some of the neurological alterations induced by Al and also to some of the neurological alterations in Alzheimer's disease. Deloncle & Guillard (1990) propose that aluminium-induced increase of ammonia contributes to the mechanisms of the pathogenesis of Alzheimer's disease.

A role for ammonia as a pathogenetic factor in Alzheimer's disease has been also proposed by other authors. Fisman et al. (1985) noted in the individual Alzheimer patients fluctuations from day to day in awareness, intellectual ability, and mood suggestive of a metabolic encephalopathy. Also, a number of Alzheimer patients have triphasic waves on EEG typically encountered in hepatic encephalopathy and hyperammonemia. These observations prompted Fisman et al. (1985) to measure blood ammonia levels in patients with Alzheimer's disease and they found that it is increased over the values for controls. A similar increase in plasma ammonia levels in patients with Alzheimer's disease was also reported by Branconnier et al. (1986).

Hoyer et al. (1961) took arterial and cerebral venous blood from the internal jugular bulb to determine arterio-venous differences of ammonia in patients with Alzheimer's disease and in controls. For controls, the brain took up a small amount of ammonia. However, in patients thought to be suffering of incipient early-onset dementia of Alzheimer type, the brain released a significant amount of ammonia (256 ± 162 mg kg^{-1} min^{-1}). In patients in advanced states of Alzheimer's disease, the brain released a small amount of ammonia (27 ± 3 mg kg^{-1} min^{-1}). On the basis of these results, Hoyer et al. (1990) suggested that ammonia is endogenously generated in the brain of patients with Alzheimer's disease and that this ammonia has a role in the cascade of cell damaging events leading to Alzheimer's disease.

There are a number of biochemical, morphological and cellular alterations which are found both in hyperammonemia and in Alzheimer's disease. Some of the experimental evidences in favour of the idea that ammonia plays a role in dementia of the Alzheimer type have been reviewed by Seiler (1993 and 1997).

Looking for the biochemical mechanisms leading to increased ammonia in Alzheimer's disease, Sims et al. (1998) studied whether AMP-deaminase may be the source of this ammonia. AMP deaminase converts adenosine monophosphate to inosine monophosphate with the release of ammonia. Sims et al. (1998) found that the activity of AMP deaminase in postmortem brain tissue from Alzheimer's disease is $\approx$2-fold greater than in controls, suggesting that increased activity of this enzyme may augment ammonia levels in the brain in Alzheimer's disease.

Although the results reported are not enough to confirm the hypothesis, the possible interaction between Al, ammonia and Alzheimer's disease would deserve some attention and the design and realization of some studies to clarify the role of hyperammonemia in Alzheimer's disease would be useful.

Aluminium, Glutamate Metabolism and Another Glutamatergic Mechanism for Al Toxicity

As discussed above, Deloncle et al. (1990) proposed that Al would form a complex with glutamate and lead to altered glutamate metabolism and hyperammonemia (see

above). Taking into account the alterations in glutamatergic neurotransmission reported in Alzheimer's disease, Zielke et al. (1993) tested the hypothesis that one of the mechanisms of Al neurotoxicity is mediated by altering glutamate metabolism in brain. Since glial cells are central to glutamate metabolism, possessing both a high affinity uptake for glutamate and the enzyme glutamine synthetase, Zielke et al. (1993) used primary cultures of astrocytes to assess the effect of Al on glutamate metabolism. They treated the astrocytes with different concentrations of Al-lactate for 2–4 days and measured the 'in vitro' activities of glutamine synthetase and of glutaminase. High concentrations of Al (1–7.5 mM), lead to increased activity of glutamine synthetase (≈200% of controls at 7.5 mM Al) and reduced activity of glutaminase (≈35% of controls at 7.5 mM Al). At these high Al concentrations, the intracellular concentration of glutamate was reduced and glutamine was increased. Zielke et al. (1993) propose that these changes may alter the availability of neurotransmitter glutamate in vivo and may be a mechanism for Al neurotoxicity. However, as indicated above, Al concentration in brains of people with aluminium-related neurological disorders and of people with Alzheimer's disease range between 30 and 250 μM (Lai & Blass, 1984; Crapper et al., 1976). Although these Al concentrations reported in the literature may not be directly comparable with those present in the studies of Zielke, at these concentrations, the results reported by Zielke et al. (1993) show no significant changes in glutaminase and a decrease in glutamine synthetase.

In a recent study, Struys-Ponsar et al. (2000) studied the effects of Al treatment in rats (i.p. injection for 2 months) and in cultured astrocytes on glutamate and glutamine content and on glutamine synthetase activity. They found that Al treatment increased glutamine content in rat brain and in cultured astrocytes and increased the activity of glutamine synthetase in astrocytes (44% to 140% increase for 200–800 μM Al). These results indicate that Al interferes with glutamate metabolism in astrocytes, which can contribute to altered glutamatergic neurotransmission.

Indirect Effects of Al on Glutamatergic Neurotransmission. Interaction of Al with ATP

In addition to the direct effects of Al on the different steps or components of glutamatergic neurotransmission (transporters, receptors, associated signal transduction pathways, etc), Al may also interfere with glutamatergic neurotransmission in a more indirect way, by altering molecules or processes which modulate it. For example, Exley (1999) proposed that Al, acting as a complex with ATP, may act as an excitotoxin at physiological concentrations by potentiating the activity of receptors involved in neurotransmission. ATP plays important roles in extracellular signal transduction. Extracellular ATP binds to ionotropic and metabotropic P2 receptors and modulates glutamatergic neurotransmission. Exley (1999) hypothesized that Al interferes with receptor desensitization by increasing the stability of the metal-ATP-receptor complex. This would result in increased activation of the NMDA type of glutamate receptors, for example, when glutamate and ATP are released into the synaptic cleft, they will act upon their respective receptors and induce the entry of Ca^{2+} into the post-synaptic neuron through the NMDA receptor. In the presence of Mg-ATP (the normal conditions) the subsequent rise of

intracellular Ca^{2+} will trigger an immediate inactivation of the NMDA receptor. Exley proposed that in the presence of Al-ATP inactivation of NMDA receptors is diminished, thus leading to increased activation of NMDA receptors which can lead to excitotoxicity and neuronal degeneration. As far as we know, this hypothesis has not been experimentally assessed yet, but is an example of one of the many indirect mechanisms by which Al may alter glutamatergic neurotransmission.

Informed Opinion

Disparity of Results on Al Effects on Glutamatergic Neurotransmission

It is clear from the above summary that there is a disparity in the literature on the effects of Al on most of the steps of glutamatergic neurotransmission studied. The reasons for this disparity are not clear. The chemistry of Al in solution should be taken into account. Al has complex effects on pH and forms a mixture of different Al hydroxydes, which can exert their own independent effects, thus complicating the interpretation of the results and the comparison of studies carried out under different conditions.

One important point to be considered concerning these studies is that a wide range of different experimental systems have been used (slices or subcellular components from different brain areas, primary cultures of neurons or astrocytes from different brain areas, animals in vivo, etc.). Moreover, treatments with Al were also very different (addition of Al in vitro for different time periods; ex vivo experiments with slices or cell cultures from animals treated with Al; in vivo experiments with animals treated with Al). The way of administering Al to the animals was also different in different studies (in the drinking water; by injection into the brain), also the Al salts and the doses used were different. The duration of the treatments were also different (acute, chronic). In some studies the animals were treated with Al prenatally and in others at different post-natal ages.

It has been shown that the effects of Al are different depending on the experimental design used, for example the effects of acute and chronic treatment with Al on the glutamate–nitric oxide–cGMP pathway are very different (Cucarella et al., 1998). Also the effects of prenatal or post-natal exposure are different (Hermenegildo et al., 1999; Llansola et al., 1999). The effects of Al on the parameters under study will depend therefore on the experimental conditions used. This may explain some of the discrepancies found in the literature about the effects of Al on some aspects of glutamatergic neurotransmission.

Although all the experimental systems may provide useful information on the mechanisms of neurotoxicity of Al, for the study of the possible contribution of Al to the alterations of glutamatergic neurotransmission in Alzheimer's disease, it seems that the more appropriate experimental system may be an animal model in which Al is chronically administered in vivo and the concentration of Al reached in the brain is similar to that found in the patients and is maintained for a long period of time.

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Aluminium and Alzheimer's Disease
The Science that Describes the Link
C. Exley (Editor)

CHAPTER 18

Aluminum-Induced Alteration of Phosphoinositide and Calcium Signaling

William R. Mundy [1,*] and Timothy J. Shafer [2]

[1]*Cellular and Molecular Toxicology Branch,* [2]*Neurophysiological Toxicology Branch, National Health and Environmental Effects Research Laboratory, Office of Research and Development, USEPA, Research Triangle Park, North Carolina, 27711, USA*

* Corresponding author, email: mundy.william@epa.gov

Abbreviations: IPs – inositol phosphates; IP_3 – inositol 1,4,5-trisphosphate; PIP_2 – phosphatidylinositol 4,5-bisphosphate; Pi-PLC – phospholipase C; PKC – protein kinase C; Aβ – β-amyloid; NFTs – neurofibrillary tangle

Summary

Receptor-stimulated phosphoinositide hydrolysis results in the formation of the second messenger inositol trisphosphate and the release of calcium from intracellular stores, and is a major cholinergic signaling mechanism in the brain. Recent evidence suggests that aluminum can impact the phosphoinositide signaling pathway at several levels including G-proteins, phospholipase C, and protein kinase C. Aluminum can also alter calcium homeostasis via actions at voltage-sensitive ion channels and Ca^{2+}/Mg^{2+}-ATPases. Because deficits in cholinergic transmission and the deregulation of calcium-dependent processes represent biochemical events which may contribute to the dementia and pathology of Alzheimer's disease, these actions of aluminum may serve to exacerbate an ongoing disease process.

Historical Perspective

Cellular signaling, particularly receptor- and channel-mediated changes in intracellular calcium concentration, is an important mechanism by which neurons and other cells regulate enzymatic activities (e.g. kinase and phosphatase activity), neurotransmitter release, and gene transcription. Disruption of calcium signaling pathways can have a variety of adverse ramifications for neurons which range from altered function to cell death (Nicotera et al., 1992). A number of neurotoxic compounds including lead (Gilbert, 1997), methylmercury (Denny & Atchison, 1996), polychlorinated biphenyls (Mundy et al., 1999) and aluminum (Shafer & Mundy, 1995) perturb various aspects of cellular signaling and calcium homeostasis. Aluminum ion (Al^{3+}) and various aluminum

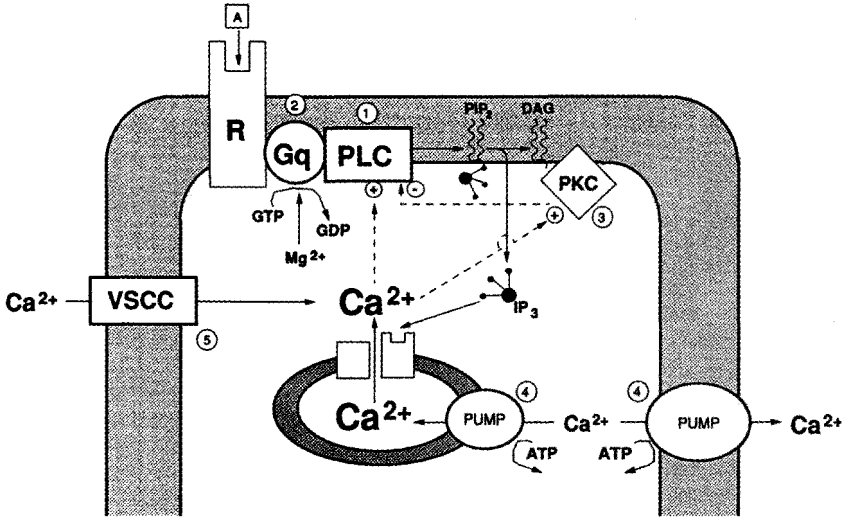

Fig. 1. Inositol phosphate and intracellular calcium regulation in the neuron. Positive ⊕ and negative ⊖ modulation are indicated by dashed lines. Identified sites of action (encircled numbers) for aluminum include: 1 = phosphatidylinositol-specific phospholipase C (PLC); 2 = G-protein (G_q); 3 = protein kinase C (PKC); 4 = Ca^{2+}/Mg^{2+}-ATPase (calcium pump); 5 = voltage-sensitive calcium channels (VSCC).

complexes disrupt cellular calcium homeostasis and signaling both *in vivo* and *in vitro*, and may act at multiple sites within neurons to exert these effects. These sites include receptor-activated phosphoinositide signaling, receptor- and voltage-gated ion (calcium) channel function, and intracellular calcium store function.

The inositol phosphate signaling pathway is a major target of aluminum. This pathway consists of a receptor-coupled to a G-protein (G_q) and phospholipase C (Pi-PLC). Activation of the receptor results in hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP_2) to inositol 1,4,5-trisphosphate (IP_3) and diacylglycerol (DAG) (Fig. 1). Hydrolysis of PIP_2, and hence activity of this pathway, is easily measured by collection of ^{3}H-inositol phosphates (IPs) from cells or slices of CNS tissue. Both intracellular calcium concentration and phosphorylation of receptors or G_q by protein kinase C (PKC) can modulate the activity of this system (for review, see Fisher et al., 1992). Effects of aluminum on IP signaling are well understood mechanistically as they have been studied thoroughly in clonal cell lines and brain slices, and following *in vivo* administration of aluminum. In neuroblastoma cells grown in culture, acute exposure to aluminum chloride inhibited IP accumulation stimulated by activation of muscarinic (Wood et al., 1994) and bradykinin (Shi & Haug, 1992) receptors. In slices from different areas of rat brain, including neocortex, hippocampus and cerebellum, acute treatment with aluminum chloride ($\geq$100 μM) inhibited IP accumulation stimulated by activation of muscarinic (Johnson & Jope, 1986; Shafer et al., 1993), adrenergic and metabotropic glutamatergic (Shafer et al., 1993) receptors. In addition, receptor-stimulated IP accumulation is inhibited by acute exposure to aluminum chloride in both neonatal and adult rat brain slices (Mundy et al., 1995). There also is evidence from *in vivo* studies that phosphoinositide signaling is disrupted;

a 3 month exposure to aluminum sulfate (0.3%) in drinking water resulted in a 15% decrease in the levels of IP_3 in treated animals (Johnson et al., 1992). A previous study in adult male rats exposed to aluminum sulfate (0.3%) in the drinking water for 1 month found a small (<10%) but statistically significant increase in the number of muscarinic receptors in the hippocampus (Connor et al., 1988). However, neither cortical muscarinic receptor expression nor cholinergic nor adrenergic stimulation of IP turnover in slices made from treated animals were altered (Connor et al., 1988). Aluminum sulfate exposure (0.3% in drinking water) for 4 months has also been associated with increases in the phosphorylation of endogenous proteins in cortical homogenates and increased activity of PKC in the particulate fraction of adult rat cortical homogenates (Johnson et al., 1990). In addition, a 3 month exposure to 0.3% aluminum sulfate in drinking water also decreased MAP-2 content in both adult and developing animals, as well as increased hippocampal cyclic AMP (Johnson et al., 1992). Tubulin, tau and neurofilament protein levels were not changed in these studies in rats (Johnson et al., 1992). An increase in cyclic AMP activity has also been reported in neuroblastoma X glioma cells cultured in the presence of 2 mM aluminum lactate for 4 days (Singer et al., 1990). This study also reported a 41% reduction in muscarinic receptors and a 37% increase in the activity of choline acetyltransferase, but no changes in neurofilament quantity or ultrastructure (Singer et al., 1990). Thus, following both acute *in vitro* and more prolonged *in vivo* studies, aluminum exposure perturbs phosphoinositide signaling, expression of related receptors and activity of related kinases.

In vitro studies have been extremely useful in determining the mechanisms of action of aluminum on receptor-stimulated phosphoinositide signaling. There are numerous potential targets of aluminum action on this system. It is virtually certain that the disruption of phosphoinositide signaling by aluminum is not mediated through actions on receptors. Several lines of evidence support this conclusion. First, as discussed above, aluminum disrupts phosphoinositide signaling stimulated by a variety of different receptor types, including cholinergic, adrenergic, glutamatergic and bradykinin receptors. While it is unlikely that aluminum would interact directly with many different receptor types, it could be acting indirectly via a modulatory site (e.g. aluminum interactions with extracellular ATP; Exley, 1999). Secondly, and more conclusively, is the demonstration that aluminum disrupts phosphoinositide signaling even if receptor activation is bypassed by direct activation of G-proteins; aluminum chloride inhibits IP accumulation in brain slices when NaF (Jope, 1988; Shafer et al., 1993) or GTPγS are utilized to activate directly G-proteins (Shi & Haug, 1992; Shi et al., 1993). Thus, effects of aluminum on phosphoinositide signaling are thought to occur at a site downstream of receptor activation.

G-proteins appear to be extremely sensitive substrates for aluminum action (Exley & Birchall, 1992), and aluminum has an affinity for the Mg^{2+} binding site in G-proteins which exceeds that of Mg^{2+} by approximately seven orders of magnitude (MacDonald et al., 1987). Not surprisingly, aluminum has been demonstrated to alter the activity of a number of G-proteins including inhibiting transducin (Kanaho et al., 1985; Miller et al., 1989) and tubulin (MacDonald et al., 1987) activity, and activating the adenylate cyclase-stimulating G-protein G_s (Johnson et al., 1989). An action of aluminum on G_q cannot be ruled out as contributing to the decreased agonist-induced IP accumulation

in the presence of this metal, but there is substantial *in vitro* evidence that aluminum does not mediate inhibition of phosphoinositide signaling at this site. Aluminum chloride inhibited GTPγS stimulated IP accumulation in permeabilized neuroblastoma cells in a non-competitive manner; neither high concentrations of GTPγS nor Mg^{2+} were able to reverse the inhibitory action of aluminum (Shi et al., 1993). In addition, aluminum chloride was without effect on adrenergic stimulation (via G_s) and muscarinic inhibition (via G_i) of cAMP formation in brain slices under conditions identical to those in which aluminum inhibited receptor stimulated IP accumulation (Shafer et al., 1994). Given the high affinity of aluminum for the Mg^{2+} binding site of transducin (MacDonald et al., 1987), it is unlikely that aluminum would have actions on G_q, but not G_s or G_i in brain slices. The non-competitive inhibition of IP turnover in permeabilized neuroblastoma cells may be due to one or more of the following: (i) permeabilization allows aluminum to have access to sites not available under normal conditions, (ii) the high affinity of aluminum for the Mg^{2+} binding site on G-proteins makes the interaction of aluminum essentially irreversible, or (iii) the actions of aluminum occur at a site downstream from G-protein activation. Examples of the latter include disruption of G-protein/Pi-PLC interactions, or disruption of hydrolysis of PIP_2 by Pi-PLC.

In fact, hydrolysis of PIP_2 by Pi-PLC is likely to be the critical site of action for aluminum on phosphoinositide signaling systems. If the activation of G proteins is bypassed by stimulating Pi-PLC activity with calcium, IP formation is inhibited by aluminum chloride in both synaptosomes (Shafer et al, 1994) and neuroblastoma cells (Shi et al., 1993). These data suggest that the site of action of aluminum is Pi-PLC activity, not G-protein function and/or G-protein/Pi-PLC interactions, since an effect of aluminum on these sites would be overcome by direct stimulation of Pi-PLC in the above experiments. Contrary to overcoming aluminum effects, the magnitude of inhibition of aluminum on calcium-stimulated (direct stimulation of Pi-PLC) IP accumulation was nearly identical to its inhibition of receptor-stimulated IP accumulation (Shafer et al., 1994). Additional evidence confirms that the action of aluminum is to decrease hydrolysis of PIP_2. Aluminum chloride inhibits Pi-PLC activity ($IC_{50} \sim 100$ μM) in cortical homogenates (Shafer et al., 1994) and does so in a manner which is competitive with the substrate, PIP_2 (Nostrandt et al., 1996). Macdonald and Mamrack (1988; 1995) had previously reported that Pi-PLC activity isolated from heart was also inhibited by aluminum, and a subsequent report has demonstrated that aluminum binds to PIP_2 in liposomes with a 1:1 stoichiometry (Jones & Kochian, 1997).

The decrease in PIP_2 hydrolysis by Pi-PLC in the presence of aluminum may in fact be due to specific interactions of aluminum with PIP_2. Acetylcholinesterase is attached to rat erythrocytes via a phosphatidylinositol bond which can be cleaved by Pi-PLC (Low & Finean, 1977; Futerman et al., 1985), but this activity is not inhibited by aluminum chloride at concentrations much higher (>400 μM) than those which inhibit PIP_2 hydrolysis (Nostrandt et al., 1996). Studies of Pi-PLC activity isolated from heart (McDonald & Mamrack, 1988) or in neuroblastoma cells (Shi et al., 1993) suggest that hydrolysis of PIP is less susceptible to inhibition by aluminum. Thermodynamically, the preferential binding of aluminum by PIP_2 is consistent with the ability of multiple phosphate groups on macromolecules to participate in cooperative binding of aluminum (Birchall and Chappell, 1988).

Aluminum also has other actions on important components of cellular signaling in neurons. PKC, an important enzyme activated by hydrolysis of PIP_2 by Pi-PLC, is also inhibited by low micromolar (50–100 μM) aluminum chloride (Katsuyama et al., 1988; Cochran et al., 1990; Shafer et al., 1994) when PKC activity is examined in brain homogenates. However, actions of aluminum on this enzyme may be due to complexation of the ATP substrate in these assays by aluminum, as agonists and antagonists of PKC activity did not influence the inhibition of receptor-stimulated IP accumulation by aluminum chloride (Shafer et al., 1994). In contrast to these effects on PKC activity, aluminum concentrations between 10 nM and 10 μM have also been reported to increase the phosphorylation of human tau protein by kinases, including P34 and PKC (El-Sabae et al., 1993). Higher concentrations of aluminum (100–500 μM) inhibit phosphorylation of tau by these kinases (El-Sabae et al., 1993). Aluminum chloride also inhibits the extrusion and sequestration of calcium by Ca^{2+}/Mg^{2+}-ATPases (Kodavanti et al., 1993; Mundy et al., 1994); once again, these assays are in broken cell preparations and require exogenous ATP, which may potentially be complexed by aluminum. However, aluminum does alter intracellular calcium homeostasis in more intact systems. In permeabilized neuroblastoma cells, aluminum inhibited both IP release and calcium mobilization (Wood et al., 1994). More dramatic alterations occur in non-neuronal cells, as aluminum citrate (10 μM) perturbed agonist-induced calcium oscillations in hepatocytes by first increasing the frequency of oscillations but later increasing basal calcium levels and causing oscillations to broaden and become irregular (Schöfl et al., 1990). These alterations in calcium oscillations are not due to effects of aluminum on 3-kinase or 5-phosphatase activity, as aluminum did not affect activity of these enzymes isolated from rat liver microsomes (Shears et al., 1990). Aluminum also impairs calcium mobilization from intracellular stores in pancreatic acinar cells (Wakui et al., 1990; Petersen et al., 1992). By contrast, while a sixty minute treatment with aluminum chloride (10–1000 μM) had no effect on intracellular calcium on its own, aluminum potentiated glutamate-induced intracellular calcium increases in primary cultures of cerebellar granule cells (Mundy et al., 1997). In cortical neurons grown in culture, aluminum altered the frequency of spontaneous intracellular calcium oscillations following exposure for 22 days. This measure, used as an index of synaptic connectivity, preceded aggregation of cell bodies and fasciculation of neuritic processes following 48 days of exposure to 100 μM aluminum (Kawahara et al., 1992). While the studies discussed above vary widely in the effects of aluminum and the particular endpoint examined, they do demonstrate that aluminum consistently affects the mechanisms responsible for calcium homeostasis and protein phosphorylation in a variety of cell types.

In vivo studies have also indicated potential alterations in neuronal calcium homeostasis. Injection of 100 μl of 0.05 M aluminum lactate into the tail vein of mice led to an increase in $^{45}Ca^{2+}$ content in brain tissue of treated animals when assessed 24 hours after exposure (Anghileri 1992). In addition, direct injection of 100 mM aluminum lactate (total 10 μmol aluminum) into the ventricles of the brain were associated with an increase in tissue calcium content contiguous with the onset of seizures in treated animals (Farnell et al., 1985). Perhaps the most complete *in vivo* study regarding effects of aluminum exposure on calcium homeostasis mechanisms and related signal transduction enzymes was conducted by Julka and Gill (1996). Rats received intraperitoneal injections of 10 mg/kg aluminum each day for 4 weeks. *Ex vivo* examination revealed that treated rats had

lower Ca^{2+}-ATPase activity, increased synaptosomal intracellular calcium concentrations but decreased depolarization-dependent calcium influx, decreased membrane fluidity, and decreased activities of calmodulin and PKC (Julka & Gill, 1996). Thus, there is evidence from both *in vitro* and *in vivo* studies that aluminum alters calcium homeostasis and related signaling in neurons.

In addition to intracellular calcium homeostasis, aluminum also disrupts the function of a variety of ion channels important to regulation of membrane excitability and/or calcium entry. Aluminum chloride ($\geq$50 μM) blocked calcium entry into rat brain synaptosomes (Koenig & Jope, 1987) and blocked voltage-gated calcium channel current (Büsselberg et al., 1993; Platt & Büsselberg, 1994). Block of voltage-gated calcium current is most likely to be due to Al^{3+} ion, as increasing the concentration of this ion by decreasing pH increases the potency of block by aluminum (Platt et al., 1993). Effects of aluminum on other ion channel types which might influence electrical excitability and calcium homeostasis are not clear. Aluminum was reported to biphasically enhance then inhibit $GABA_A$ receptor gated currents in olfactory bulb neurons grown in culture (Trombley, 1998), but to only cause slight reductions in $GABA_A$ current in dorsal root ganglion cells (Ma & Narahashi, 1993). The former paper also reported that aluminum was without significant effects on voltage- as well as glutamate-gated ion channel currents (Trombley, 1998) but others have reported that aluminum inhibits glutamate (Platt et al., 1994), and enhances (Csòti et al., 1999) or inhibits (Kanazirska et al., 1997) voltage-gated sodium currents.

Informed Opinion

From the research described above it is evident that aluminum can have multiple effects on calcium homeostasis, phosphoinositide signaling and protein phosphorylation. Much of this work was performed using *in vitro* preparations, with effective concentrations of aluminum ranging from 10 to 1000 μM. Total brain aluminum has been measured in patients suffering from dialysis dementia (concentrations up to 500 μM) and Alzheimer's disease (concentrations up to 80 μM) (Ganrot, 1986). However, the question remains as to whether the effects of aluminum observed experimentally can participate in the etiology of Alzheimer's disease or contribute to the loss of cognitive function which characterizes its early manifestation. The question is made more difficult by the lack of a well accepted mechanism for the cause of Alzheimer's disease. One theory is based on the fundamental importance of calcium signaling in the brain. Calcium plays a pivotal role in many neuronal processes including neurotransmitter release, neuronal growth and plasticity, activation of kinases and phosphatases, and cell death. Thus, alterations of calcium signaling could be involved in the cellular processes which are disturbed in the aging brain and in Alzheimer's disease.

The 'calcium hypothesis' proposes that deregulation of intracellular calcium homeostasis and the associated changes in calcium signaling are the central defects which underlie brain aging and Alzheimer's disease (Disterhoft et al., 1994; Khachaturian, 1994). In its original form, the calcium hypothesis postulated that, due to excessive influx, reduced efflux, or impaired intracellular buffering, intracellular calcium levels were increased.

Thus, sustained increases in intracellular calcium would contribute to impaired function and eventually cell death in later stages of the disease (Thibault et al., 1998). Consistent with this hypothesis, increased expression of L-type voltage-gated calcium channel has been demonstrated *in vivo* in hippocampus of aged animals (Herman et al., 1998). In primary cultures of hippocampal neurons, increasing expression of L-type voltage-gated calcium channel with age *in vitro* is correlated with decreasing neuronal survival (Porter et al., 1997). The calcium elevation hypothesis is also supported by experimental results on the effects of β-amyloid (Aβ) on calcium signaling. The brain of Alzheimer's patients has large accumulations of Aβ, a main component of the amyloid plaques which are a pathological hallmark of Alzheimer's disease (Hardy & Higgins, 1992). In artificial lipid bilayers and in cell culture, Aβ can interact directly with the membrane, forming calcium channels and increasing intracellular calcium (Mattson et al., 1992; Arispe et al., 1993; Ueda et al., 1997). Further support for the calcium elevation hypothesis comes from the finding that calpain, a calcium-activated neutral protease (Nixon, 1989), is over-activated in brains from Alzheimer's patients (Saito et al., 1993). Calpain is involved in the dynamic turnover of tau, a protein which is the main component of the paired helical filaments. Paired helical filaments form neurofibrillary tangles (NFTs), which are the other pathological hallmarks of Alzheimer's disease. If the actions of aluminum tended to increase intracellular calcium, it would act in concert with the increase in calcium induced by Aβ. Interestingly, an inherited form of early-onset Alzheimer's disease has been linked to mutations in the presenilin-1 (PS-1) gene on chromosome 14 (Kim & Tanzi, 1997). Cells expressing mutant PS-1 have perturbed calcium homeostasis and, at high levels of expression, show enhanced elevations of intracellular calcium (Guo et al., 1996). Thus, it is possible that genetically linked forms of Alzheimer's disease could also be impacted by effects of aluminum to increase intracellular calcium.

However, the calcium elevation hypothesis has been recently challenged. Because calcium-mediated processes (e.g. cell growth and plasticity, neurotransmission) are diminished during aging and Alzheimer's disease, it has been proposed that there is actually a *deficit or reduction* of intracellular calcium during the early stages of the disease, with rises occurring only at the later stages of the disease (Müller et al., 1996; Chen, 1998; Chen & Fernandez, 1999). Direct measurement of global changes in resting intracellular calcium concentrations in the brain are not possible. However, there are several reports using peripheral tissues from Alzheimer's patients and brain cells isolated from aging animals indicating reductions in intracellular calcium levels. Peterson and colleagues have reported that there is a decrease in the resting level as well as in agonist-stimulated levels of cytosolic intracellular calcium in skin fibroblasts from Alzheimer's patients as compared to age-matched controls (Peterson et al., 1986; Peterson et al., 1988). In acutely isolated brain cells from aging mice, both resting and depolarization-stimulated intracellular calcium levels were lower compared to young controls (Hartmann et al., 1996).

A reduction of calcium signaling can be linked to the formation of amyloid plaques and NFTs. Aβ is derived from the inappropriate processing of the β-amyloid precursor protein (APP). APP is processed by two mutually exclusive pathways; the actions of α-secretase lead to a soluble form of APP, while the β-/γ-secretases result in the production of Aβ. A reduction in the activity of α-secretase, a calcium dependent enzyme, has been

proposed to result in a decrease in soluble APP and an increase in Aβ in Alzheimer's disease (Lannfelt et al., 1995; Chen, 1997). Likewise, the formation of NFTs could result from a decrease in calcium signaling. The hyperphosphorylation of tau is the primary mechanism underlying NFT accumulation, and is mainly the result of the inactivation of phosphatases, including the calcium-dependent phosphatase calcineurin (Trojanowski and Lee, 1995). Thus, a decrease in calcium-dependent calcineurin activity could lead to increased levels of phosphorylated tau and an increase in NFT accumulation. These data suggest that the primary cause of Alzheimer's disease may be a reduction in calcium signaling, which could ultimately lead to the formation of amyloid plaques and NFTs. In this scenario, actions of aluminum which tend to reduce intracellular calcium levels would contribute to the disease state. Such actions of aluminum have been described above, including effects to decrease calcium mobilization from intracellular stores and inhibition of calcium currents and calcium influx.

Regardless of whether elevated brain aluminum is causative of or pivotal to the development of pathology and progressive neurodegeneration in Alzheimer's disease, alterations of calcium and phosphoinositide signaling could exacerbate the widespread, neurotransmitter-based cognitive disturbances observed in Alzheimer's disease. One of the earliest and most consistent findings was the breakdown in cholinergic transmission. Early studies showed that cholinergic neurons in the basal forebrain degenerate in Alzheimer's patients, and marked parallels were noted between the loss of memory and cognitive function and the loss of acetylcholine that occurred in the cerebral cortex, hippocampus, and amygdala of these patients (Bartus et al., 1982; Coyle et al., 1983). Studies in both humans and animals strongly supported the idea that decreases in cholinergic function result in the loss of memory and cognition (Coyle et al., 1983; Bartus et al., 1985; Arendash et al., 1987; Mundy & Tilson, 1988) and supported the "cholinergic hypothesis of memory dysfunction". This hypothesis asserts that significant functional deficits in cholinergic signaling underlie the memory loss and cognitive impairment associated with aging and Alzheimer's disease. A majority of the cholinergic receptors in the brain are of the muscarinic subtype, and cholinergic signaling through the muscarinic receptor is linked to phosphoinositide hydrolysis. This suggests that aluminum effects on phosphoinositide hydrolysis could impair further an already compromised cholinergic system in Alzheimer's disease.

In light of the relationship between the cholinergic system and cognitive function, there have been a number of studies on the phosphoinositide hydrolysis pathway in Alzheimer's disease. There is now considerable evidence that regulation of this pathway is severely disrupted in Alzheimer's disease at the level of receptor/G-protein coupling, G-protein activation of Pi-PLC, PKC activation, and actions of IP_3 at its cognate receptor. While it does not appear that there is a significant decrease in muscarinic receptor number in Alzheimer's disease (Mash et al., 1985; Svensson et al., 1992), the interaction of the muscarinic receptor with G-proteins is altered. Using receptor binding techniques, Flynn et al. (1991) showed a loss in high-affinity agonist binding in post-mortem brain tissue from Alzheimer's patients, indicative of receptor/G-protein uncoupling. Receptor/G-protein coupling can also be examined by measuring receptor-stimulated GTPase activity. Using this technique, Joseph et al. (1993) found significant decreases in carbachol-stimulated GTPase activity in the basal ganglia and hippocampus of Alzheimer's brains that cor-

related with disease severity and duration. Similarly, Ladner et al. (1995) demonstrated muscarinic receptor/G-protein uncoupling that was restricted to brain regions showing abundant amyloid plaque and NFT pathology.

Several studies have investigated the activation of Pi-PLC in postmortem tissue from Alzheimer's disease brains following stimulation by neurotransmitters and G-protein activators. Using a mixture of radiolabeled exogenous phosphoinositides in the presence of GTP, Ferrari-DeLio and Flynn (1993) reported a decreased muscarinic receptor-stimulated Pi-PLC activity in frontal cortex. Similar findings in frontal cortex were reported by Crews et al. (1994). Jope and colleagues reported a significant reduction in carbachol stimulated, GTPγS-dependent Pi-PLC activity in prefrontal cortex from Alzheimer's brains (Jope et al., 1994; Greenwood et al., 1995). These studies suggest a deficit in muscarinic-stimulated phosphoinositide hydrolysis in Alzheimer's disease. However, phosphoinositide hydrolysis in post-mortem brain from Alzheimer's patients has also been shown to be decreased after stimulation with other neurotransmitters including glutamate, histamine, and serotonin (Greenwood et al., 1995), as well as in response to direct G-protein activation (Crews et al., 1994; Greenwood et al., 1995). This data points to a general dysfunction in G-protein activation of PLC as well as a more specific defect in muscarinic receptor/G-protein coupling. Because expression of the G-proteins involved in phosphoinositide hydrolysis appears to be unaltered in Alzheimer's patients (Greenwood et al., 1995; Shanahan et al., 1995; Cowburn et al., 1996), the decreased activation of Pi-PLC by G-proteins in Alzheimer's disease probably results from an altered function of G-proteins, rather than a simple decrease in their levels.

Stimulation of Pi-PLC activity leads to the formation of the second messengers DAG and IP_3, which activate PKC and release intracellular calcium, respectively. Thus, impairment of phosphoinositide hydrolysis could lead to further deficits in downstream signaling pathways. Decreases in PKC activity and regulation have been observed in Alzheimer's disease (Cole et al., 1988; Masliah et al., 1991; Wang et al., 1994). In addition, decreases in the levels of various PKC isoforms including PKC βII (Masliah et al., 1990; Shimohama et al., 1993) and PKCζ (Greenwood et al., 1995) have been reported. As well as impairing neuronal signaling, a decrease in PKC activity has implications for the formation of amyloid plaques. It has been reported that activation of PKC via muscarinic receptors stimulates the secretion of the soluble form of APP and reduces the formation of Aβ (Hung et al., 1993; Nitsch & Growdon, 1994). Similarly, stimulation of metabotropic glutamate receptors also appear to regulate the secretion of APP via a PKC-associated mechanism (Jolly-Tornetta et al., 1998). Therefore, impairment of the Pi-PLC/PKC signaling pathway would be expected to allow a greater accumulation of Aβ. Studies in Alzheimer's brains have also shown a loss of IP_3 receptor sites. Receptor binding studies in homogenates have shown significant decreases in IP_3 receptors in hippocampus, cerebellum, and cortex from Alzheimer's brain (Young et al., 1988; Garlind et al., 1995). Using an antibody directed against the IP_3 receptor, Haug et al., (1996) confirmed the loss of IP_3 receptors in temporal cortex of Alzheimer's brains, and demonstrated a correlation with the severity of Alzheimer's pathology based on the numbers of amyloid plaques and NFTs. The loss of IP_3 receptors in Alzheimer's disease would be expected to contribute to the alterations in calcium signaling and homeostasis described previously.

Because most studies on phosphoinositide signaling are performed using post-mortem tissue, it should be cautioned that they may be giving a picture of events which are occurring near the end of the disease process. However, the ability of aluminum to inhibit phosphoinositide hydrolysis clearly suggests this effect could interact with the disruption of phosphoinositide signaling observed in Alzheimer's disease, regardless of when these effects first take place. Changes in phosphoinositide signaling occurring during early stages of the disease could be related to the manifestation of cognitive deficits. This notion is supported by evidence showing cognitive effects after human exposure to high levels of aluminum via long-term kidney dialysis or occupational exposure. Aluminum has been shown to be the causative agent for dialysis encephalopathy (Alfrey et al., 1976), a syndrome resulting in impaired cognitive function and memory loss which is correlated with plasma aluminum content (Bolla et al., 1992). Cognitive deficits including memory loss were also observed following occupational exposure to aluminum dust (Rifat et al., 1990) or fumes (White et al., 1992). Changes in phosphoinositide signaling could also promote formation of amyloid plaques by interfering with PLC/PKC signaling and subsequent formation of Aβ; however, this hypothesis is not supported by data showing that exposure of neuroblastoma cells or rats to aluminum had no effect on the processing of APP (Neill et al., 1996). As described above, the primary site of action of aluminum is most likely at the level of the Pi-PLC-substrate (PIP_2) interactions (although effects at the level of the G-protein cannot be ruled out), while the major defect in Alzheimer's disease appears to be at the level of the G-protein. Thus, it is unlikely that the changes observed in phosphoinositide hydrolysis observed in Alzheimer's disease are the direct result of aluminum. However, because the actions are at two separate but sequential sites in the phosphoinositide signaling pathway, there is the potential for a synergistic effect of aluminum to inhibit signaling further in an already compromised system.

While a number of plausible mechanisms have been described by which aluminum-induced alterations in calcium and phosphoinositide signaling could have an impact on the etiology of Alzheimer's disease, the uncertainties involved make conclusions on the role of aluminum difficult. These uncertainties include 1) lack of knowledge about the underlying cause of the disease, 2) lack of good animal models which encompass both the cognitive and neuropathological changes characteristic of the disease, and 3) biochemical measurements which are by necessity made *in vitro* or *ex vivo*, or in post-mortem tissue. In order to examine further the hypothesis that aluminum effects on calcium and phosphoinositide signaling are involved in the etiology of Alzheimer's disease, the following recommendations outline some basic principles which should be considered in addressing these uncertainties.

(i) Development and use of an appropriate animal model, which shows the progressive development of an Alzheimer's disease-like state. Such a model might be based on the recent development of transgenic mice with copies of the human mutant APP and PS-1 genes. These mouse strains are predisposed to form neuritic plaques within 9–10 months.

(ii) Chronic, low-level dosing of the animal model with aluminum, along with periodic testing to examine early and late stage changes in calcium and phosphoinositide signaling in relation to the development of cognitive and pathological abnormalities.

(iii) Proof-of-principle studies designed to show that alteration of calcium and phosphoinositide signaling can result in cognitive and/or pathological changes. These studies could use pharmacologic agents, antibodies, or antisense technology to specifically target sites of action which are proposed to be altered by aluminum, including calcium channels, G-proteins and Pi-PLC.

(iv) Development of new imaging techniques that will allow determination of signaling parameters in the intact brain. By their very nature, signaling pathways are part of a complicated web, with changes in one parameter of signaling affecting many others. This complex system will function differently when studied in isolated elements, and the true nature of toxicant effects may only be assessed in the intact system.

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Aluminium and Alzheimer's Disease
The Science that Describes the Link
C. Exley (Editor)

CHAPTER 19

The Interaction of Aluminium with Peptides and Proteins

Tamás Kiss [1,*] and Miklós Hollósi [2]

[1] *Department of Inorganic and Analytical Chemistry, University of Szeged, P.O. Box 440, H-6701 Szeged, Hungary*
[2] *Department of Organic Chemistry, Eötvös Loránd University, P.O. Box 32, H-1518 Budapest, Hungary*

* Corresponding author, e-mail: tkiss@chem.u-szeged.hu

Abbreviations: Aβ – β-amyloid; AD – Alzheimer's disease; Ala – alanine; APD – amyloid precursor protein; Asn – asparagine; Asp – aspartic acid; ATP – adenosine triphosphate; BBB – blood brain barrier; CD – circular disroism; CNS – central nervous system; Cys – cysteine; EDTA – ethylenediaminetetraacetic acid; EM – electron microscopy; FTIR – Fourier transform infrared; GDP – guanosine diphisphate; Gln – glutamine; Glu – glutamic acid; Gly – glycine; GTP – guanosine triphosphate; His – histidine; Hyp – hyroxyproline; IDA – iminodiacetic acid; IRP – iron regulatory protein; LFER – linear free energy relationship; Lys – lysine; Met – methionine; NF – neurofilament; NFH – high-size neurofilament subunit; NFL – low-size neurofilament subunit; NFM – mid-size neurofilament subunit; NFT – neurofibrillary tangle; NMR – nuclear magnetic resonance; NOESY – nuclear Overhauser effect spectroscopy; NTA – nitrilotriacetic acid; pGlu – pyroglutamic acid; PHF – paired helical filament; Pro – proline; Ser – serine; Ser(P) – phosphorylated serine; SP – senile plaque; TF – transferrin; TFE – trifluoroethanol; TFR – transferrin receptor; Thr – threonine; Tyr – tyrosine

Summary

The chapter provides a chemical background and a basis for an understanding of the possible role of Al(III) in biology by reviewing the coordination chemistry of the interactions of Al(III) with peptides and phosphorylated peptides and their building blocks: amino acids and phosphorylated amino acids. It then discusses the interactions of Al(III) with enzyme and non-enzyme proteins in general, but not those proteins which may be more directly involved in Alzheimer's disease (these are covered in other chapters of this book).

Historical Perspective

General Characterization of Al(III)–Protein Interactions

Proteins, and to a lesser extent their building blocks, peptides and amino acids, may be the most important materials of living systems. They are not only bulk constituents

of organs and tissues, but as reactive organic compounds are important regulators of biological processes. They all contain electron pair donor functional groups, which are able to bind electron pair acceptor metal ions. Their interactions are essentially important in the functions of metalloproteins and metalloenzymes, but may be detrimental when metal ion coordination interferes or even blocks biological reactions.

The basic metal-binding mode of amino acids is through both the amino and the carboxylate groups. Monodentate carboxylate coordination often occurs with relatively small and/or highly charged metal ions (called hard metal ions), such as Ca^{2+}, Mg^{2+}, Al^{3+}, *etc.*, while monodentate amino coordination is frequently observed with relatively large metal ions with more diffuse electron shells (called soft metal ions), such as Ag^{+}, Pt^{2+}, Hg^{2+}, *etc.* However, chelation involving both the amino and the carboxylate groups is the most common mode of binding of amino acids with metal ions (Kiss, 1990). Besides these groups, the donor atoms in the side chain offer further potential sites for interaction. Depending on the nature of the metal ion and their spatial arrangements within the amino acid molecule, the histidine (His)-imidazole, cysteine (Cys)-thiolate and aspartate (Asp)-carboxylate groups are the most frequent additional binding sites. The tyrosine (Tyr)-phenolate, lysine (Lys)-amino and glutamate (Glu)-carboxylate groups are less important binders for steric reasons, while the serine (Ser)- or threonine (Thr)-alcoholic-OH, methionine (Met)-thioether and asparagine (Asn)- and glutamine (Gln)-amide groups are less important because of their weaker donor strengths. Alcoholic-OH and amide functionalities can be significantly stronger binders of metal ions when they are in the deprotonated form.

The metal binding capability of oligopeptides is highly influenced not only by the nature (basicity, charge, *etc.*) of the C- and N-terminal donors, but also by the presence and relative positions of suitable side-chain donors, which may be able 'to anchor' the metal ions (Sóvágó, 1990). In the case of oligopeptides consisting of only a few amino acids, the metal ion-induced deprotonation and subsequent coordination of the peptide-NH group play a crucial role in the efficiency of metal binding. With Al(III), no metal ion-promoted deprotonation of the amide can be detected. The reasons are the lack of suitable anchoring donors in the peptide chain and the rather hard character of Al(III) as compared with the much more borderline character of the amide group. For larger peptides or even proteins a suitable spatial arrangement *i.e.* some degree of preorganization of appropriate donor groups, becomes much more important in metal binding, and the possibility of amide coordination is generally subordinate. Accordingly, a suitable arrangement of the donors, *i.e.* the necessary conformation of the peptide/protein molecule, has a decisive role in Al(III) binding. The extent of preorganization of a peptide/protein may of course increase with the molecular weight as the possibility of structure-making weak interactions within the molecule increases.

The behaviour of Al(III) species in cells and biological fluids can be described by four different forms: (i) free or mononuclear ions, (ii) low molecular mass complexes, (iii) reversible macromolecular complexes, and (iv) irreversible macromolecular complexes (Macdonald & Martin, 1988).

As a highly charged small cation, Al^{3+} is easily hydrolysed in aqueous solutions in the absence of competing ligands. Neutral solutions give a precipitate of $Al(OH)_3$, which redissolves with the formation of $Al(OH)_4^-$, the primary soluble Al(III) species at pH >7

at μM concentrations of total Al(III). However, solutions which are supersaturated with respect to amorphous $Al(OH)_3$ are frequently formed.

Al^{3+} is a typical hard metal ion. The most likely binding sites of $Al(H_2O)_6^{3+}$ in biosystems are therefore O donors, and especially negatively charged O donors. Carboxylate, phenolate, catecholate and phosphate are the strongest Al(III) binders. Biomolecules containing such functions may be involved in the uptake and transport processes of Al(III).

Al(III) interacts with a large number of proteins, glycoproteins and carbohydrates, but very little is known about the chemistry, binding strength and binding mode of these complexes. Al(III)–transferrin (TF) complexes are the best characterized. Because of the lack of quantitative information, it is not easy to assess the biological relevance and possible biological role of such interactions.

The fourth major component of Al(III) binding in biological systems is irreversible macromolecular complexes. Although Al(III) is regarded as a rather sluggish metal ion, the practically irreversible binding of the metal ion in biological systems is rather rare. It may occur, however, in molecular aggregates, when the exchange reactions of Al(III) are slowed down because of the formation of hydrolysed oxo- or hydroxo-bridged Al clusters enclosed by organic compounds. Al(III) accumulated in the brain in neurofibrillary tangles (NFTs) and senile plaques (SPs) may represent such complexes.

The Al(III)-binding potential of bioligands may be characterized by $pAl = -\log[Al^{3+}]$, *i.e.* the negative logarithm of the free Al^{3+} concentration. These values are computed from the thermodynamic stability constants at known total concentrations of the metal ion and the ligand. For biologically relevant Al(III) complexes, the value of pAl is commonly calculated for a solution containing 1 μM total metal and 50 μM total ligand. Table 1 lists the pAl values for several bioligands at the physiological pH of 7.4. The higher the pAl value, the stronger the binding of the biomolecule.

The data in Table 1 reveal that simple bidentate carboxylic ligands such as lactic acid, oxalic acid or amino acids cannot keep Al(III) in solution via complex formation, but that $Al(OH)_3$ precipitates or forms metastable solution at physiological pH. Tridentate hydroxycarboxylates or organic phosphates are stronger Al(III) binders and keep Al(III) in solution (Kiss et al., 1991). Amino acids are weak binders, whereas an oligopeptide is strong enough to prevent the precipitation of $Al(OH)_3$, while a protein with a specific donor group arrangement can be an extremely strong binder. The natural siderophore desferrioxamine is one of the strongest Al(III) binders known.

In addition to the stabilities of metal ion complexes, an important and often overlooked feature is the rate of ligand exchange out of and into the metal ion coordination sphere. Al(III) displays a relatively low ligand exchange rate with a first-order rate constant of up to 10^4 s^{-1} (Orvig, 1993). The rate of exchange of inner sphere water with bulk water, measured for many metal ions, gives the following sequence:

$$Al^{3+} \ll Fe^{3+} < Ga^{3+}, Be^{2+} \ll Mg^{2+} < Fe^{2+} < Zn^{2+} < Ca^{2+}$$

Each inequality sign indicates an approximate 10-fold increase in rate constant, from 1.3 s^{-1} for Al^{3+} to 10^8 s^{-1} for Ca^{2+} at 25°C (Martin, 1991). The 10^5 times higher rate for Mg^{2+} furnishes an explanation for the inhibition by Al^{3+} of enzymes with Mg^{2+} cofactors. Processes involving rapid Ca^{2+} exchange would obviously be totally thwarted

Table 1. Most likely Al(III) complexes and pAl values at pH 7.4 and 25°C [a]

Bioligand	Species present [b]	pAl [c]
Lactic acid	Metastable solution + $Al(OH)_3$ (s)	10.7
Oxalic acid	$Al_2(OH)_2L_4$ + $Al(OH)_3$ (s)	10.7
Catecholamines	AlL_3	12.5–13.4
Citric acid	$Al(LH_{-1})L$, $Al_3(LH_{-1})_3(OH)$	12.9
Hydroxide	Amorphous $Al(OH)_3$	10.7
Phosphate [d]	$Al(PO_4)_n(OH)_{3(1-n)}$ (s) (composition variable from $n = 0$ to 1)	11.4
Silicate [e]	$Al_2(OH)_4Si_2O_5$ (s)	12.6
Desferrioxamine	AlL	20.9
Phosphoserine (Ser(P))	$AlL(OH)$, $Al(OH)_4$	10.8
2,3-dpg [f]	AlL, AlL(OH)	12.1
ATP	AlL_2, $AlL_2(OH)$	12.2
Amino acids	$Al(OH)_3$ (s)	10.7
Heptapeptide [g]	$AlL(OH)_2$	11.6
Transferrin	Al_2L	15.3

[a] Taken from Harris et al., 1996 and Kiss & Farkas, 1996.
[b] L is an abbreviation for ligand and (s) denotes a solid precipitate. For the citrate complexes, $H_{-1}L$ represents a coordinated citrate ligand in which the hydroxy group and all three carboxylate groups have been deprotonated. The overall charges on the complexes have been omitted.
[c] $pAl = -\log[Al^{3+}]$, calculated for 1 μM total Al^{3+} and 50 μM total ligand.
[d] 2 mM total phosphate, typical of plasma.
[e] 5 μM total $Si(OH)_4$, typical of plasma.
[f] 2,3-Diphosphoglyceric acid.
[g] See text later.

by substitution of the 10^8-fold slower Al^{3+}. Partially hydrolysed Al(III) often undergoes faster substitution, while chelated ligands exchange more slowly than monodentate ligands. Reactions of oligonuclear and/or mixed hydroxo complexes of Al(III) can be extremely slow, resulting in a non-equilibrium state of Al(III)–ligand systems in biology.

Complexes with Amino Acids and Derivatives

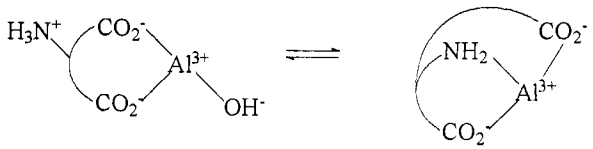
The α-carboxylate group of amino acids is weakly basic (pK ~ 2.2), which suggests a rather weak Al(III)-binding capability. The strength of complexation to Al(III) decreases strongly in the sequence dicarboxylic acid ≫ hydroxycarboxylic acid > carboxylic acid ≫ amino acid. The weakening effect of amino substitution can be explained in terms of the electrostatic repulsive effect of the $-NH_3^+$ group. Use of an LFER (linear free-energy relationship) approach led to the estimation of a stability constant of log K ~ 5.8–5.9 for the interaction between Al(III) and glycine (Gly), the simplest α-amino acid. Simple α-amino acids such as Gly, alanine (Ala), Ser, Thr, Asn and Gln have been found unambiguously to influence the speciation of $Al(H_2O)_6^{3+}$ (Kiss et al., 1997). Formation of the mononuclear species $Al(LH)(OH)^{2+}$ (LH denotes an amino acid protonated at the amino end) and $AlL(OH)^+$, and a carboxylate and dihydroxo-bridged dinuclear complex $Al_2LH(OH)_2^{4+}$ may be assumed. The stability constants obtained from direct pH-metric

measurements for the interactions of Al(III) with simple bidentate amino acids are in the range log K =5.5 to 5.9, which is in good agreement with the value derived from LFER calculations (*vide supra*). ^{27}Al, ^{1}H and ^{13}C NMR studies (Jons & Johansen, 1988; Kiss et al., 1997) in the weakly acidic pH range indicated that in the complexes $Al(LH)(OH)^{2+}$ and $Al_2(LH)(OH)_2^{4+}$ the simple amino acids bind in a monodentate way through the carboxylate group, and the amino group is in the protonated form. At pH $\geq$4.5, the solution becomes opalescent and $Al(OH)_3$ precipitates slowly. With a less basic amino group ($pK(NH^+) \sim 5.2$), complexation becomes conveniently observable with the pyridinecarboxylate α-picolinate, which yields mono and bis complexes AlL^{2+}, AlL_2^+ and $AlL_2(OH)$ (Jons & Johansen, 1988; Feng et al., 1990b).

The tridentate Asp ($(COOH)CH_2CH(NH_2)COOH$), containing two COO^- and one central NH_2 binding donors, is a significantly stronger Al(III) binder. The stability of the 1 : 1 complex is about 2 orders of magnitude higher than that of any simple amino acid, indicating the involvement of both carboxylates in metal binding (Kiss et al., 1997). It is also interesting that no such strong complexation has been detected with either succinic acid ($HOOCCH_2CH_2COOH$) or N-acetylaspartic acid (both lack the central amino binding site), which would indicate involvement of the $-NH_2$ group in the binding mode of Al(III)–Asp complexes. The possible binding isomers of such species are shown in Scheme 1. The relatively strong interaction between Al(III) and Asp can be detected by means of ^{27}Al NMR: a relatively sharp signal at $\sim$ 10 ppm (as compared to $Al(H_2O)_6^{3+}$) suggests octahedral Al(III) in a fairly symmetrical chemical environment. This spectral behaviour is reminiscent of that of the trinuclear Al(III)–citrate complex (Feng et al., 1990a; Lakatos et al., 2000).

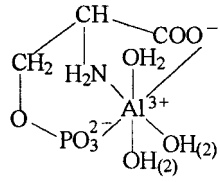
The tridentate coordinating ability of Glu ($HOOCCH_2CH_2CH(NH_2)COOH$) is much weaker, due to the lower stability of the seven-membered chelate ring which should be formed with participation of the terminal carboxylate group. Thus, Glu rather acts as a bidentate ligand, displaying similarity with simple amino acids. These results may suggest that, besides negatively charged O donors such as COO^-, alcoholic–O^- and phenolic–O^- and in the event of a favourable steric arrangement, the amino group can also participate in binding to Al(III).

As the number of potential binding donors increases in the aminopolycarboxylates (such as iminodiacetic acid (IDA) and nitrilotriacetic acid (NTA)) and polyamino-polycarboxylates (such as ethylenediaminetetraacetic acid (EDTA) and others), they become more and more efficient Al(III) binders (Iyer et al., 1989; Öhman, 1990; Clevette & Orvig, 1990). In consequence of the large number of coordinating donors, these ligands, with the exception of the tridentate IDA (Iyer et al., 1989), form only 1 : 1 complexes. Co-



AlL

Scheme 1



$$AlL \longrightarrow AlL(OH) \longrightarrow AlL(OH)_2$$

Scheme 2

ordination of the N-donor groups as well as the carboxylate functions was demonstrated by X-ray crystallography (Valle et al., 1989) and NMR methods (Iyer et al., 1989).

The alcoholic-OH or phenolic-OH side-chain groups of the amino acids in Ser, Thr or Tyr-containing peptides can be easily phosphorylated and these derivatives occur in biological systems. It has been found, for example, that the NFTs observed in the neurons of AD patients are especially rich in abnormally and overphosphorylated proteins (Sternberger et al., 1985; Grundke-Iqbal et al., 1986). As the phosphate groups of these proteins are fairly basic, with $pK_a \sim 6$–7, they can bind Al(III) and other hard metal ions quite strongly (Martin, 1988; Martin 1994). As concerns the metal-binding capabilities of the building blocks of these proteins, the phosphorylated amino acids, a comparison can be made between the potential binding sites. As discussed above, the equilibrium constant characteristic of the Al(III)–amino acid interaction is log K $\sim$ 5.9 to 6.2, while that of the Al(III)–phosphate interaction is log K = 6.1 (Atkári et al., 1996), *i.e.* they are comparable, and hence it is reasonable to assume that with hard metal ions the phosphate moiety is competitive with the aminocarboxylate chelating site for binding Al(III). This was clearly demonstrated by the parallel pH-metric and ^{31}P NMR monitoring of complex formation between Al(III) and the phosphorylated derivatives of Ser (Ser(P)) (Kiss et al., 1998). It was interesting to observe that Ser(P) was capable of keeping Al(III) in solution, preventing precipitation even at pH $\sim$ 8. The most probable binding mode of Ser(P) is its tridentate chelation through (OPO_3^{2-}, NH_2, COO^-) donors (Scheme 2). At pH >6, one or two OH^- are also involved in the coordination. The Ser(P) residue of various peptides and proteins is involved in Al(III) binding in the brain (*vide infra*), but also in other organs, *e.g.* in the gastric protein casein (Danielsson et al., 1995).

Speciation and Multinuclear NMR Studies on Model Oligopeptides

In the systems Al(III)–AspAsp and Al(III)–AspAspAsp, the presence of the numerous negatively charged COO^- functions makes the ligands able to bind to Al(III) in the weakly acidic pH range. Various mononuclear 1 : 1 complexes are formed in different protonation states; however, depending on the metal ion to ligand ratio, precipitation occurs at pH $\sim$ 5 to 6. This indicates that the terminal and side-chain COO^- groups of Asp (and also Glu) at such a low level of preorganization in small peptides are not sufficient to keep Al(III) in solution and to prevent the precipitation of $Al(OH)_3$ at

physiological pH (Kiss et al., 2000). To achieve this, a more specific arrangement of the side-chain donors is necessary.

Several penta- and heptapeptides of neurofibrillary origin have also been studied (Kiss et al., 2000). In the first group, the studied pentapeptides contained Glu and Ser residues, and had a protected C-terminus and a free or protected N-terminus, *e.g.* Ac-ProGluValSerGly-NH_2 and its non-protected analogue HProGluValSerGly-NH_2. It was found that the Al(III)-binding capabilities of these peptides were stronger when they had a free Pro function at the N-terminus. This is rather surprising, as Al(III) is known (Martin, 1991; Kiss et al., 1997) to have a fairly low affinity for amines, and the N-terminal Pro-NH group is therefore not expected to be an efficient binding site. As the C-terminal COO^- was blocked in both peptides, we would expect coordination via the side-chain Glu-COO^- and Ser-O^- groups. Instead, coordination seems to be much more likely through the N-terminal donor groups. In the second group, the peptides had a free C-terminus, but a blocked N-terminus. With these heptapeptides, various 1 : 1 complexes could again be detected by pH-metry. For one of these peptides, Ac-LysSerProValVal GluGly (the formula is given in Fig. 2), 1 : 1 complexes in various protonation states were detected by pH-potentiometry. ^{1}H NMR indicated a weak interaction of the peptide with Al(III) in the slightly acidic pH range. Fig. 1 depicts the ^{1}H NMR spectra of the peptide at pH $\sim$ 4 in the absence and the presence of equimolar Al(III).

The significant selective line broadening indicates interaction between the peptide and the metal ion. The rate of the ligand-exchange reaction is roughly comparable with the ^{1}H NMR time scale; hence, there is practically no shift in the position of the resonances, and there is no increase in the number of signals. Interestingly, mostly the signals of the protons near the C-terminus undergo broadening, which suggests that coordination starts at the terminal COO^- (complex $AlLH_2$), and Al(III) then chelates through the terminal COO^- of the peptide and the side-chain COO^- of Glu^6 (the number in superfix indicates the position of the particular amino acid in the peptide sequence), with participation of the central peptide-carbonyl, through the formation of a joint chelate system (complex AlLH).

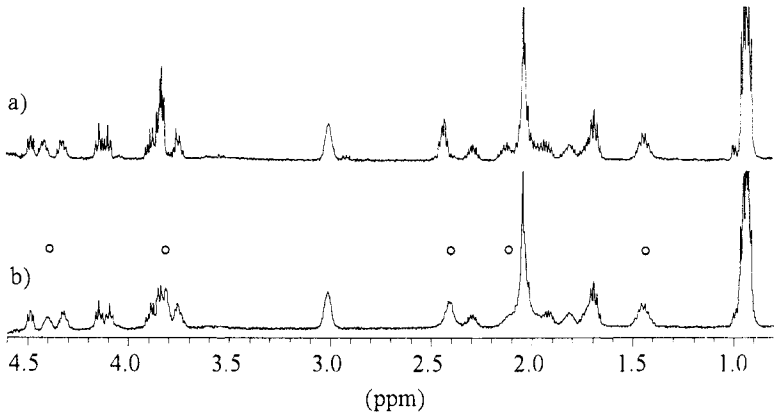


Fig. 1. 1H NMR spectra of Ac-LysSerProValValGluGly (H_2L) at 4.0 mM at pH 4.0 (a) in the absence and (b) in the presence of 4.0 mM Al(III).

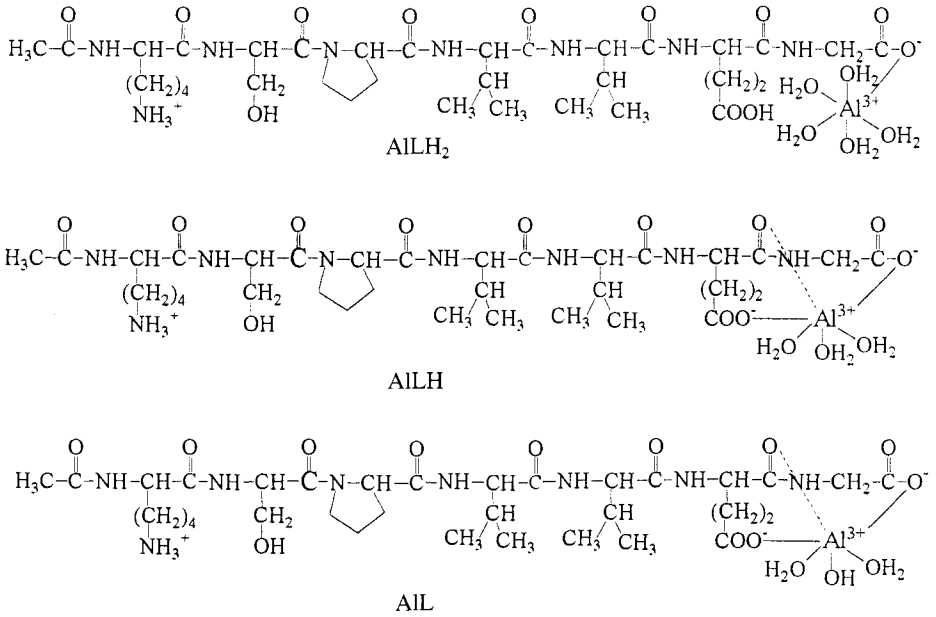


Fig. 2. Suggested binding modes in the Al(III) complexes of Ac-LysSerProValValGluGly (H_2L).

Parallel overlapping deprotonations of the non-coordinating protonated $Lys^1NH_3^+$ and two coordinated water molecules subsequently take place (AlL, $AlLH_{-1}$ and $AlLH_{-2}$) (see Fig. 2). Molecular dynamic calculations indicate favoured arrangements of the terminal Gly^7COO^- and the side-chain Glu^6COO^- for the chelation of Al(III). It is interesting, however, that at pH $\sim$ 7 the two spectra (in the presence and absence of Al(III)) barely differ and the direct coordination of Al(III) is therefore not confirmed. However, since no precipitation is observed in the solution, it is very likely that Al(III)-hydroxo species in some metastable state (which is fairly common in aqueous Al(III) solutions) form outer-sphere complexes through hydrogen-bonding with the hydrated oligopeptides.

The interactions of several other biologically important oligopeptides, among others a nonapeptide fragment of collagen, GlyLysHypGlyGluHypGlyProLys (CP9) (Gervais et al., 1987), and thymulin, pGluAlaLysSerGluGlyGlySerAsn (pGlu: pyroglutamyl), a linear oligopeptide of thymic origin, isolated from the serum (Laussac et al., 1988), have also been studied by multinuclear NMR techniques. In both cases, the formation of similar types of species with 1 : 2 metal ion to ligand stoichiometry has been suggested, with a fairly high association constant. The presence of Al(III) hardly affected the ^1H NMR signals of the two peptides, similarly as in the case of the heptapeptide discussed above. This may again indicate extremely labile complex formation in aqueous medium. However, Al(III) significantly perturbed the ^{13}C frequencies. The fact that the magnitudes of the Al(III)-induced chemical shift perturbations increased with increasing metal ion concentration indicates that the peptide undergoes fast exchange (on the ^{13}C NMR chemical shift time scale) between its free and complexed states.

In the case of CP9, the presence of a Pro and two Hyp in the peptide chain resulted in a fairly well-defined conformation with a certain amount of folded structure stabilized by intramolecular hydrogen bonds (γ-turn conformation). The presence of Al(III) induced a large conformational change along the peptide chain, and coordination of the C-terminal Lys^9COO^- and the Glu^5COO^- sites of both peptides led to the formation of a 1 : 2 complex (Gervais et al., 1987). The two OH groups of Hyp^3 and Hyp^6 might achieve coordination around the metal ion or could stabilize the complex through the formation of intramolecular hydrogen bonding.

The key role of the C-terminal Asn^9COO^- group was found to lie in the coordination of the Al(III)-thymulin complex in dimethylsulfoxide (Laussac et al., 1988). NOESY data suggested that some interaction between the terminal pGlu and the Lys side-chain brings Ser^4OH into a favourable position for binding to Al(III), creating a compact cavity for metal-binding. This involves the Asn^9COO^- terminal group and the Ser^4OH residue in a 1 : 2 stoichiometry.

The results discussed above clearly confirm the preferential role of the C-terminal COO^- group, the COO^- of the Asp and Glu residues and the –OH groups of the Ser side chains of oligopeptides in Al(III) binding. Some preorganization of such functional groups might be beneficial, but not a prerequisite for efficient binding.

Al(III) coordination has been detected for larger oligopeptides too. ^{27}Al NMR was used to study the interaction of the soluble amyloids Aβ(1–40) and Aβ(6–25) with Al(III) (Vyas & Duffy, 1995a). The occurrence of a new, relatively broad ^{27}Al signal ($\Delta\nu \sim 850$ Hz) on addition of the peptides to $Al(H_2O)_6^{3+}$ indicated the presence of peptide-bound Al(III) undergoing slow exchange on the ^{27}Al NMR time scale. From the relative intensities of the signals, the coordination of at least 4 Al(III) to each Aβ(1–40) peptide molecule was assumed in the pH range 4–6. Asp-COO^- and His-N donors were assumed to bind Al(III), which induced conformational changes leading to the aggregation of the peptide. At pH >6, a precipitate was formed, which was soluble in acid and contained only Al(III); this indicates that the peptide ligand is displaced by OH^-. (It should be mentioned here that these experiments were carried out in rather strong solutions, at 10 mM Al(III), which is irrelevant physiologically. Accordingly, at lower Al(III) concentrations and especially in the physiologically more relevant μM range, interactions of Al(III) with oligopeptides and proteins can be assumed in the neutral pH range too (*vide supra*)).

CD and FTIR Spectroscopic Studies on the Fragments of Brain Proteins

Circular dichroism (CD) and Fourier transform infrared (FTIR) spectroscopic studies on peptides representing the repeating region of human neurofilament (NF) protein mid-size subunit (NFM) were prompted by the possible roles of cytoskeletal proteins and Al(III) in the aetiology of AD (Crapper-McLachlan, 1986). The dense NF masses found in the brains of Al(III)-treated animals are composed of constituents of the normal NF triplet including low-size (NFL; 65 kDa), NFM (160 kDa), and high-size (NFH; 200 kDa) molecular mass subunits. *In vitro* experiments have demostrated that Al^{3+} alone, or in combination with maltol or other chelators, increases the phosphorylation of the

NFM and NFH subunits and induces the aggregation of NFs (Leterrier et al., 1992). Crucial points in this hypothetical mechanism include the chemical 'composition' of the Al(III)-binding sites and the conformational effect of Al(III) complexation. Interestingly, there is an acidic domain of the amyloid precursor protein (APP) that shares sequential similarities with the three NF subunits (Delamarche, 1989).

The human NFM protein contains a 13-mer sequence, LysSerProValProLysSerPro ValGluGluLysGly (NFM13), which is repeated 6 times near the C-terminus (the tail region of the protein) (Myers et al., 1987). The tetramer LysSerProVal, repeated 12 times in this domain, was found to be the C-terminal repeating phosphorylation site of NFM (Lee et al., 1988). The microtubule-associated protein tau, the main protein component of NFTs, appears to acquire AD-like properties upon phosphorylation with a specific brain kinase (Biernat et al., 1992). One of the potential phosphorylation sites of the tau (LysSerProVal) is identical with the repeating phosphorylation site of NFM.

The results of early CD and FTIR studies on peptides and phosphopeptides representing the repeating C-terminal domain of NFM have been reported in a series of papers (Ötvös et al., 1988; Hollósi et al., 1992, 1993; Holly et al., 1993).

Phosphorylated and non-phosphorylated fragments of NFM proteins (such as NFM13) and GluGluLysGTlyLysSerProValProLysSerProValGluGluLysGly (NFM17)) were titrated with Ca(II) and Al(III) ions (Hollósi et al., 1992, 1993, 1994, Shen et al., 1994) in trifluoroethanol (TFE), a solvent with unique solvating properties (see *e.g.* Bodkin & Goodfellow, 1996). Na^+, used as reference, generally gave rise to negligible or no spectral shifts, even at $n_{Na} > 10$ (n_M is the concentration ratio $[M^{n+}]/[\text{peptide}]$) in dilute (0.1–1 mM) solutions in TFE. In contrast, Ca(II) induced CD spectra resembling those measured in water or TFE/water mixtures (type U spectra featuring an intense negative band near 200 nm, with a shoulder or a negative band of decreased intensity near 220 nm).

The effect of Al(III) was found to depend strongly on the solvent and the sequence of the peptide. In aqueous solution at pH <7, Al(III) up to $n_{Al} \sim 10$ caused no definite change in the almost general type U spectrum. This shows that, even if it binds to side-chain functionalities, Al(III) does not have a significant effect upon the backbone conformation. In TFE solution, however, Al(III) resulted in diverse CD spectral effects. The unphosphorylated peptide NFM13, which displays a marked spectral change in the presence of 1–2 equivalents of Ca^{2+}, did not undergo any significant spectral shifts, even at higher Al^{3+} to peptide ratios ($n_{Al} = 4$). The possible role of side-chain functionalities in the binding of Al(III) was investigated with the help of Ser to Ala and Glu to Ala substituted derivatives (Hollosi et al., 1994; Shen et al., 1994). It was found that Ca(II) binding to the carboxylate function of Glu does not play a significant conformational role. By contrast, Al(III) binding results in a spectral shift reflecting β-pleated sheet conformation only for NFM17($Ala^{6,11}$), which contains carboxylate, but not hydroxyl side-chain groups. In the case of NFM17($Ala^{6,11}$), the Al(III)-induced β-pleated sheet formation seems to be directly connected with Al(III) complexation, probably through Al(III) bridges between carboxylates from different molecules.

CD-monitored titrations with Ca(II) and Al(III) have also been performed on a variety of Ser-phosphorylated NF fragments (Hollósi et al., 1992, 1994). Titration of the phosphorylated species with either Ca(II) or Al(III) caused significant conformational

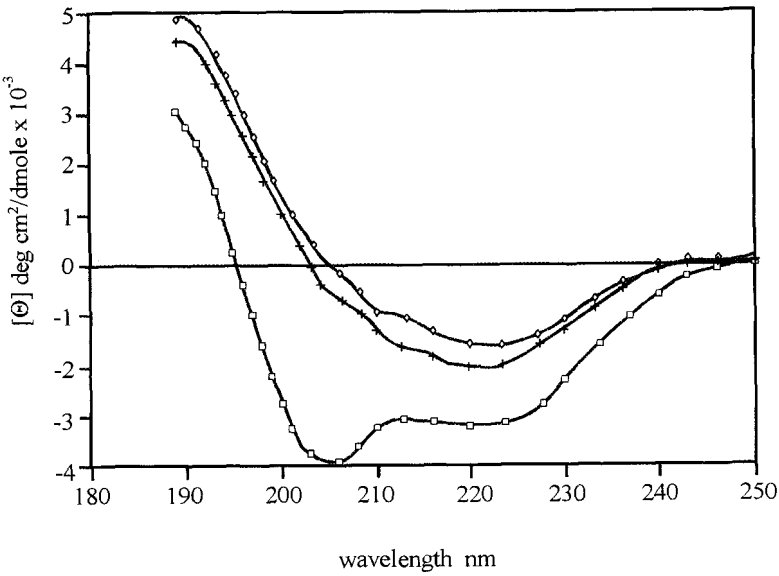


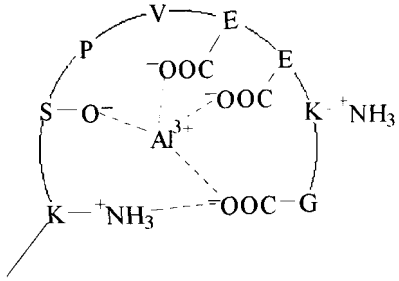
Fig. 3. Citric acid back-titration experiments on the Al^{3+} complex of NFM13[S(*P*)]$_2$ in TFE, $c_{peptide}$ = $2.17 \cdot 10^{-4}$ M. □ = no Al(III) or citric acid; + = $c_{Al(III)}/c_{peptide} = 12$; ◊ = n_{Al}=12 and $c_{citric\ acid}/c_{Al(III)} = 4$. Note the typical low-intensity β-sheet spectrum, resistant to citric acid, due to the formation of β-aggregates.

changes, yielding a high content of β structure (Fig. 3). These results suggest that the critical metal-binding sites of NFM17, yielding the β-pleated sheet structure, are the four Glu or phosphorylated Ser, especially the C-terminal Ser(P). In contrast, peptides and phosphopeptides with a Ser in special sequential position probably form a stable intramolecular complex with Al(III) rather than a β-sheet structure. Back-titration with citric acid fails to reverse the Al(III)-induced conformational changes in the phosphorylated peptides, while Ca(II)-induced spectral and consequently structural changes can be completely reversed by citric acid or other chelators (Hollósi et al., 1994; Shen et al., 1994). The above results, and particularly the higher stability of Al(III) complexes, may be relevant to the molecular mechanism through which the neurotoxin Al(III) could give rise to the formation of NFTs.

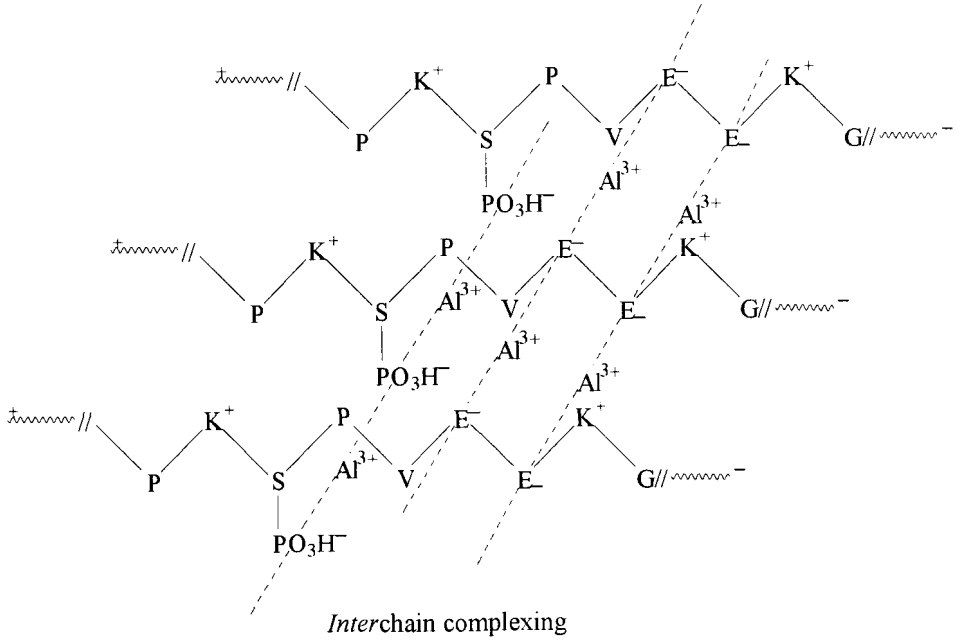
FTIR measurements confirmed the above-mentioned findings (Hollósi et al., 1995). The amide I region in the FTIR spectra in TFE revealed that the peptide backbone and the protonation states of the carboxyl groups of NFM17 and NFM17($Ala^{1,11}$) participate in different changes in the presence of Al(III). This suggests the formation of Al(III) complexes with different structures and stabilities. The Al(III) complex of NFM17($Ala^{1,11}$) is likely to be less stable.

Of the spectroscopic data discussed above, the possible interactions of Al(III) with these protein fragments are depicted in Fig. 4 (Hollósi et al., 1993).

Conformational studies on fragments of the tau protein were prompted by the finding that enzymatic phosphorylation converts tau into a paired helical filament (PHF)-like form, as found in AD (Roder et al., 1993). The effects of phosphorylation upon the



*Intra*chain complexing



*Inter*chain complexing

Fig. 4. Hypothetical model of an intrachain Al(III) complex of NF protein fragments and an interchain Al(III)-bridged complex of phosphorylated fragments.

fragments of human tau protein have been studied (Láng et al., 1992a; 1992b; Láng & Ötvös, 1992). The fragment consisting of amino acids 408–421 was synthesized in both unphosphorylated and Ser^{416}-phosphorylated forms. CD in a TFE–water mixture indicated a β-turn → β-pleated sheet conformational transition upon phosphorylation. Immunological studies in combination with CD spectroscopy (Láng et al., 1992b) confirmed this finding.

A great number of new protected and unprotected fragments of NF and tau proteins have recently been synthetized in our laboratories. The main goal of this work was to find a lead compound for the design of a selective peptide-based Al(III) chelator. CD spec-

troscopy revealed a difference in the conformational behaviour of some phosphorylated peptides as compared to their unphosphorylated parent molecules. The phosphorylated peptide GlyGluGlyGluGlySer(P)GlyGly proved to be a promising Al(III) binder. The Al(III)–tau interaction is discussed in detail in Chapter 21.

Recent experimental data point to the basic contribution of β-amyloid (Aβ) aggregation to the aetiology of AD (Carrell & Gooptu, 1998; Selkoe, 1999). The ability of the longest Aβ peptides (Aβ(1–42) and Aβ(1–43) to form amyloid most easily has been well documented (Lansbury, Jr., 1996). Amyloid fibril formation generally occurs via nucleation-dependent oligomerization. An ordered nucleus ('seed') is formed only after a lag phase and in a 'supersaturated' solution, in which the concentration of the amyloidogenic species exceeds a critical level. After nucleation or seeding, the growth of the fibril proceeds rapidly. Accordingly, addition of a preformed nucleus or seed to a supersaturated solution accelerates aggregation, finally leading to fibril formation. Aβ(1–40) a less amyloidogenic (fibrillogenic) fragment of Aβ, can be seeded *in vitro* by Aβ(1–42). We consider that inorganic compounds, such as free or ligand-bound metal ions, may also behave as seeds for aggregation.

At a molecular level, the first step of fibril formation is a conformational change in the largely unfolded, low-molecular mass Aβ to β-sheet-enriched amyloid fibrils (Rochet & Lansbury, Jr., 2000). The β-sheet-forming tendency of Aβ is localized to its C-terminal part. X-ray crystallography, electron microscopy (EM), cryo-EM, FTIR and CD spectroscopy, solid-state NMR spectroscopy and molecular modelling have been used to study the structures of amyloid fibrils and their prefibrillar intermediates (Rochet & Lansbury, Jr., 2000).

Asp/Asn → isoAsp (-NH-CH(COOH)-CH_2-CO-) changes are frequent side-reactions during peptide synthesis. It is noteworthy that elevated levels of isoAsp residues have been detected in proteins involved in NFTs (Roher et al., 1993). Upon the substitution of Asp^1 and Asp to isoAsp residue, the intermolecular β-pleated sheet content was found to increase markedly for the post-translationally modified peptide as compared with that in the corresponding unmodified human or rodent Aβ sequences, both in aqueous solutions in the pH range 7–12, and in membrane-mimicking solvents (Fabian et al., 1994).

In addition to isoAsp, D-Asp has been detected in the degenerated proteins from AD plaques (Anderson et al., 1989). The purified core amyloid was found to contain relatively large proportions of D-Asp and D-Ser (Shapira et al., 1988).

The CD spectra of the D-Asp-substituted analogues of Aβ(6–25) and Aβ(1–40) underwent a distinct blue shift on Al(III) complexation (Vyas & Duffy, 1995b). The influence of Al(III) coordination was most significant on the triply substituted peptide Aβ(1–40)($D\text{-}Asp^{1,7,23}$). The spectral changes suggested that the interaction of Al(III) with D-Asp induces an increase in the antiparallel β-sheet character of the peptide backbone. D-Asp substitution and chelation with Al(III) leads to increases in stability of higher molecular mass species of Aβ40, and could thereby increase the toxicity of the AD amyloid protein.

In long-lived mammalian proteins, racemization may be catalysed by local factors such as changes in pH, elevated temperatures due to inflammatory processes, the imbalance of cation homeostasis, *etc*. Certain metal ions, and primarily Al(III), have been observed to catalyse the racemization of amino acids in both the free and the protein-bound state

(Anderson et al., 1989). Ser and particularly Asp proved to be the fastest racemizing amino acids. Long-lived proteins accumulate D-Asp at a rate of approximately 0.15% per year (see Shapira et al., 1988, and references cited therein).

CD and FTIR spectroscopic studies have been performed on two series of Aβ fragments: (2–11) and (20–29) (Majer et al., 1999). The CD data indicated that Ac-AlaGluPhe ArgHisAspSerGlyTyrGlu-NH_2 (A1), Ac-PheAlaGluAspValGlySerAsnLysGly-NH_2 (A2) and their derivatives are structureless in water and in an aqueous buffer at pH ~ 7. In the structure-promoting solvent TFE, peptides A1 and A2 furnish CD spectra indicative of a definite shift in the conformational equilibria, and suggest the predominance of the α-helix conformation (~ 60%, calculated from the FTIR spectrum of A1). The spectra of the derivatives (D-Asp^7)A1, (iAsp^7)A1 and (D-Asp^7D-Ser^8)A1 clearly demonstrate a decreased helicity and the presence of a mixture of conformers. FTIR experiments in TFE have confirmed this finding. The conformational effects of Na^+, Ca^{2+}, Mg^{2+} and Al^{3+} have also been monitored.

The spectroscopic data clearly indicated that D-amino acids weaken the helical order of Aβ peptides and bring about a gradual defolding of the chain, even in a helix-promoting environment. The gradual increase in the D-amino acid content in APP and Aβ may provide an explanation for the progression of late-onset forms of dementia and also for the elevated amyloid level in the brain of non-demented elderly people. Risk factors of AD, *e.g.* elevated levels of metal ions such as Al(III), may catalyse the racemization of amino acids in β-amyloid and other proteins, and increase the probability of non-genetic, early-onset forms of neurodegenerative diseases. A detailed discussion of the possible role of amyloid and Al(III) in AD can be found in Chapter 22.

Complexes with Proteins

In consequence of the chemical similarities of the metal ions, proteins which bind and/or transport Ca(II), Mg(II) or Fe(III) are expected to interact with Al(III) (Martin, 1988; 1991). Although interactions of Al(III) with a large number of proteins have been detected, mostly via the effects of the metal ion on their biochemical or biological processes, relatively few thermodynamic or structural data are to be found in the literature as concerns the binding mode of Al(III) to proteins.

D-Xylose isomerase is one of the few enzymes for which crystallographic results are available. This enzyme catalyses the reversible conversion of D-xylose to D-xylulose and of other sugars from aldose to ketose, and is widely used industrially for the conversion of D-glucose to D-fructose. It has two metal-binding sites: one of them has a structure-making role, while the other has a catalytic role. The enzyme requires divalent metal ions (Mg^{2+}, Co^{2+} or Mn^{2+}) for activation, while others (*e.g.* Ca^{2+}, Ba^{2+} and Al^{3+}) inhibit the reaction. X-ray diffraction studies have revealed that the xylose isomerases exhibit a high degree of structural homology: each subunit of the tetrameric enzyme folds into a main domain, which has a typical 'triose phosphate isomerase barrel' motif, with an eight-stranded parallel β-sheet surrounded by eight α-helices, and a C-terminal helical domain, which forms a large loop embracing a neighbouring subunit. The structure of a double mutant of *Arthrobacter* xylose isomerase with Al^{3+} at both metal-binding sites

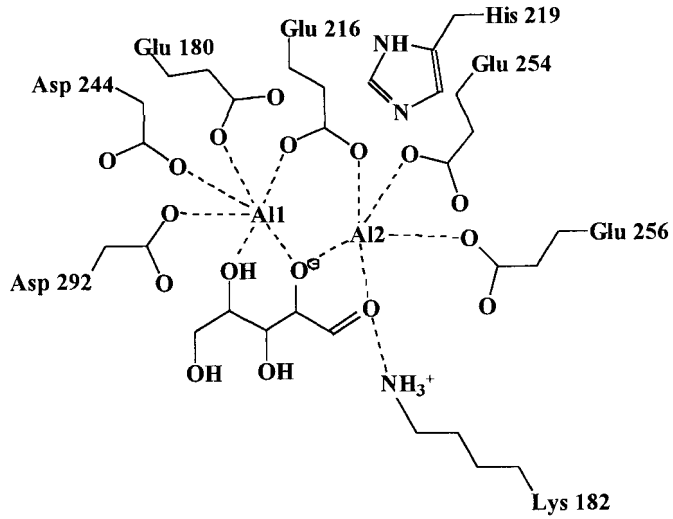


Fig. 5. Schematic representation of the Al(III)-binding sites of the double mutant D-xylose isomerase D-xylitol complex. The sixth ligand of Al 2 is one of the N atoms of His 219.

has been determined by the molecular replacement method (Gérczey et al., 1999). It was found that the binding of the two Al^{3+} ions does not significantly alter the overall structure of the enzyme. A comparison of the Mg(II)- activated wild-type and the mutant structures revealed that the coordination of the structural metal ion remains qualitatively the same. The catalytic metal ion is coordinated to a His-N, a Glu-O, an Asp-O and a water O atom, while a second Asp donates both of its carboxylate oxygens for metal binding (Fig. 5). The octahedral co-ordination of Al^{3+} by the enzyme is similar to that observed in the wild-type structures binding the activating Mg^{2+}; thus, it is not the different stereochemistry that is responsible for the inhibitory effect of Al^{3+}.

In most biological studies, no clear information is given as regards the exact solution state of the Al(III) used in the *in vitro* or *in vivo* investigations, albeit (as shown above) this may strongly influence its biological effects. In many biological studies, for example, aqueous solutions of simple inorganic Al(III) salts are used, which will hydrolyse at physiological pH, resulting in a metastable, ill-defined state *(vide supra)*.

Interactions with Non-Enzyme Proteins

The chemical speciation of Al(III) is obviously a critical point in attempts to assess which specific compounds cross the blood–brain barrier (BBB) and contribute to the neurotoxicity of Al(III). There is a broad consensus that most of the Al(III) (80–90%) in the serum is bound to protein (Harris, 1996, Harris et al., 1996). It appears that practically all this amount is bound to the iron transport protein TF. This is known to bind Al(III) at both high-affinity iron-binding sites through the coordination of two Tyr, one His, one Asp and a bidentate carbonate ion in pseudo-octahedral geometry (Aramini et

al, 1996, Congiu-Castellano et al., 1997). Interestingly, the binding strengths of the two sites display a pH dependence, the C terminal site being preferred at physiological pH (Harris & Sheldon, 1990, Kubal et al., 1992a, 1992b). For Al(III) to be transported in the blood, its displacement of the 10^9 times more strongly binding Fe(III) is not necessary (Tang et al., 1995), because only 30% of the TF binding sites are occupied by iron and the concentration of unoccupied sites in plasma TF is about 50 μM. The corresponding Fe(III)–TF equilibrium constants are log $K_1 = 21.4$ and log $K_2 = 20.4$ (Harris, 1996; Sun et al., 1997), while the constants are in the range log K = 12 to 14 for Al(III) (Clevette & Orvig, 1990); most probably, log $K_1 = 13.5$ and log $K_2 = 12.5$ (Harris & Sheldon, 1990). A wide range of metal ions of natural, therapeutic, diagnostic and toxic interest are thought to be transported by serum TF. It has been shown that the strength of binding of divalent and trivalent metal ions to human serum TF correlates with the metal ion acidity (and therefore with the strength of binding to hydroxide, $K_1(OH)$) (Sun et al., 1997). Study of the location of Al(III) and Ga(III) in human neuroblastoma cells after treatment with appropriate metal complexes demonstrated that 30–35% of the cytosolic metal was associated with proteins, mostly with TF, but partly with ferritin (Dobson et al., 1998a). The possible involvement of Al(III) in iron homeostasis is discussed in detail in Chapters 11 and 15.

The ^{27}Al NMR spectra of Al(III) added to albumin indicated relatively weak binding of about three Al(III). It was also observed that Al(III) does not bind at the N-terminal Cu(II) binding site. Instead, it is bound to six O ligands (probably carboxylates and waters) in octahedral geometry, which has led to the suggestion that Al(III) might be bound at Ca(II)- binding sites (Fatemi et al., 1991, 1992). A direct competition between Al(III) and Ca(II) is consistent with a report that the binding of Al(III) to albumin is eliminated by the addition of 0.2 mM Ca(II) (Harris et al., 1996). The Ca(II)-binding constants are in the range 10^2–10^3 (Peters, 1985). There are a few reports of stronger binding of Al(III) to albumin (Fatemi et al, 1991), but it is unlikely that albumin can compete with TF for Al(III). There are also reports of two small Al(III)-binding proteins (Khalil-Manesh et al., 1989a, 1989b; Favarato et al., 1992a, 1992b), with apparent molecular masses of about 18 kDa and 8 kDa.

Al(III) interferes with the metabolism of Ca(II)-containing proteins. Al(III) was found to reduce the vitamin D-dependent Ca(II) absorption in chicks, rats and humans: the amount of calbindin D-28K, a Ca(II)-binding protein in the intestine, was reduced by added dietary $AlCl_3$ (Dunn et al., 1993). Al(III) may have a similar effect in other tissues containing substantial levels of calbindin.

Phosphophoryn, an anionic nucleation activator protein isolated from dentin, serves as an 'acidic' template for Ca(II) biomineralization. It possesses high-affinity Ser(P)-rich and low-affinity Asp-rich binding sites for Ca(II). Other proteins associated with bones and teeth are osteocalcin and osteoporin. These three proteins bind Al(III) with dissociation constants, K_D, of 10^{-7} M or greater at pH ~ 7 (Rowatt et al., 1997). The number of Al(III) ions bound is related to, but not equal to the number of Ser(P) residues in phosphophoryn and osteopontin. Osteocalcin binds a single Al(III) tightly, presumably through the γ-carboxyl Glu residue.

The enzyme-regulating small Ca(II) protein calmodulin also interacts with Al(III). The binding of Al(III) presumably does not involve the four specific Ca(II)-binding

domains; it generates considerable conformational changes (a ~ 30% reduction in α-helix content) (Haug & Vitorello, 1996, Wolf et al., 1998). Calmodulin bearing negatively charged carboxylate residues is assumed to bind Al(III) non-specifically, merely as a polyelectrolyte (Martin, 1992). Such interactions may lead to an impairment of the flexibility of the protein and to the loss of its ability to bind to other proteins, thereby decreasing or inhibiting the regulatory character of calmodulin (Levy et al., 1998). The involvement of Al(III) in calmodulin biology is discussed in detail in Chapter 20.

The effects of Al(III) upon structural proteins have similarly been examined. Collagen is a family of fibrous proteins, which serve to hold cells together in discrete units. It has been demonstrated that, even under *in vivo* conditions, metal ions link to collagen structures and influence their metabolism. Electron microscopy has revealed that Al(III) compounds result in the development of the transverse striation of collagen from rat tail tendons and in an increased temperature of contraction of collagen. An inhibitory effect of Al(III) on the proliferation of fibroblast cultures has been established. In chick embryos, Al(III) caused a decline of the hydroxyproline concentration and hence a reduced collagen synthesis in practically all investigated tissues (Hulejovla et al., 1994).

Interestingly, the interaction of Al(III) with protein may sometimes be beneficial. For instance, it has recently been found that pretreatment of the abundant structural protein elastin with $AlCl_3$ makes it completely resistant to calcification; this is a consequence of the irreversible Al(III) binding and subsequent structural alterations (reduction of β-sheet structure and increase in coil-turn conformations) (Vyavahare et al., 1999).

The results concerning the interaction of Al(III) with brain proteins are rather controversial. As an example, it is assumed that the neurotoxic effect of Al(III) is mediated through interaction with the synthesis or processing of amyloid precursor protein (APP). Immunological studies on neuroblastoma cells and rat brain cells provided no evidence of any effect of Al(III) on APP expression or processing (Neill et al., 1996). At the same time, the precursor of the non-Aβ component of amyloid plaques (NACP or α-synuclein) was shown to interact with Al(III). NACP, which is a presynaptic protein possibly involved in neuronal biogenesis, including synaptic regulations, was suggested to serve as a seed for the formation of amyloid plaques (*vide infra*). Spectroscopic studies have revealed that Al(III) generates an increase in helicity in the precursor protein (Paik et al., 1997). This structural change makes NACP resistant to proteases such as trypsin, α-chymotrypsin and calpain.

Ubiquitin, a small (8.5 kDa) highly conserved protein present in all eukaryotic cells, plays an important role in tagging proteins for destruction. The C-terminal Gly of ubiquitin covalently links to the ϵ-amino group of the Lys residues of proteins destined to be degraded. Ubiquitin is also a component of SP in AD. At low concentration (10 μM), Al(III) stimulated the levels of immunoreactive Aβ and ubiquitin in a neuroblastoma cell culture, while at higher concentrations (100 and 500 μM), Al(III) failed to influence Aβ, whereas the ubiquitin level continued to increase. No changes in Aβ and ubiquitin content were found in glioma cells, regardless of the concentration of Al(III) (Campbell et al., 2000).

Al(III) interacts with mucin, a ubiquitous glycoprotein of the gastrointestinal tract (Exley, 1998). The predominant reaction suggested is the intercalation of freshly formed $Al(OH)_3$ into the macroreticular network of the hydrated mucin polymer. Al(III) probably

cross-links mucin molecules at the carbohydrate moieties through oxo and hydroxo bridges. Such complexation reactions may regulate the gastrointestinal absorption of Al(III) (Powell et al., 1999a, 1999b).

Interactions with Enzyme Proteins

Table 2 lists enzymes on which the effects of Al(III) have been investigated. For a compilation of such studies, see, for example the paper by Ganrot (1986) and the special issue of Life Chemistry Reports (Nicolini et al., 1994). It is seen from Table 2 that Al(III) influences (inhibits or activates) not only acid–base enzymes, but also some redox enzymes. Most of them are either activated by Mg(II) or Ca(II) or use nucleotides (mostly ATP) as a cofactor. The effect of Al(III) in these enzymatic processes is generally the displacement of metal ions or its strong interaction with the nucleotides, through their phosphate functions.

Al(III) has been shown to be toxic to the haemopoietic system (Zaman, 1994). Its effect is rather complex as it influences haeme biosynthesis by inhibiting cytosolic enzymes such as δ-aminolaevulinic acid dehydratase, ferrochelatase and uroporphyrogen decarboxylase. It also affects iron levels through interactions with proteins involved in the iron metabolism: TF, ferritin and the iron regulatory protein IRP2. Al(III) may have effects on red blood cells in addition to haeme biosynthesis. The best-documented effect is probably the inhibition of hexokinase (Exley et al., 1994), due to the tendency of ATP to form stronger complexes with Al(III) than with Mg(II) and to the fact that Al(III)–ATP complexation comprises a strong competitive inhibition with respect to Mg(II)–ATP. The interaction between Al(III) and ATP may imply that Al(III) can affect many other enzyme reactions in which ATP is a substrate (Martin, 1992). It has been shown that Al(III) influences the activities of acetylcholinesterase, glucose-6-phosphate dehydrogenase, glutathione reductase, lactate dehydrogenase and dihydropteridine reductase in the bone marrow and erythrocytes (Zaman, 1994). Further, the maturation of erythrocyte precursors could be impeded via the interference of Al(III) with cellular actions of calmodulin, thereby inhibiting Ca-ATPase, with an increase in the cytosolic free Ca(II) level (Drueke, 1994).

Numerous examples of Al(III) inhibition of Mg(II)-dependent enzymes have been reported. Besides the already-mentioned hexokinase or protein kinase C (Cochran et al., 1990; Meiri et al., 1993), other enzymes that are inhibited by Al(III) are adenylate cyclase, 3,5-cyclic nucleotide phosphodiesterase, acid and alkaline phosphatases, acetylcholinesterase, *etc.* In most cases of Al(III)-mediated enzyme inhibition, substitution of Mg(II) for Al(III) is documented or at least assumed. Although the mechanism of inhibition is not well characterized, it may involve conformational alteration of the protein by the binding of Al(III) at allosteric sites (*e.g.* in phosphatases). The Al(III)-mediated reduced activity of phosphorylases and cholinesterase in mice could be reversibly restored by the combined administration of vitamins C and E and Ca(II) (Chinoy & Patel, 1999). In the case of catechol-O-methyl transferase, metal binding to the substrate leads to enzyme inhibition (Martin, 1988).

The levels and activities of cholinergic enzymes in Al(III)-treated animals have been extensively investigated in attempts to determine whether Al(III) causes a cholinergic

Table 2. The effects of aluminium on the activities of enzymes

Enzyme	Effect of Al on activity [a]	Application of Al(III)	Reference
Kinases			
Calpain II	I	*in vitro* [b] (effect on substrate)	Nixon et al., 1990
	I	*in vitro* [b]	Zhang & Johnson, 1993
cAMP-dependent kinase	A	*in vitro*	Sihag & Nixon, 1990
Glycerokinase	I	*in vitro*	Furumo & Viola, 1989
Hexokinase	I	*in vitro*	Furumo & Viola, 1989
	I	*in vitro*	Exley et al., 1994
Protein kinase A	–	*in vitro* (soluble or particulate)	Johnson et al., 1990
Protein kinase C	–	*in vivo* (soluble)	Johnson et al., 1990
	A	*in vivo* (particulate)	Johnson et al., 1990
	I	*in vitro*	Cochran et al., 1990
	I	*in vitro*	Sihag & Nixon, 1990
Tyrosine kinase	–	*in vitro*	Johnson et al., 1990
Phosphatases			
Inositol polyphosphate phosphates	A	*in vitro*	Ali et al., 1995
ATPases	A	*in vitro*	Sankaran et al., 1997
Proteolytic enzymes			
Apopain	A	*in vitro*	Guo Ross et al., 1998
Calpain	A	*in vitro*	Guo Ross et al., 1998
α-Chymotrypsin	A	*in vitro*	Clauberg & Joshi, 1993
	I	*in vitro*	Angeletti et al., 1993
Lysosomal proteases	A	*in vitro* (implicated in APP cleavage)	Dobson et al., 1998b
Trypsin	I	*in vitro*	Zatta et al., 1993
Trypsin-like protease (from cerebral cortex of rats)	–	*in vitro*	Guo Ross et al., 1998
Dehydrogenases, redox enzymes			
Catalase	A (in the presence	*in vitro*	
Superoxide dismutase	of vanadium)	(red blood cells)	Abou Seif, 1998
Glucose-6-phosphate dehydrogenase	I	*in vitro*	Meiri et al., 1993
Monoamine oxidase B	A	*in vitro*	Zatta et al., 1999
Cholinergic enzymes			
Acetylcholine esterase	–	*in vivo*	Gulya et al., 1990
	A	*in vitro*	Zatta et al., 1994
Choline acetyltransferase	I	*in vitro*	Meiri et al., 1993

[a] I = inhibition; A = activation; – = no effect.
[b] Different conformational effects of Ca(II) and Al(III).

deficiency in the central nervous system (CNS) similar to that described in AD patients. Differing results were reported by various investigators: a significant reduction in the activity of choline acetyltransferase, a key enzyme involved in the synthesis of acetylcholine was found in some laboratories, and a lack of any change in enzyme activity in others (Meiri et al., 1993; Dobson et al., 1998b). One possible reason for the contradictory results may be the use of different Al(III) salts and different routes of administration.

The injection of Al(III) salts into experimental animals did not induce changes in the activity of acetylcholinesterase, the enzyme decomposing acetylcholine, in various parts of the CNS (Gulya et al., 1990). In contrast with the lack of an effect of Al *in vivo*, Al(III) did depress the activity of the enzyme *in vitro* (Zatta et al., 1994b).

Many neurological diseases in which Al(III) is implicated involve defects in the cytoskeleton (Macdonald et al., 1987, Troncoso, 1994). Al(III) has been shown to affect the polymerization of tubulin. The tubulin assembly is the principal subunit of the microtubules and requires Mg(II), which is thought to bind at receptor sites for GTP and GDP. It is inhibited by Ca(II), which, is believed to associate at a distinct binding site. Al(III) at a nM concentration level promotes the polymerization of tubulin, but inhibits GTP hydrolysis and the Ca(II)-induced depolymerization in Al(III) microtubules (Macdonald et al., 1987). Al(III) or Be(II) has been found to be a necessary cofactor for the fluoride activation of guanine nucleotide-binding proteins (G-proteins), including tubulin (Sternweis and Gilman, 1982) and transducin (Antonny & Chabre, 1992). Although the mechanism of activation is not fully understood, AlF_4^- has been postulated to possess structural homology with PO_4^{3-} and may therefore bind at the terminal phosphate receptor site when the nucleotide site is occupied by GDP. In this way, it mimics GTP, but without the potential for hydrolysis (Martin, 1988). However, complex equilibrium calculations (Martin, 1996), confirmed by multinuclear NMR measurements (Nelson, 1996), predicted that, under physiological conditions the hydroxo complex $AlF_3(OH)^-$ predominates over AlF_4^-, and all binary Al(III)–F^- complexes should be hexacoordinated with water molecules at the free sites. By contrast, $AlF_3(OH)^-$ could be tetrahedral, hence appearing to be a better candidate as a phosphate analogue (Antonny and Chabre, 1992). This phosphate analogue model has been extended to adenine nucleotide/binding enzymes such as actin (Combeau & Carlier, 1988, Exley, 1999), myosin (Werber et al., 1992, Maruta et al., 1998) and the ATPases (Bragadin et al., 1994, Sankaran et al., 1997, Braig et al., 2000).

Mitochondria, as the basic energy/producing organelles of cells, may be easy targets for the toxic effects of metal ions, including Al(III). Several lines of evidence indicate that the neuronal mitochondria are affected by degenerative disorders related to aging (Blass et al., 1995). As discussed above, some reactions involved in glycolysis, which is strongly correlated with the mitochondrial Krebs cycle, are inhibited by Al(III) (Cho & Joshi, 1988, Xu et al., 1990). Accordingly, it is reasonable to assume that Al(III) could interfere with the bioenergetics of mitochondria. It was recently demonstrated that, among the enzymes that participate in the Krebs cycle, α-ketoglutarate dehydrogenase and succinate dehydrogenase are activated by Al(III) (Zatta et al., 2000). (This is in apparent contradiction with the earlier report that the α-ketoglutarate dehydrogenase activity is reduced in AD brain tissue (Butterworth & Besnard, 1990).) At the same

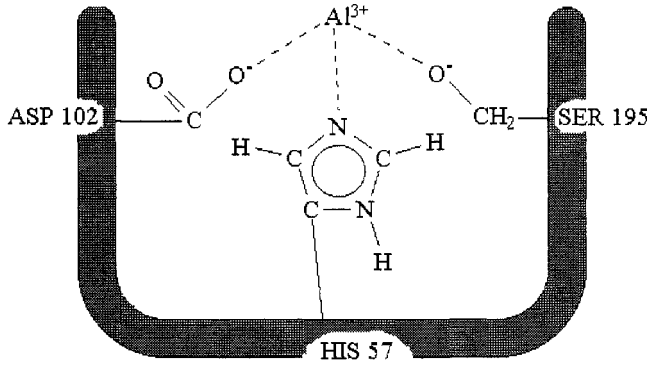


Fig. 6. Hypothetical binding site of Al(III) in trypsin.

time, aconitase, probably as a result of complexation with the substrate isocitrate, exhibits a decreased activity in the presence of the metal ion. Al(III) also inhibits glutamate dehydrogenase, an enzyme converting Glu to α-ketoglutarate, and thus closely linked to the Krebs cycle.

Proteases hydrolyse peptide bonds. Hence, it may reasonably be assumed that hydrolytic processes are influenced by Al(III), a strong Lewis acid. Of the proteolytic enzymes, the serine proteases trypsin and α-chymotrypsin are inhibited by Al(III) at mM concentration level. The inhibitory reaction can be reversed in the presence of EDTA, a strong Al(III) chelator (Zatta et al., 1993). The active site of trypsin involves Ser195, His57 and Asp102, which are topologically in close proximity in the folded protein. As shown in previous sections of this chapter, Al(III) can bind strongly to COO^- and alcoholate-O^- functions. Furthermore, when N-donor groups from both sides of the molecule are adjacent to strong Al(III) binders, even N can participate in metal binding (see Al(III)–Asp and Al(III)–NTA interactions). A hypothetical model (a slightly modified version of that proposed by Zatta (Zatta et al., 1993)) with the blocked active centre of the enzyme is seen in Fig. 6.

The inhibitor metal ion can be extracted from the active centre with a stronger Al(III) binder molecule, such as EDTA. In a more recent paper (Angeletti et al., 1997), Al(III) in μM concentrations has been demonstrated to act as an activator of α-chymotrypsin, promoting binding of the substrate to the catalytic site. The Al(III) binding to the enzyme displayed an approximately threefold decrease in affinity for some of its inhibitors. This suggests the occurrence of an Al(III)-linked conformational change in the enzyme molecule, which brings about marked structural changes at the primary and secondary recognition sites for substrates and inhibitors.

The Ca(II)-activated neutral proteinase (calpain II) purified from human brain is particularly active toward NF proteins. Calpain-mediated proteolysis of NF, tubulin and glial fibrillary acidic protein was inhibited by $AlCl_3$ and Al(III)-lactate (Nixon et al., 1990). Al(III) salts inhibited proteolysis principally by affecting the substrates directly. Fluorescence spectroscopy suggests conformational changes which are significantly different from those caused by Ca(II) (Zhang and Johnson, 1993).

Effects of Al(III) on the Blood–Brain Barrier

In order to exert its harmful effects, for instance by reacting with neuropeptides and thereby inducing conformational changes leading to the formation of toxic protein aggregates, Al(III) must somehow reach the brain. The brain is defended by a continuous layer of cell membranes, the BBB, which not only hinders the entry of large molecular mass substances into the CNS, but additionally regulates all the exchanges of nutrients, hormones, toxins and therapeutic agents that enter the CNS from the blood or the blood from the CNS. Accordingly, any dysfunction of such a major regulator of the CNS could have serious consequences for the activity of the brain. Al(III) has been shown to affect several functions of the BBB: among others, it increases the transmembrane diffusion of lipophilic substances and it also influences some of the more specific saturable transport systems, although it does not disrupt the membrane integrity (Banks & Kastin, 1989).

Al(III) is known to enhance the blood-to-brain passage of many compounds, including various peptides. This increased penetration of peptides into the brain is a result not of direct interactions of Al(III) with the peptides themselves, but of its effect on the transmembrane diffusion permeability. It has recently been demonstrated that Aβ peptides can also cross the BBB. Al(III) has further been found to alter the structures and functions of Aβ peptides and in this way to inhibit metalloproteases associated with the processing and degradation of amyloids. It may be assumed, therefore, that Al(III) can alter the access to the CNS of Aβ peptides produced by the peripheral tissues, either by changing the permeability of the BBB or by affecting enzymatic degradation (Banks et al., 1996). An enhanced permeability of the BBB could account for the increased transport of bioligand-bound Al(III) into the brain (Deloncle & Guillard, 1990). In respect of the access of Al(III) to the brain, the most important factor is its binding to TF and its receptor (TFR). As discussed above, TF is the main protein carrier of Al(III) in the plasma. Cells in the brain have specific high-affinity TFRs. These receptors are specific for TF and not Fe(III), *i.e.* they are independent of the metal ion being transported. The TF–TFR system is postulated to be the route whereby the brain gains access to Fe(III) and other hard metal ions such as Al(III) (Roskams & Connor, 1990). Calculation of the rate of Al(III) distribution into the brain, however, indicates that TFR-mediated endocytosis cannot be the sole mechanism enabling Al(III) to enter the brain. The steady-state brain/blood Al(III) ratio measured in animal experiments was always significantly less than 1 and was influenced by the Al(III) form administered. This suggests an active process moving Al(III) out of the brain (Yokel et al., 1994). Genetic differences may result in variations in the efficiency of these processes and the permeability of the BBB, which supports the hypothesis that individual susceptibility to Al(III)-induced neurobehavioural toxicity may in part be genetically determined (Fosmire et al., 1993). It was recently assumed (Yokel et al., 1999) that the rapid brain emptying of citrate-bound Al(III), for instance, is achieved by a special mechanism via the monocarboxylate transporter system.

How can Al(III) affect the functions of the BBB at a molecular level? As a highly charged and relatively small cation, Al^{3+} can bind strongly to the negatively charged membrane surface sites, such as the phosphate groups of the phospholipids or the carboxylate functions of the membrane proteins. At the same time, Al(III) is known to have a high tendency to form oligonuclear binary and ternary hydroxo species with

various bioligands. It is assumed that such oligonuclear complexes, with structurally arranged positive charge centres able to interact with more than one negatively charged membrane site, will bind more strongly (Flaten & Garrutto, 1992) and are thus more effective than mononuclear Al(III) species in the increasing membrane rigidity and permeability. This binding is likely to induce membrane changes that enhance Al(III) entry by diffusion from the blood to the brain, where it can accumulate and exert secondary toxic effects. Others consider that the Al(III) uptake is not dependent upon on alterations in BBB permeability (Walton et al., 1995).

Informed Opinion

After the mid-1990s, there was a rapid decline in intensity of the research relating to the role of Al(III) in the aetiology of AD. We believe that this was mainly due to the relatively sparse X-ray crystallographic or NMR spectroscopic evidence indicative of the occurrence of Al(III) complexes of peptides and proteins involved in the formation of SPs and NFTs, the two common markers of AD. The X-ray crystal structures of selected Al(III) compounds which may be of relevance for an understanding of Al(III) binding in biological contexts have been reviewed (Powell & Heath, 1996). X-ray data on complexes of Al with porphyrins and phthalocyanines are discussed in that review, but not a single Al(III) complex of a protein or peptide is mentioned. Another reason is the complexity of the coordination chemistry of Al(III), which complicates the planning of the experiments, from the selection of the Al compound to be applied to the evaluation of the results. The strong tendency of Al(III) to hydrolyse and its rather sluggish complexation kinetics cause severe difficulties as concerns an exact description of the solution state of Al(III) and its existing forms in biological fluids and tissues (Harris et al., 1996). In an excellent review dedicated to amyloid fibrillogenesis (Rochet & Lansbury, Jr, 2000), Al(III) is not listed as a risk factor of AD. Amyloid fibrils, including those constituting the SPs in AD, are suggested to be formed by a common self-assembly pathway. However, environmental factors (including temperature, ionic strength, pH and oxidation potential, but not metal ions) are mentioned as influencing protein unfolding, nucleation and protofibril elongation. Moreover, the role of surfaces in inducing aggregation is stressed, as fibrillization may occur in the proximity of membranes *in vivo*.

At a molecular level, the first step of fibril formation is a conformational change in the largely unfolded, low-molecular mass Aβ proteins to form β-sheet enriched structures which more readily aggregate to SPs and NFTs. The question arises of whether Al(III) (and other toxic metal ions) can induce such conformational changes and enhance peptide/protein aggregation processes (Savory et al., 1996). On the basis of the previously discussed speciation studies and spectroscopic (CD, IR and multinuclear NMR) measurements, we consider that the answer to this question is undoubtedly that it can.

Stability constant determinations have revealed that the interactions of Al(III) with oligopeptides (modelling specific fragments of brain proteins involved in AD) rich in negatively charged side-chain binding sites such as COO^-, alcoholic-O^- or OPO_3^{2-} (in phosphorylated derivatives) are strong enough to bind Al(III) (Kiss & Farkas, 1996).

Spectral studies have indicated that such metal ion binding can induce intramolecular or intermolecular conformational changes in these protein fragments (Hollósi et al., 1996).

There are several crucial points where Al(III) in most of its chemical forms, if present and if its concentration exceeds a critical level, can interfere and promote fibril formation. Al(III) may play a crucial role in the nucleation process of β-amyloid. Long-term exposure to an elevated level of Al(III) in the brain could accelerate the assembly of β-strands into two-dimensional β-sheets and three-dimensional protofibrils by pasting them together through cation bridges. The tiny but stable Al(III)-bridged seeds may initiate the aggregation of Aβ peptides and other amyloidogenic debris of normal or aberrant metabolic processes. Hyperphosphorylation of the tau protein is one of the suggested causes of the formation of NFTs, the other hallmark of AD. The binding of Al(III) to the phosphate groups of hyperphosphorylated tau protein may promote aggregation and NFT formation. As demonstrated above, depending on its concentration and the actual presence of small bioligands, Al(III) may exist in various chemical forms. At low concentration it preferentially occurs in mononuclear forms, while at higher concentrations oxo- or hydroxo-bridged oligonuclear forms may predominate under physiological conditions. These various forms certainly have different tendencies to induce or enhance protein-aggregation processes. Additionally, insoluble Al(III) compounds such as aluminium silicates may serve as templates for the initiation or acceleration of the aggregation of amyloid (see *e.g.* Fasman, 1996). This might explain the differences in the morphology of such aggregates observed in the brain of AD patients (who might undergo long-term exposure to low concentrations of Al(III)) and in acute *in vivo* Al(III) toxicity studies.

Al(III) has been shown to interfere with many enzymatic key processes of the brain which basically control cell functions. These include (i) the phosphorylation–dephosphorylation of proteins, (ii) proteolytic reactions, (iii) neurotransmitter transformations, *etc.*, all of them implicated in the aetiopathology of AD. Much less is known, however, about the mechanistic and structural aspects of these Al(III)-modulated enzymatic processes. Information is needed to explain the stimulatory or inhibitory actions of Al(III). It is clear that in many cases the amount of Al(III) will determine whether it has a stimulatory or inhibitory effect (Exley, 1999). Further the chemical form of the metal may strongly influence the bioavailability of Al(III) and thus its biological effects. The importance of the actual chemical form of Al(III) as regards its *in vitro* effects on enzyme systems (Exley et al., 1994) and cell cultures (Zatta et al., 1994a; Kobayashi et al., 1996) has been emphasized and would need further investigation.

Long-lasting elevated levels of Al(III) may have a strong influence on both the fibrillogenic proteins and peptide fragments and the proteolytic and phosphorylation–dephosphorylation enzymes responsible for their overproduction. It was proposed in 1994 that neuronal death creates a proteolytic imbalance that generates pathological lesions (Smith & Perry, 1994). Following the initial cell damage, a self-perpetuating cycle of degeneration might arise that results in gradually increasing amounts of NFTs and SPs. This hypothesis emphasizes accidental macroscopic (*e.g.* brain trauma) or microscopic (*e.g.* local overload of neurotoxins) events which may cause neuronal death in critical brain areas. The interactions between Al(III) and enzymes are likely to be mediated by calmodulin (Haug & Vitorello, 1996). The possible effects of Al(III) upon proteolytic

enzymes have been studied in detail (Clauberg & Joshi, 1993). It has been proposed that Al(III) may accelerate the proteolytic processing of APP by suppression of the inhibitor domain. Thus, Aβ may accumulate and initiate SP formation. To our knowledge, the effects of Al(III) upon β- and γ-secretase, involved in the formation of the fibrillogenic fragment Aβ, have not yet been investigated. Inhibitors and activators of the enzymes responsible for the overproduction of Aβ and in particular the most fibrillogenic 42 and 43 residue forms, and of hyperphosphorylated tau protein, are promising candidates as future drugs.

The therapeutic prospects of AD were earlier surveyed by Allen & Burns (1995). Among the therapeutic options (including transmitter replacement, acetylcholinesterase inhibition, neuropeptide treatment, calcium channel blocking and, monoamine oxidase and serotonin re-uptake inhibition), chelation therapy was listed. The treatment of Al(III) toxicity is discussed somewhat more generally by Yokel (Yokel et al., 1996). Desferrioxamine has been applied for its ability to chelate Fe(III) and Al(III). It has been shown to be efficient in the treatment of Al(III) overload (Chang & Barre, 1983), and in one paper (McLachlan et al., 1991) (which received rather contradictory opinions) it was claimed to be effective in slowing down AD dementia. Accordingly, this aspect would appear to merit careful and thorough investigations.

Future targets for the drug therapy of the disease are the conformational transition from the native state to the β-structured form of Aβ and the process of self-assembly, which may have several stages, including the formation of prefibrillar intermediates and mature fibrils (Rochet & Lansbury, Jr, 2000). A peptide-based approach has recently been used to inhibit fibrillogenesis in an animal model of AD (Soto et al., 1998). Short peptide homologues, containing a substituted Pro as a β-strand breaker, have been shown to block the formation of amyloid fibrils and prevent neuronal death in cell cultures. Phosphopeptide chelators may be promising candidates for the solubilization of Al(III)-induced NFTs or SPs (*vide supra*). Peptides, however, are extremely difficult both to administer and to target for therapeutic use.

Accordingly, our overall opinion is that Al(III) is probably not a primary and major risk factor, but (as shown above) has numerous ways to interfere with enzymatic processes or interact with various proteins, and it could therefore act as an important co-factor able to induce or aggravate the late-onset form of AD. Studies on the interactions of the various chemical forms of Al(III) with biologically relevant model peptides and natural enzymatic and non-enzymatic proteins should be pursued with much higher intensity than at present because they are relatively unexplored and may be key-issues in clarifying or confirming the role of Al(III) in AD.

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Aluminium and Alzheimer's Disease
The Science that Describes the Link
C. Exley (Editor)

CHAPTER 20

Calmodulin, Aluminium and Alzheimer's Disease

Beka Solomon *

Department of Molecular Microbiology and Biotechnology, George S. Wise Faculty of Life Sciences, Tel-Aviv University, Ramat Aviv 69978, Tel-Aviv, Israel

* Corresponding author, email: beka@ccsg.tau.ac.il

Abbreviations: AβP – beta amyloid peptide; AD – Alzheimer's disease; Al – aluminium; BSA – bovine serum albumin; CaM – calmodulin; mAb – monoclonal antibodies; ELISA – enzyme linked immunosorbent assay; PKC – protein kinase C

Summary

Aluminium was found in senile plaques and neurofibrillary tangles in brains affected by Alzheimer's disease. However, a mechanism for the entry of aluminium into the cells of the central nervous system has yet to be found. In vitro studies showed that Al^{3+} binds to calmodulin, one of the most versatile Ca^{2+} binding proteins, inducing structural alterations which affect its biological activities. In spite of the large volume of data regarding calmodulin behaviour in vitro, little information exists on its possible involvement in introducing aluminium into the cells and triggering the development of Alzheimer's disease.

Historical Perspective

Calcium Dysregulation in Neuronal Aging and Alzheimer's Disease

Changes in the expression and function of Ca^{2+}-binding proteins observed in Alzheimer's affected diseased brains may serve to elucidate some of the complex neurological disturbances associated with the disease. Some of the important biochemical pathological findings related to Alzheimer's disease, e.g. overproduction, proteolytic cleavage and/or phosphorylation of β-amyloid precursor protein and/or τ-protein, are attributed to alterations in Ca^{2+}-binding protein functionality (Abu-Soud & Stuehr, 1993; Drewes et al., 1993; Fisher & Cyr, 1993; Gandhi & Keenan, 1983; Heizmann & Braun, 1992; Houenou et al., 1993; Mattson, 1992; Peterson et al., 1986; Thibault, et al., 1998).

There seems to be wide agreement regarding the basic tenet of the 'Ca^{2+} hypothesis of aging and dementia', namely, that some form of subtle Ca^{2+} dysregulation is a critical factor in brain aging and AD. In most, but not all cases, the specific form of dysregulation found has tended to suggest a condition of elevated Ca. However, beyond this

there appears to be little consensus on critical questions regarding the nature of the key changes, the interaction between alterations in multiple systems, which changes are cause and which are effect, or even for that matter, on the direction of change in a given process.

The view that Ca^{2+} dysregulation is involved in neuronal aging/Alzheimer's disease arose in the early 1980s (Khachaturian, 1984) out of a background that included, on the one hand, growing awareness of the potentially neurotoxic effects of elevated Ca^{2+} (Schanne et al., 1979), exemplified by excitotoxicity and Ca^{2+} influx during activation of glutamate receptors and, on the other hand, several initial studies on Ca^{2+} regulation in animal models of brain aging (Choi et al., 1987; Mayer & Westbrook, 1987; Rothman & Olney, 1987). However, it was apparent even in those early stages that multiple sites and mechanistic pathways of Ca^{2+} regulation were altered in aging.

In some of the initial studies on this subject, Ca^{2+} uptake was examined in brain synaptosomes from young and aged animals and was found to be reduced with aging under various conditions of stimulation (Gibson & Peterson, 1987; Peterson & Gibson, 1983). A separate study by these investigators examined Ca^{2+} uptake in fibroblasts isolated from AD patients and found that the uptake was reduced in these cells as well (Peterson et al., 1985). Another laboratory also used brain synaptosomal preparations, but in this case to examine Ca^{2+} extrusion by membrane pumps/proteins which they found to be reduced in aging animals (Michaelis et al., 1984; Michaelis, 1989). A third group utilized hippocampal slices in conjunction with electrophysiological recording to study Ca^{2+}-dependent potentials and processes e.g. synaptic frequency facilitation, and found evidence of enhanced voltage-dependent Ca^{2+} influx like the Ca^{2+}-dependent after hyperpolarization (AHP) (Landfield & Pitler, 1984; Landfield et al., 1986; Landfield, 1987).

Based on previous studies showing a role for the Ca^{2+}-activated protease calpain in long-term potentiation (LTP), investigators correlated differences in the amount of calpain with the rate of aging across species (Lynch et al 1986; Baudry et al., 1986). Although these early studies focused on very different processes, they shared in common the implication that relatively small changes in Ca^{2+} regulation with aging might gradually and cumulatively result in neuronal deterioration.

The regulation of intracellular Ca^{2+} has been extensively studied (Blaustein, 1988; Carafoli, 1987) and is well recognized to involve an enormously complex and integrated set of systems that includes endogenous buffering mechanisms and Ca^{2+}-binding proteins, membrane channels, intracellular storage sites, and intracellular release channels/receptors.

Recently it was suggested that β-amyloid peptide (AβP) toxicity is mediated directly via Ca^{2+}-permeable pores made from *fresh*, non-aggregated AβPs (Zhu et al., 2000). It has been shown that fresh and nonfibrillar $A\beta P_{1-40}$ induces rapid cellular reorganization, including loss of cytoskeletal network, cell–cell connections, and the retraction of cellular processes. Such cellular degeneration is mediated by elevating the level of intracellular calcium, most likely through cation-permeable AβP channels, and not by its interaction with the tachykinin receptors or by AβP induced and enhanced responsiveness to free radicals (Zhu et al., 2000). Consistent with this assertion was 1) in the Ca^{2+}-free medium, AβP incubation did not induce any cellular degeneration, and 2) AβP induced cellular degeneration was blocked by *zinc* and *Tris*. This may explain the modulation of conductances in AβP reconstituted vesicles and various cell types (Michaelis et al, 1984;

Michaelis, 1989 Landfield et al., 1986). This behaviour was shown in the significant and sustained increase in intracellular Ca $^{2+}$ level in cells treated with $A\beta P_{1-40}$, but not in the absence of extracellular calcium. An AβP-induced, sustained, elevated Ca^{2+} level is, most likely, a result of Ca^{2+} uptake from the external medium via AβP pores and AβP channel-mediated calcium uptake was reported for other cell types. Thus, it is likely that $A\beta P_{1-40}$ acts via existing ion channels and by making its own channels. A presence of AβP in the whole cell plasma membrane will be consistent with the possibility of $A\beta P_{1-40}$ forming cation selective channels in the plasma membranes.

The major Ca^{2+}-binding proteins, e.g. calbindin, calmodulin, parvalbumin, have been shown to be affected by aging (Baimbridge et al, 1992; de Jong et al., 1996; Gao et al., 1998; Iacopino & Christakos, 1990; Miller, 1995). Although declines in neuronal parvalbumin content have been detected in aged animals (Maguire-Zeiss et al., 1995), more prominent reductions have been observed for calbindin. Calbindin mRNA levels in brain decrease both with aging and in specific neurodegenerative diseases (Iacopino & Christakos, 1990). Declines in hippocampal calbindin have been reported to occur with AD (Iacopino & Christakos, 1990; Maguire-Zeiss et al., 1995).

There is considerable evidence that aging can affect Ca^{2+}-dependent intracellular signalling at several different levels. For example, alterations in receptor sensitivity, coupling efficacy, e.g. G-proteins, effector function, and second messenger generation have all been demonstrated with age and may lead to an altered biological response (Battaini et al., 1990; Foster & Norris, 1997; Jin & Saitoh, 1995; Magnoni et al., 1992; Pascale et al., 1998).

The Ca^{2+}-phospholipid-dependent protein kinase C (PKC) was shown to be impaired in both physiological and pathological aging as PKC activity or levels decline in aging rat (Battaini et al., 1990; Miller, 1995; Pascale et al., 1998) and AD affected brain (Cole et al., 1988; Horsburch et al., 1991). In addition, several studies have demonstrated a role for PKC in the neuropathology of AD. For example, activation of PKC inhibits β-amyloid production (Gabuzda et al., 1993; Hung et al., 1993). Thus, the decrease in PKC activity seen with AD may be a key factor in the overproduction of β-amyloid, one of the hallmark neuropathological feature of the disease.

Calmodulin

One of the most abundant and versatile Ca^{2+}-binding brain proteins, calmodulin (CaM) regulates a large number of cellular processes and target proteins in response to Ca^{2+} signalling (Cheung, 1980; Cheung, 1982; Klee et al., 1980; Linse et al., 1991; Means & Dedman, 1980; Vogel, 1994; Weinstein & Mehler, 1994; Zhang et al., 1995).

Calmodulin is a primary Ca^{2+}-binding protein found in all eukaryotic cells (Bachs et al., 1992).. It couples the intracellular Ca^{2+} signal to many essential cellular events by binding and regulating the activities of more than 40 different proteins and enzymes in a Ca^{2+}-dependent manner. CaM contains two structurally similar domains connected by a flexible central linker. Each domain of the protein Ca^{2+} binds two Ca^{2+} ions with positive cooperation. The binding of Ca^{2+} transforms the protein into its active form through a reorientation of the existing helices of the protein. The binding of Ca^{2+} results

in conformational changes of these Ca^{2+}-binding proteins, thus enabling these proteins to interact with their respective partner proteins and/or enzymes. The formation of complexes between Ca^{2+}-binding proteins and their targets induces further conformational changes in both components of the complexes, thereby allowing functional regulation of the targets (Klee, 1988; Weinstein & Mehler, 1994).

Calmodulin itself is not active; its active form is the calmodulin–Ca^{2+} complex. Ca^{2+} organizes and stabilizes the four-domains structure of calmodulin in a helical active conformation that can bind to its target; the central helix remaining flexible is an essential condition for its recognition by target proteins (Andersson et al., 1983; Ikura et al., 1983; James, et al., 1995).

Kinetic studies indicate that the active form of calmodulin contains four Ca^{2+} binding sites, the limiting factor for its activation being the cellular concentration of Ca^{2+} and/or the ability of calmodulin to undergo the conformational changes required for Ca^{2+}-binding.

Various calmodulin regulated enzymes interact with and are activated by distinct conformers of calmodulin containing different amounts of bound Ca^{2+}. Such a mechanism will allow calmodulin to translate a quantitative change in Ca^{2+} concentration into qualitatively different biochemical responses.

Binding of Ca to the Ca-binding sites may expose the central helix, which may be the conformational change that exposes the hydrophobic domain responsible for activation of target proteins (O'Neil & DeGrado, 1990; Pascual-Ahuir et al., 1991; Weinstein & Mehler, 1994) or interacting with fluorescent and photoactive drugs (O'Neil & DeGrado., 1989; Rao et al., 1992). Apparently Ca must be bound at three or more binding sites in order for this conformational change to occur (Seaton et al., 1985; Starovasnik et al., 1992).

Calmodulin and Alzheimer's Disease

The versatility of biological roles of calmodulin in binding and activation of a large number of target proteins has been widely investigated (Weinstein & Mehler, 1994). Many of the important biochemical pathological findings related to Alzheimer's disease, e.g. overproduction, proteolytic cleavage and/or phosphorylation of β-amyloid precursor protein (βAPP), τ-proteins and regulation of clathrin-coated vesicles can be attributed to alteration in calmodulin functionality as a response to intracellular Ca^{2+} concentration (Potter et al., 1993; Weiss & Haug, 1987).

In the presence of Ca^{2+}, calmodulin interacts with different cytoskeletal proteins, binds to microtubule associated proteins (Houenou et al., 1993) and to τ-proteins (Baudier et al., 1987), contributes to the dimer–polymer equilibrium of tubulin, mediates the calcium effect on the mitotic apparatus and plays an important role in chromosome movement. The mechanism by which calmodulin differentially activates its target enzymes is unknown.

Calmodulin regulates and activates more than twenty brain enzymes in the presence of Ca^{2+}, as in acetylcholine esterase biogenesis (Houenou et al., 1993), dolikol kinase production (Gandhi & Keenan, 1983), which is responsible for cellular level of glycoprotein synthesis, NO synthase activation (Abu-Said & Stuehr, 1993), activation of kinase and phosphatases involved in phosphorylation of β-amyloid and/or τ-proteins (Drewes et al., 1993; Gabuzda et al., 1993; Hung et al., 1993). Recent studies show that calmodulin

is involved in the regulation of ion channels, restoring the Ca^{2+}-dependent K^+ current channels and many other important biochemical pathways related to brain functions.

In vitro aging of calmodulin, as well as other degradative reactions, enables understanding of the relationship between the conformational stability of calmodulin and its biological activity. The affected sites identified in the calmodulin molecule after *in vitro* aging or degradation coincide with the high affinity Ca^{2+} binding sites, interfering with its efficiency in activating a whole cascade of biological events (Malencik & Anderson, 1987; Potter et al., 1993). The target proteins interact with and are activated by different conformations of calmodulin containing different amounts of bound Ca^{2+}. The conformation of calmodulin and its efficacy to interact with other proteins is profoundly altered in the presence of metal ions other than calcium. Binding of aluminium results in a loss of the α-helical content and regulatory function of calmodulin (Haug & Vitorello, 1996).

Calmodulin–Aluminium Complex

The attitude in the literature towards Al^{3+} binding by CaM is disputable. While Siegel et al. (1983) reported that binding of Al^{3+} to apoCaM causes conformational changes in the molecule, and Cox (1988) suggested that Al^{3+} ions bind to secondary sites on the CaM molecule, You and Nelson (1991) failed to detect such binding to spin-labelled CaM. Richardt et al. (1985) found that Al^{3+} ions were bound to holoCaM only when their concentrations in the reaction mixture exceeded 100 μM.

Equilibrium dialysis and atomic absorption studies indicate that Ca^{2+} remained bound to calmodulin following the addition of Al^{3+}. The lack of Al^{3+}–Ca^{2+} exchange is not surprising since metal coordination of protein is generally governed by ionic size and charge. This is in accordance with Siegel et al., 1983, who stated that it is doubtful whether the aluminium-binding sites on CaM are identical to those of calcium, but there can be overlapping of calcium and aluminium binding regions and the curvature of the respective binding loops may also vary. Moreover, the fact that the crystal and hydrated radii of Al and Ca, as well as their charge, are different makes it very improbable that Al-binding sites are identical to those for Ca^{2+}.

Aluminium causes breaking of protein helices, leads to the rearrangement of water molecules and promotes a more open structure which favours the access of quenching molecules to melittin's tryptophanyl residue, agreeing with experimental observations. This aluminium-induced mismatch in turn may be instrumental in malfunctions of calcium and calmodulin-dependent processes when aluminium ions enter the cell (Siegel & Haug, 1982; Weinstein & Mehler, 1994; Haug & Vitorello, 1996).

Presumably not involving the four specific Ca^{2+} binding domains on calmodulin, binding of Al^{3+} to calmodulin generates considerable dehydration entropies. Al^{3+}-triggered changes in the α-helix content of calmodulin are involved in the mismatch between calmodulin and its target protein. By altering calmodulin's internal dynamics, Al^{3+} apparently interferes with the protein's capacity to search out conformational substrates suitable for proper docking with a specific target protein. Aluminium is a known neurotoxic substance. Aluminium administration to sensitive experimental animals such as cats and rabbits produced neurological and neuropathological changes in the central nervous sys-

tem (Martin, 1997). However, the mechanism of aluminium neurotoxicity is unknown. In epidemiological studies, low level exposure to aluminium has been postulated as a possible contributing factor in several neurodegenerative disorders, such as Alzheimer's disease (McLachlan et al., 1991). Because the role of aluminium in the pathogenesis of these diseases remains unclear, studies on the neurotoxic mechanism of aluminium are needed.

An interesting study of Llansolo et al. (1999) shows that prenatal exposure to Al reduced significantly the content of nitric oxide synthase and guanylate cyclase and increased the content of calmodulin both in cultured neurons and in the whole cerebellum (Cucarella et al., 1998; Llansolo et al., 1999).

Although the role of aluminium in AD is uncertain, circumstantial evidence implicates aluminium in the pathogenesis of AD (Birchall & Chappell, 1988). This evidence includes the detection of aluminium in some AD lesions as well as in tangle-bearing neurons; the ability of aluminium to induce phosphorylation of normal τ protein (Abdel-Ghany et al., 1993; Scott et al., 1993; Shin et al., 1994) and the ability of aluminium to promote aggregation of Aβ (Exley et al., 1993; Mantyh et al., 1993; Kuroda & Kawahara, 1994).

The presumed relationship of Al to AD arises from comparative aluminium analyses of brain autopsies of AD patients and controls (Eichhorn, 1993). In the past by far the most active laboratory in pursuing this evidence was that of McLachlan (Crapper et al., 1976; Crapper et al., 1980). That laboratory has repeatedly demonstrated highly localized concentrations of Al in AD brains, and the work has been confirmed in other laboratories. Contrary evidence has been obtained from Ehmann et al. (1986) who have published results indicating age-dependent increases of Al in brain autopsies, but they have found no significant difference between AD and control brains. McLachlan attributes this difference to variations in sample size, because only in small samples can one be sure that the AD sample contains neurofibrillar tangles while the control samples do not (McLachlan et al., 1987).

Since Al^{3+} does not remove Ca^{2+} from its specific coordination domain, there are indications that Al binds to the N-terminal α-helices of each of the four Ca^{2+} binding domains and probably in the central α-helix connecting the two main lobes, leading to considerable decrease in the α-helix content of the protein (Haug & Vitorello, 1996; Glusker, 1991). Since structural fluctuations play a crucial role in protein dynamics and catalysis, critical interactions are modified to such an extent in aluminium–calmodulin that a proper fit of calmodulin with target proteins cannot take place. The presence of Al^{3+} apparently induces a mismatch between calmodulin and its partners preventing binding of Ca-activated CaM with its target proteins (Haug & Vitorello, 1996).

Sequential addition of Al^{3+} simultaneously caused an almost complete inhibition of the Ca^{2+} calmodulin-dependent cyclic 3′ : 5′-nucleotide of phosphodiesterase activity when the molar ratio of (Al)–(calmodulin) reached a value 4 : 1 (Richardt et al., 1985). Since Al^{3+} apparently does not remove Ca^{2+} from its specific coordination domains, one can only speculate about the location of putative Al^{3+} binding sites on calmodulin. Al^{3+} application inhibited the Ca^{2+} and calmodulin-dependent 3′,5′-cyclic nucleotide phosphodiesterase activity by impairing calmodulin rather than the basal activity of the enzyme examined in the absence of calmodulin (Siegel & Haug, 1982).

To fully understand the regulatory properties of multifunctional Ca^{2+}-calmodulin, the systematic features of the protein's space–time dynamics must be clarified (Haug & Vitorello, 1996). Links must be established between the critical conformational substrate

at the onset of ligand binding, e.g. Ca^{2+} coordination, and the final conformational substrate(s) responsible for correct docking between calmodulin and its respective target. By the same token, the temporal and structural gap must be bridged between the initial substrate of the Al^{3+}-coordinated protein and the dysfunctional substrate, non-operative for docking, to understand the molecular details of the Al^{3+}-induced lesion on calmodulin. By employing bioengineered calmodulin with a single intrinsic fluorescence emitter (tryptophan) located at selectively defined sites along the protein backbone, fluctuations of the local state can be monitored at various regions of calmodulin subjected to Al^{3+} coordination (Haug & Vitorello, 1996).

Informed Opinion

Immunological Characterization of Aluminium–Calmodulin Complex Conformation

As shown in previous studies, changes in the conformation of antigen or antibodies under various experimental conditions are illustrated by alterations in their immunological biorecognition (Katchalski-Katzir & Kenett, 1988). We followed conformational changes occurring in the calmodulin molecule after aluminium binding, using highly specific monoclonal antibodies (mAbs), was able to differentiate between the conformational states of calmodulin, as well as mAbs which recognize aluminium free or bound to proteins. Such conformational mAbs raised against Ca^{2+} calmodulin, namely CAM1 and CAM4, and mAbs raised against Al, were used for the characterization of the conformation of calmodulin imposed by aluminium binding (Levi et al., 1998a; Wolf, 1995).

Calmodulin possesses a phylogenetically highly conserved amino-acid sequence (Klee & Vanaman, 1982; Van Eldik & Watterson, 1981). As mentioned, a few monoclonal antibodies against calmodulin have been produced by immunization of mice with a mixture of mammalian calmodulin and phosphodiesterase (Hansen & Beavo, 1986) or by *in vitro* immunization of mouse-spleen cell culture (Pardue, et al., 1983) and by immunization with plant or invertebrate calmodulin (Jablonsky et al., 1991; Kobayashi et al., 1991). Preparation of a mAb against vertebrate calmodulin using a thyroglobulin-linked peptide of 20 amino acids as an antigen was reported (Sacks et al., 1991). In most cases, the antibodies obtained possessed low affinities towards native bovine calmodulin, or failed to distinguish between different functional conformations of calmodulin.

Highly specific mAbs against bovine calmodulin were prepared and characterized by the injection of a glutaraldehyde-cross-linked preparation of bovine brain extract. The addition of Ca^{2+} and cross-linking by glutaraldehyde were aimed at maintaining the functional conformation of calmodulin by complexing it with some of its target proteins (Wolf et al., 1995).

Out of nine anti-calmodulin mAbs isolated, three, CAM1, CAM2 and CAM4 were purified and characterized. Mab CAM1 was identified as an IgG1 while mAbs CAM2 and CAM4 belong to IgM class. Additivity ELISA showed that mAb CAM1 binds to an epitope located far from the epitopes recognized by the other two mAbs, while mAbs CAM2 and CAM4 recognize close epitopes. Mab CAM1 was found to be especially sensitive to the conformational state of calmodulin in the presence of Ca^{2+} ions. The

interaction of mAbs CAM2 and CAM4 with calmodulin are only slightly affected by Ca^{2+} removal. In addition, mAb CAM1 failed to recognize various species of calmodulin molecules, such as spinach and various plant recombinant calmodulin, while mAbs CAM2 and CAM4 share common epitopes with the above molecules. To examine whether and how metal ions other than Ca^{2+} affect CaM, we studied the immunological recognition of CaM–metal complexes by mAbs CAM1 and CAM4 (Wolf et al., 1995).

Mab CAM1, fully recognized CaM in the presence of Ca^{2+} or Tb^{3+} but not in the presence of Al^{3++}. As mentioned above, CAM1 is capable of detecting conformational changes that occur in the molecule upon Ca^{2+} binding. In a series of experiments, EGTA-treated CaM was incubated with Ca^{2+}, Mg^{2+}, Tb^{3+} or Al^{3+} ions (120 μM in 0.05 M MOPS buffer, pH 6.5), adsorbed onto wells of a polystyrene plate and allowed to interact with either CAM1 or CAM4 mAb. The interaction of CAM4 with all CaM–metal complexes tested exhibited similar patterns. CAM1, in contrast, fully recognized CaM in the presence of Ca^{2+} or Tb^{3+} but not in the presence of Al^{3+} (Wolf et al., 1998). The differences in the immunological recognition by the two antibodies may reflect either selective conformational changes, which result from binding of different target proteins to distinct regions in the C- and N-termini, or steric hindrance effects caused by the complexed target molecules. Nevertheless, it is pertinent to note that both mAbs reacted similarly with melittin and phosphodiesterase, regardless of the difference in their molecular weights (2.6 and 160 kDa respectively). The observation that the two antibodies react with the two extreme ends of the CaM molecule and are capable of a simultaneous interaction with holoCaM suggests that the differences in the immunological recognition of the different complexes reflect specific conformational alterations in the CaM–target molecule complexes. We investigated the loss of immunorecognition by mAbs CAM1 while increasing aluminium concentrations were added to calmodulin (Levi et al., 1998b).

Development of mAbs against Al enables the identification of aluminium when bound to calmodulin in a quantitative manner (Levi et al., 1998a). Mabs raised against aluminium bound to BSA successfully recognized aluminium or aluminium complexed with other protein or non-protein carriers. When compared with their Al-deprived counterparts, mAbs prepared against BSA–Al showed a clear positive reactivity with conjugates of CaM–Al and S100b–Al, as well as with aluminosilicate. CaM–Al and S100b–Al showed a 3–4-fold increased of reactivity compared to the protein alone.

Although the ability of CaM to complex aluminium ion is well-documented, the intense reaction between anti-aluminium mAbs G(10) and S100b–Al seen in this study demonstrates for the first time that Al is bound by the S100b protein as well. The S100b proteins are Ca^{2+}-binding modulator proteins that are involved in cell differentiation, cell-cycle progression and cytoskeletal–membrane interactions (Landfield, 1983). Both CaM and S100b protein families share structural similarities.

The loss of CaM recognition by mAbs CAM1 occurred concomitantly with the increasing concentration of aluminium added to calmodulin and with a simultaneous increase of G(10) anti-Al mAbs binding to CaM–Al (Levi et al., 1998b). A substantial reversal of these phenomena at higher Al concentrations may be explained by a) the 'exit' of Al from the solution due to precipitation, or b) inducing novel conformational changes which prohibit the mAbs interaction with Al. The epitope of mAb CaM1 was identified and located in an α-helical area between residues 130–148 of calmodulin. This epitope is

located around the fourth Ca^{2+} binding loop, an area which is very sensitive to structural changes as a response to Ca^{2+} binding (Wolf et al., 1995).

The ability of Al^{3+} to bind CaM in the presence of Ca^{2+} ions has a particular biological significance as it was shown that the damage caused by Al^{3+} in neurodegenerative diseases is associated with an increase in both Ca^{2+} and Al^{3+} ions (Drewes et al., 1993). Failure of these antibodies to recognize the new conformation of CaM imposed by Al binding suggests the alteration of calmodulin conformation which apparently interferes with its capacity to dock properly with a specific target protein. The ability of Al^{3+} to bind CaM in the presence of Ca^{2+} ions may have a particular biological significance to Al toxicity related to Alzheimer's disease and/or other neurodegenerative disorders.

Our study using mAbs proved that Al^{3+} ions bind to calmodulin even in the presence of Ca^{2+} ions at the region of the C-terminus on the calmodulin molecule. Upon binding, these ions induced conformational changes in the calmodulin molecule which led to a complete disappearance of the immunological recognition of calmodulin by mAb CAM1.

We have shown that specific mAbs can detect specific conformational changes which occur in CaM upon its binding to metal ions as well as to target proteins. However, more structural data are required to gain better understanding of how different target molecules bind to CaM and affect its conformation.

Aluminium–Calmodulin Complex and Alzheimer's Disease

Immunohistochemical methods allowed characterization at the morphological level of the localization of calmodulin in brain tissues (Caceres et al., 1983; Crapper et al., 1987). Calmodulin immunoreactivity in normal brains, revealed by both mAbs CAM1

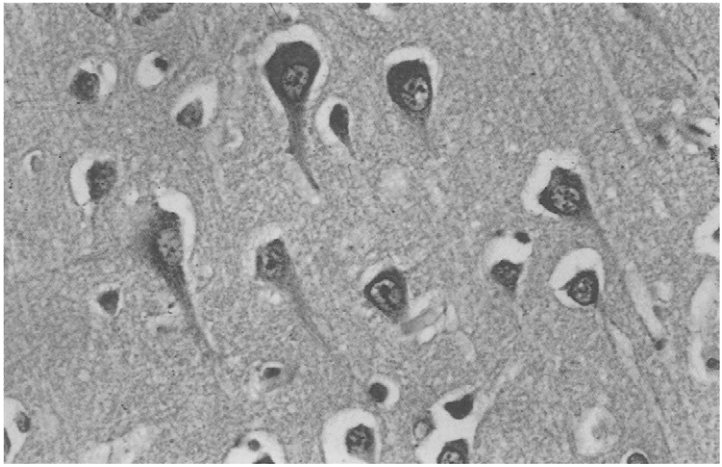

Fig. 1. Immunohistochemical localization of calmodulin in normal cortical brain. Calmodulin immunoreactivity characterized all cortical neurons which separated them from the remaining elements. Calmodulin was stained with anti-calmodulin antibodies CAM1 and/or CAM4 and visualized with Avidin–Biotin–Peroxidase complex (magnification ×400).

and CAM4, was found in both neurons and in glial cells showing abundant evidence in the cytoplasm. Oligodendroglia stained faintly, microglia and astrocytes moderately and neurons stained most intensively. The latter exhibited uniform homogenous microgranular distribution of the calmodulin inside the cytoplasm reflecting exactly the cellular outline. Slight immunoreactivity to calmodulin was seen inside the nuclei. Prominent immunoreactivity to calmodulin was unique to all cortical neurons, thus separating them from the remaining cellular elements (Fig. 1).

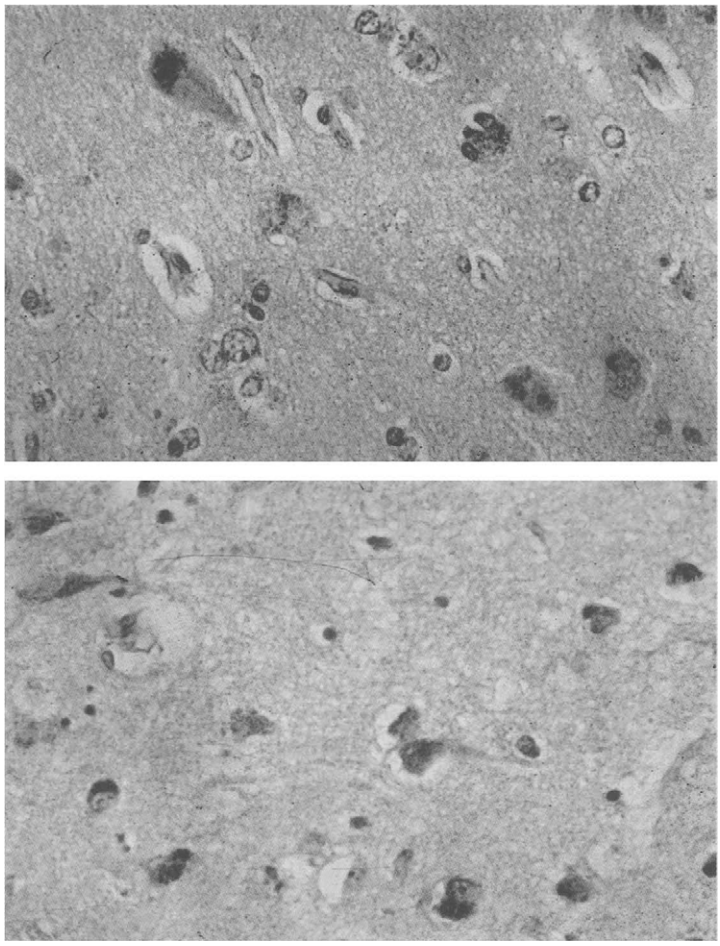

Fig. 2. Immunohistochemical localization of calmodulin in brain of Alzheimer's affected patients with mAb CAM4. Calmodulin immunostained with Avidin–Biotin–Peroxidase complex (magnification ×400). Top: Frontal cortical brain biopsy of 51 year old female Alzheimer's patient. Neuronal depletion and degeneration are accompanied by clumps of calmodulin inside surviving neurons or reactive glial cells. Coarse granules or clumps appeared deposited in different poles of the neurons, whereas other zones remained unstained. Bottom: Frontal cortical post mortem brain sections of 61 year old male Alzheimer's patient. The brain of the male Alzheimer patient presents microscopically marked neuronal depletion and uneven distribution of calmodulin inside neurons forming coarse granules or clumps deposited at various sites of the cells.

A significant difference in the distribution and amount of calmodulin was noted in neurons of demented subjects, together with marked neuronal depletion of cerebral cortex, replaced by reactive gliosis, senile plaques and neurofibrillary tangles of affected neurons. No immunoreaction was observed with mAb CAM1. The immunohistochemical staining with mAb CAM4 showed marked decrease of immunoreactivity to calmodulin in most of the neurons, with clumping and coarse granulation inside the cytoplasm. Coarse granules or clumps appeared deposited in different poles of the neurons, whereas other zones remained unstained (Fig. 2).

The presence of aluminium in brain sections of normal and AD affected brains was demonstrated using mAbs raised against Al. As shown in Fig. 3, Al is present in normal brains, however it is more abundant in AD affected brains.

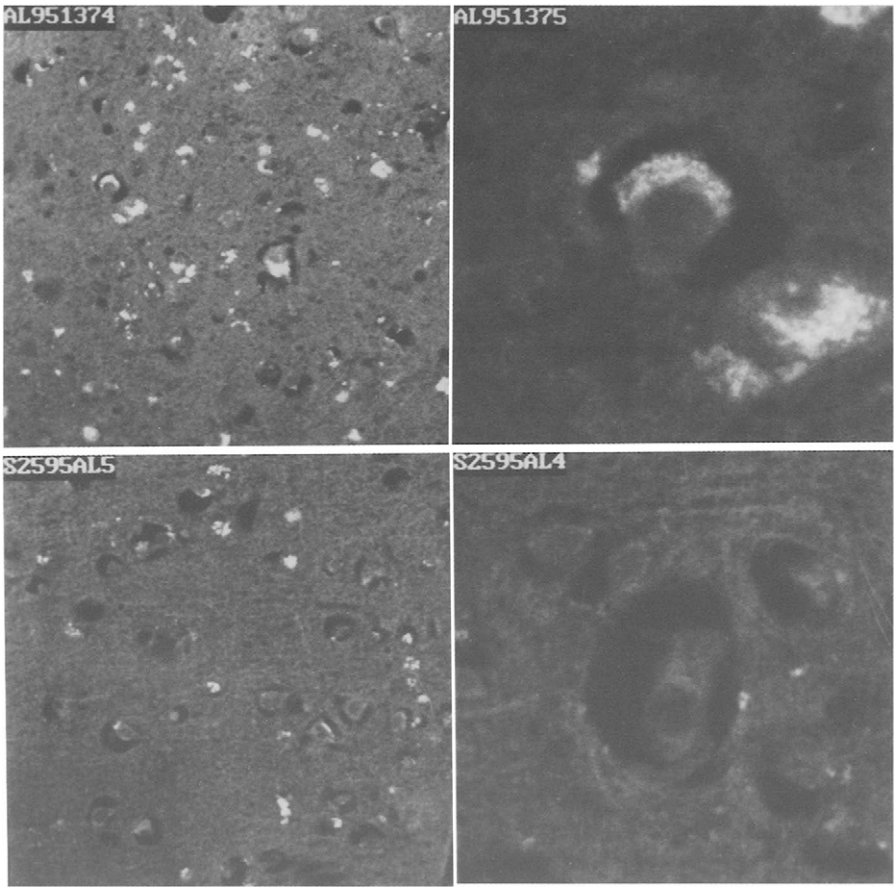

Fig. 3. Immunofluorescence visualized by confocal microscopy of aluminium in normal and affected AD brains by anti-aluminium antibodies. (upper) Frontal cortical post mortem brain sections of 61 year old male Alzheimer's patient – magnification ×200 (left), magnification ×1000 (right). (lower) Normal cortical post mortem brain section – magnification ×200 (left), magnification ×1000 (right).

The reduction of calmodulin content in Alzheimer affected cerebral cortex has been previously reported (Caceres et al.,1983; Crapper McLachlan et al., 1987). Moreover, the functional competence of the detected calmodulin was found to be less than the values estimated by radioimmunoassay techniques.

The ability of Al^{3+} to bind CaM in the presence of Ca^{2+} ions may have a particular biological significance to Al toxicity related to Alzheimer's disease and/or other neurodegenerative disorders. Alteration of the conformation of calmodulin imposed by Al binding may have possible implications in the neurotoxicity mechanism related to Alzheimer's disease. If the presence of Al in the brain is related to the loss of calmodulin compatibility, which prevents fulfilment of its vital functions, it may be suggested that calmodulin is a vehicle for Al binding into the cells and that aluminium neurotoxicity is involved in AD.

The cellular distribution and functionality of calmodulin, as well as the presence of Al ions in post mortem human brains affected by AD compared to age matched control brains shed some light on the implications of the Al–calmodulin complex in Alzheimer's disease

Current knowledge of Alzheimer's disease has not yet established one primary and central role for any of the pathological manifestations associated with the disease. None of the pathological changes is sufficient alone and it is the concomitance of all of them that confirms the diagnosis of Alzheimer's disease. What triggers the disease, what the required steps are and what is only a consequence are still unanswered questions.

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Aluminium and Alzheimer's Disease
The Science that Describes the Link
C. Exley (Editor)

CHAPTER 21

Aluminum, Tau and Neurofibrillary Degeneration

Ryong-Woon Shin *

Department of Neurological Science, Tohoku University School of Medicine, 2-1 Seiryo-machi, Sendai 980-8575, Japan

* Corresponding author, email: shin@mail.cc.tohoku.ac.jp

Abbreviations: Al – aluminum; AD – Alzheimer's disease; NFD – neurofibrillary degeneration; SP – senile plaques; NFTs – neurofibrillary tangles; PHFs – paired helical filaments; DFO – desferrioxamine; Tf – transferrin; TfR – transferrin receptor

Summary

Aluminum in the environment has established routes of access to neurons in the brain, and accumulates in affected neurons bearing neurofibrillary tangles characteristic to the brain of Alzheimer's disease. Aluminum was shown to selectively bind with hyperphosphorylated form of tau which constitutes the neurofibrillary tangles. Thus Al appears to be integrated into these lesions through association with hyperphosphorylated tau. The Al binding induces hyperphosphorylated tau to aggregate and retards its in vivo proteolysis. The aggregated Al–tau complexes might accumulate and form the neurofibrillary lesions during the neurodegeneration in Alzheimer's disease.

Historical Perspective

Tau (τ) Pathology in Alzheimer's Disease

The major histopathologic abnormalities that characterize the brains of patients with AD include an excess of neurofibrillary degeneration (NFD) and senile plaques (SPs), and the massive loss of telencephalic neurons (for review, see Spillantini & Goedert, 1998). The NFD includes neurofibrillary tangles (NFTs), dystrophic neurites associated with SPs, and neuropil threads. When viewed by electron microscopy, these lesions contain abnormal filamentous structures called paired helical filaments (PHFs) and the related straight filaments. These filaments are made from the microtubule-associated protein τ derived from the adult central nervous system. τ is a highly soluble protein (Cleveland et al., 1977; Lee et al., 1988) which is expressed widely in the brain, i.e., neuronal somatodendrites and axon fibers and glial cells (Papasozomenos & Binder, 1987; Migheli et al., 1988; Shin et al., 1991a), though its expression was originally confined to the axonal compartment (Binder et al., 1985). τ is normally attached to microtubules and is

involved in their assembly and stabilization. This function of τ is regulated by altering its phosphorylation state. τ protein constituting the PHFs, which is often referred to as PHFτ, differs in several biochemical and biological properties from that of the normal adult brain. One prominent feature that distinguishes τ from normal adult and AD brains derives from its phosphorylation state. In normal adult brain, τ is phosphorylated at many of the same sites as PHFτ but the extent of phosphorylation is less than that observed in PHFτ. Normal τ, however, is subject to rapid dephosphorylation during the postmortem period, yielding far less phosphorylated form of τ (Matsuo et al., 1994). In contrast, PHFτ remains highly phosphorylated in the AD brain even after a long postmortem interval. There are thus apparently quantitative and dynamic differences in phosphorylation between normal adult τ and PHFτ. Another prominent feature that characterizes PHFτ is its formation as insoluble aggregates, contrasting to the unusual solubility of normal τ (Cleveland et al., 1977; Lee et al., 1988). At the initial pretangle stage before defined NFTs are fully developed, τ begins to accumulate as nonfilamentous aggregates in a subpopulation of neuronal cells (Bancher et al., 1989; Shin et al., 1991b; Braak et al., 1994), followed by progressive and widespread accumulation as PHFs that form NFD (Bramblett et al., 1992; Shin et al., 1989; Shin et al., 1991b; Shin et al., 1992). Thus τ is highly phosphorylated and aggregated to assemble into PHFs in the AD brain. As to the causal relationship between phosphorylation and aggregation of τ, it is commonly assumed that elevated phosphorylation dissociates τ from microtubules, and then hyperphosphorylated free τ is aggregated into PHFs. A recent *in vitro* study (Schneider et al., 1999) has demonstrated that phosphorylation of recombinant τ is not directly responsible for but rather is inhibitory for its aggregation into PHFs. These unexpected results led us to consider two possible pathways involved in the aggregation of τ. First, aggregation of τ could occur prior to its phosphorylation. *In vitro* studies have shown that recombinant nonphosphorylated τ can be assembled into PHF-like structures by treating it with polyanionic factors such as heparin, RNA or poly-Glu (Friedhoff et al., 1998; Goedert et al., 1996; Hasegawa et al., 1997; Kampers et al., 1996; Perez et al., 1996). There have been no reports, however, showing that such polyanion-induced PHF assembly is relevant to that occurring in AD. Further in the brain nonphosphorylated form of τ mostly remains attached to microtubules, and such microtubule-associated τ has not so far shown to be assembled into PHFs. The pathway in which aggregation of τ precedes its phosphorylation is thus far from proved. Second, aggregation of τ occurs following its phosphorylation as widely accepted. For this pathway to be promoted, however, other factors such as aluminum might be implicated. The purpose of this review is to summarize current evidence implicating Al in the pathogenesis of AD through interaction with τ which constitutes the NFD.

Interaction of Al with τ Protein

Aluminum III (Al) is an element that has been shown to accumulate in association with the pathologic lesions, especially NFTs, of AD brain (for reviews, see Markesbery & Ehmann, 1993; McLachlan, 1995; Shin et al., 1995). Elucidation of the mechanisms for the concentration of Al in NFTs and the biological effects that Al exerts in NFTs should lead to a better understanding of the role of Al in the pathogenesis of AD. Considering

the binding ability of Al to phosphoproteins (Webb et al., 1973; Siegel & Haug, 1983; Birchall & Chapell, 1988) and the stable hyperphosphorylation state of the τ protein which constitutes the NFD, a number of studies were performed to examine the effects of Al on τ protein.

The long-term exposure of cultured neurons to Al resulted in the formation of τ-immunoreactive aggregates in cell bodies and cell processes (Kawahara et al., 1992). Isolated bovine τ protein, recombinant τ, and AD PHFτ were shown to aggregate *in vitro* following treatment with Al (Scott et al., 1993). In addition, enhancement of τ immunoreactivity was observed in Al-treated human neuroblastoma cells (Mesco et al., 1991) and in rat brains injected with Al salts (Shigematsu et al., 1992). More direct evidence for interaction of Al with τ and its aggregation was obtained using circular dichroism and ^{27}Al NMR spectroscopy (Madhav et al., 1996). These results suggest that τ is susceptible to aggregation by the association with Al

There have been studies aimed at exploring the effects of Al on the phosphorylation state of τ. Phosphorylation of τ protein was induced in neuroblastoma cells exposed to Al for several weeks as revealed by phosphorylated τ-specific antibodies (Guy et al., 1991), while similar phosphorylation of τ was not observed in cultured rat and human neurons following exposure to Al for up to 6 days (Mattson et al., 1993). Intracerebral co-injection of PHFτ and Al salts in rat brains induced neurons near the injection sites to acquire PHFτ-like properties as monitored with antibodies that recognize defined PHFτ epitopes containing phosphorylated residues (Ser202, Thr 205, Ser396 and Ser404) (Shin et al., 1994). Further, intracisternal administration of Al maltolate to rabbits produced a marked argyrophilic neurofibrillary degeneration which exhibited positive immunostaining with AT8 antibody that recognizes PHFτ epitopes containing phosphorylated Ser202 and Thr 205 residues (Savory et al. 1995). Thus Al exposed to cultured cells or to experimental animal brains appears to affect the phosphorylation state of τ. Such phosphorylation of τ elicited *in vivo* by Al might be ascribed partly to altered activity of protein phosphatases. Specifically, Al was shown to increase the phosphorylation of τ by inhibiting the activity of protein phosphatase 2A and 2B (Yamamoto et al., 1990), both of which are known to dephosphorylate τ protein *in vitro* (Goedert et al., 1992; Drewes et al., 1993; Harris et al., 1993). Al can also catalyze a non-enzymatic covalent cross-linkage that results in the incorporation of the α and γ-phosphate of ATP, GTP and CTP into τ and other proteins (Abdel-Ghany et al., 1993). Further support for a role of Al involved in the phosphorylation of τ comes from an examination of the brains from patients with renal failure who were exposed to Al during dialysis (Harrington et al., 1994). Immunohistochemical and immunochemical analyses were performed in order to investigate AD-like changes in τ protein. Although there was no significant accumulation of NFTs in the brains examined, normal τ was depleted and insoluble hyperphosphorylated τ was increased in association with elevated Al concentrations. This parallels observations showing that the production of hyperphosphorylated τ is associated with reduced levels of normal τ protein in areas of the AD brain with abundant neurofibrillary pathology (Bramblett et al., 1992; Shin et al., 1992). Thus, studies of the brains of renal dialysis patients with high levels of Al revealed abnormal τ protein similar to that seen in AD, but this abnormal τ did not appear to progress to formation of NFTs. These observations suggest that Al might play a role in the phosphorylation of τ.

Evidence outlined above for the effects of Al on τ protein indicates that, under certain conditions, this element can induce phosphorylation and aggregation of τ. A major question left unresolved is whether τ phosphorylated and/or aggregated by Al is assembled into PHFs. Previous ultrastructural studies of intracranial Al injection in experimental animals induced neuronal filamentous lesions, which are different from PHFs (Terry & Pena, 1965; Wisniewski & Soifer, 1979). Thus Al does not produce full pictures of NFD seen in AD brain, and the Al-induced aggregation and phosphorylation of τ reflect limited but significant aspects of the lesions. Important questions, however, are to be answered if such Al association to τ is indeed relevant to the formation of NFD occurring in AD brain. In our continuing efforts to elucidate the pathobiological involvement of Al in AD, we have provided evidence that interaction between Al and hyperphosphorylated PHFτ occurs in the NFD and promotes the formation of the lesions in AD brain (Shin et al., 1994; Murayama et al., 1999).

Preferential Interaction of Al with Hyperphosphorylated PHFτ

The 'chelating autoclave method' comprises pretreating tissue sections by hydrated autoclaving (Shin et al., 1991a) using trivalent-cationic chelators such as desferrioxamine (DFO) or EDTA, allowing Al removal from the sections (Murayama et al., 1999). By combining with morin histochemistry for Al (De Boni et al., 1974; Wen et al., 1985; Kihira et al., 1993) and immunohistochemical procedures, this method was used to demonstrate that Al is associated with PHFτ in the NFD of the AD brain. Morin staining of AD brain sections revealed positively stained NFTs, indicating a high level of Al in the NFTs. Application of the chelating autoclave method to AD brain sections prior to morin histochemistry attenuated the positive fluorescence of NFTs, indicating Al removal from NFTs. When this method was applied prior to immunostaining with phosphorylation-dependent anti-τ antibodies, the immunoreactivity of the NFD was significantly enhanced. Thus the chelating autoclave method was shown to remove Al from NFTs as well as to enhance in the immunostaining of these lesions. The observation that these two phenomena converged to occur concurrently at the same brain sites, i.e., the NFD where PHFτ and Al are colocalized indicates intimate association of Al to PHFτ, especially at its phosphorylated epitopes. Incubation of AD sections with $AlCl_3$, prior to morin staining, revealed Al accumulation with association to NFTs and this morin-positive illumination was attenuated after subsequent treatment by the chelating autoclave method. Similar incubation prior to immunostaining with the phosphorylation-dependent anti-τ antibodies abolished the immunolabeling of the NFD and this abolition was reversed by the Al chelation. These findings indicate that there is cumulative binding of Al to phosphorylated epitopes of PHFτ, thereby masking them from antibodies. Al binding was further documented for electrophoretically-resolved PHFτ on immunoblots, indicating direct Al binding to PHFτ. *In vitro* incubation of isolated PHFτ but not of normal adult τ with $AlCl_3$ resulted in insoluble aggregates of PHFτ, and this effect was lost by prior dephosphorylation of PHFτ. The interaction between PHFτ and Al is mediated largely by the phosphorylation of PHFτ. First it was phosphorylation-dependent but not phosphorylation-independent anti-τ antibodies

that revealed the immunoreactive alterations in PHFτ of the NFD following removal or addition of Al. Second dephosphorylation of PHFτ eliminated its interaction to Al. The aggregation of isolated PHFτ by interaction with Al was reproducibly observed (Yang et al., 1999). The *in vivo* effect of Al on PHFτ was examined by co-injecting PHFτ and Al salts into the rat brain. When PHFτ alone was used for injection, it formed proteolysis-resistant aggregates (Shin et al., 1993). However, co-injection of PHFτ with Al dramatically enhanced the resistance of PHFτ to *in vivo* degradation as evidenced by the prolonged persistence of PHFτ aggregates at the injection site (Shin et al., 1994). There has been no report showing that the Al-induced phosphorylation of τ is implicated in the formation of NFD in AD brain.

Informed Opinion

There are a number of lines of evidence implicating Al in the etiology of AD, but most of these lines of evidence appear to be circumstantial, and some are contradicted by other studies (for reviews, see Markesbery & Ehmann, 1993; McLachlan, 1995; Shin et al., 1995). Yet a straightforward link between Al and AD is suggested by the presence of Al in the pathologic lesions of AD, especially NFTs. The Al association with NFTs has been repeatedly demonstrated, although there are also some conflicting data on the association. The discrepant results are thought to derive from the relatively poor sensitivity of some of the analytical instruments used for the *in situ* microanalysis (McLachlan, 1995). Importantly, the mere presence of Al in NFTs does not necessarily imply pathological association. If Al is a pathological agent to promote AD through the association with NFTs, a route of access must be demonstrable for Al to neurons in the brain and therein Al must bind with a key ligand. An *in vitro* study has demonstrated that Al follows the normal physiological entry path into cells of the CNS via the same transferrin–transferrin receptor system as used for iron uptake by neurons and glial cells (Roskams & Connor, 1990). Through complexation with citrate in the labile iron pool, intracellularly introduced Al eventually becomes bound to phosphate ligands such as those on phosphorylated proteins (Birchall & Chapell, 1988). Several *in vivo* studies have demonstrated Al transportation to the brain via drinking water (Walton et al., 1995), or by intraperitoneal (Julka et al., 1996) or intranasal administration (Perl & Good, 1987). Thus there are several routes of access by which Al may be transported from the environment to neurons. In the degenerating neurons of the AD brain, τ becomes hyperphosphorylated by as yet unidentified mechanisms. Such hyperphosphorylated τ could serve as a ligand for Al in the neuron. This assumption is supported by the observation that abnormally phosphorylated τ acquires avid Al binding stronger than normal adult τ (Shin et al., 1994, Murayama et al., 1999). Given these features of Al and hyperphosphorylated τ, current hypothesis for how Al contributes to the formation of NFD is summarized in Fig. 1. The intracellular Al is highly likely to interact with PHFτ and induce the aggregation of PHFτ in affected neurons. The aggregated PHFτ–Al complexes might accumulate and constitute the NFD during the events leading to the neurodegeneration in AD brain. The PHFτ–Al interaction was demonstrated only indirectly by observing the Al-induced antigenic alterations in the phosphorylated epitopes of PHFτ. A more

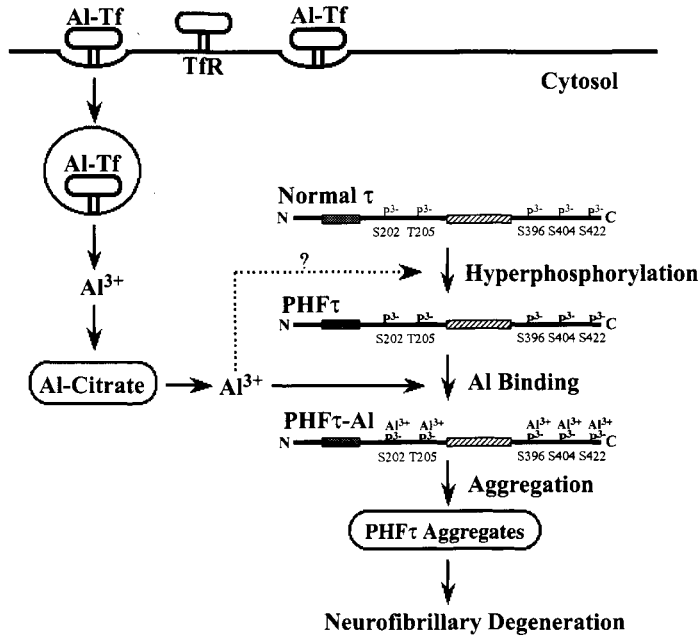

Fig. 1. Schematic illustration of how cytosolic interaction between aluminum and τ could be part of a hypothetical sequence of events that lead to the formation of neurofibrillary degeneration in Alzheimer's disease (AD). Aluminum (Al) follows the normal physiological entry route into the neuronal cell via the same transferrin (Tf)-transferrin receptor (TfR) system as used for iron uptake. Through complexation with citrate in the labile iron pool, Al eventually becomes bound to phosphate ligands such as on phosphorylated proteins. Microtubule-associated τ is a phosphoprotein with many of the same sites phosphorylated as PHFτ has at Ser202, Thr205, Ser396, Ser404, Ser422, etc., though to less degree. In the AD brain, τ is more phosphorylated due to impaired activation of phosphatases as well as excessive activation of kinases, and it is maximally phosphorylated so that it becomes PHFτ. It remains undetermined whether a series of these steps involve phosphorylation of τ by Al. After PHFτ is generated, intracellular Al avidly binds to this abnormally phosphorylated protein, and thereby induces the aggregation and delayed proteolysis of PHFτ. The PHFτ aggregates serve as nidus for the formation and maturation of neurofibrillary degeneration in AD.

direct and consolidated demonstration awaits further study using more sensitive *in situ* microanalysis for Al.

The involvement of Al in the PHFτ-associated pathology provides important aspects on therapeutic strategy of the Al chelation for the treatment of AD. There is good correlation between intracellular neurofibrillary lesions and the presence of dementia in AD patients (Arriagada et al., 1992; Neve & Robakis, 1998), and therefore these lesions could be a therapeutic target. Though the Al-induced aggregation of PHFτ represents the secondary event that follows the hyperphosphorylation of τ in the neurofibrillary pathology, the removal of Al from the NFD and/or the prevention of its further interaction with PHFτ could be used as a means to inhibit the formation and maturation of the NFD. In a clinical trial of Al chelation therapy using desferrioxamine (DFO), this agent appeared to slow the progression of dementia associated with AD (Crapper-McLachlan et al., 1991). The theoretical basis of the modest therapeutic response might be ascribed to

the possible involvement of the Al–PHFτ interaction in the NFD. Subsequent studies have confirmed the effects of DFO modulating the interaction between Al and PHFτ. In the experimental system in which phosphorylated τ-positive neurofibrillary degeneration was induced following exposure to Al in rabbits (Savory et al., 1995), co-administration of DFO partially reversed the changes in τ (Savory et al., 1998). *In vitro* aggregation of PHFτ induced by incubation with Al was significantly prevented when DFO was co-incubated with PHFτ and Al (Murayama et al., 1999). These findings indicate that DFO may be effective in modulating the interaction of Al with PHFτ. As DFO is nevertheless not an ideal chelator, particularly for long-term prevention, due to its practical limitations such as administration via injection and its expensive cost, the search for an orally effective alternative should be encouraged.

In the chelation of Al, Fe (III) must be taken into account since association and/or competition exists between Al and iron (Greger, 1993). Significantly, Fe and Al share the same protein ligands (Yokel, 1994) and have common mechanisms for both extracellular and intracellular distribution (Roskams & Connor 1990). The phenomenon that Al intoxication commonly results in anemia (Drueke et al., 1986) prompts one to assume that administration of iron could conversely lower the level of Al. Support for this assumption was derived from the observation that overload of iron in rats led to lowered level of Al in serum and brain (Cannata et al. 1991). Furthermore, a clinical study showed a therapeutic response of AD patients to long-term administration of iron, vitamin B6 and coenzyme Q10 (Imagawa et al. 1992). An extended study is required before the administration of iron is verified for its effectiveness as an Al chelation therapy. The hypothesis for the involvement of Al in the formation of the NFD will be eventually justified by the advent of an Al chelation therapy that is established to be of clinically beneficial use in patients with AD.

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Aluminium and Alzheimer's Disease
The Science that Describes the Link
C. Exley (Editor)

CHAPTER 22

The Association of Aluminium and β Amyloid in Alzheimer's Disease

Christopher Exley * and Olga Korchazhkina

Birchall Centre for Inorganic Chemistry and Materials Science, School of Chemistry and Physics, Keele University, Keele, Staffordshire, ST5 5BG, UK

* Corresponding author, email: cha38@keele.ac.uk

Abbreviations: Al – aluminium; Aβ – beta amyloid; AD – Alzheimer's disease; Fe – iron; Cu – copper; Zn – zinc; APP – amyloid precursor protein; ROS – reactive oxygen species; thT – thioflavin T; ATP – adenosine triphosphate; sAPP – soluble APP; ADP – adenosine diphosphate

Summary

The proteolytic cleavage of the amyloid precursor protein and the production of the neurotoxic and amyloidogenic beta amyloid peptide are believed to be critical events in the aetiology of Alzheimer's disease. Aluminium is bound by beta amyloid and is found co-localised with beta amyloid in the Alzheimer's disease brain. The possibility that this association between beta amyloid and aluminium might be involved in the aetiology of Alzheimer's disease is discussed and a number of mechanisms whereby physiologically significant concentrations of aluminium could instigate or accelerate the formation of neurotoxic amyloid fibrils are explored.

Historical Perspective

Over 20 years have passed since it was first shown that aluminium (Al) and β amyloid (Aβ) were co-localised in senile plaques in Alzheimer's disease (AD) brain (Duckett & Galle, 1980). Subsequent observations of Al at the centre of senile plaques (reviewed by Lovell et al., 1996) were refuted (Stern et al., 1986; Jacobs et al., 1989; Landsberg et al., 1992; Lovell et al., 1993) and the occurrence of Al was attributed to contamination of tissue samples by Al present in fixatives and staining reagents.These researchers were correct to highlight the very real problems that could arise from using materials and reagents contaminated with Al. However, their research was not sufficient by itself to rule out the occurrence of Al in Aβ deposits and the most recent research has been extremely careful to account for contamination and has used inductively coupled plasma mass spectrometry (ICP–MS) to demonstrate that Al is found in significant amounts (only iron (Fe) was found in greater amounts) in senile plaques (Beauchemin & Kisilevsky, 1998).

Al may not be present in Aβ deposits in foci of sufficient concentration to be identified by other less sensitive analytical techniques (Makjanic & Watt, 1999).

Their co-localisation in AD brain is not the only example of an association between Al and Aβ. Research has suggested a link between exposure to Al and the synthesis, transport and metabolism of Aβ's parent protein, the amyloid precursor protein (APP) (Edwardson et al., 1992; Shigematsu & McGeer, 1992; Clauberg & Joshi, 1993; Dobson et al., 1998; Exley, 1999). APP is aggregated by Al in vitro (Chong & Suh, 1995), it is found co-localised with Al in Golgi in neuronal cells exposed to Al (Lévesque et al., 1997) and APP showed increased neuronal immuno-reactivity in Al-treated rats (Shigematsu & McGeer, 1992) and rabbits (Huang et al., 1997). However, other studies using both in vitro and in vivo exposure to Al have not demonstrated any direct effect on the expression of APP (Neill et al., 1996; Campbell et al., 2000). Aβ immunoreactivity in neurones and neuroblastoma was increased following both in vivo (Varner et al., 1998; Huang et al., 1997; Rao et al., 2000) and in vitro (Kuroda et al., 1995; Campbell et al., 2000) exposure to Al. The observed effects upon the metabolism of both APP and Aβ were accompanied by many other cellular events, such as increased ubiquitination, oxidative damage and apoptosis, and these may or may not have been the result of a direct interaction with Al. The influence of Al on the proteolytic cleavage of APP (Nunan & Small, 2000) has still to be determined though precedents exist for the regulation of protease activity by Al (Clauberg & Joshi, 1993; Guo-Ross et al., 1998).

Whilst the nature of any association between Al and APP is uncertain we do know that Aβ binds Al in vitro (Exley et al., 1993). Circular dichroism was used to show that the partially helical conformation adopted by Aβ(1–40) in a membrane-mimicking solvent was abolished by Al. The loss of α-helical structure in favour of β-turn and random coil was instigated at approximately equimolar concentrations of peptide and Al and was completed when Al was present at a 6–7 fold excess.The effects of Al on the cd spectrum were not uniform over the wavelength range and this was, tentatively, interpreted as Al being bound at more than one site on the peptide. The abolition of α-helical structure by Al may help to explain how it prevented Aβ(1–40) forming calcium channels in bilayer membranes (Arispe et al., 1993).

The interaction of Al with Aβ(1–40) has been shown to induce aggregation of a physiologically significant concentration (10^{-10} M) of peptide (Mantyh et al., 1993). Zinc (Zn) and Fe (but not copper (Cu)) also induced aggregation though the huge excess of these metals (ca. 10^{-3} M Al, Fe, Zn) that was required to induce aggregation (as measured by centrifugation) may have indicated that the formation of an Aβ-metal complex was not by itself sufficient to induce aggregation of peptide but that the observed aggregation was actually the result of the incorporation of such complexes into sedimentable precipitates of metal hydroxides. The observed dependency of aggregation upon both pH and temperature would tend to support such a conclusion. Certainly high concentrations of Al (10^{-4}–10^{-3} M) will promote Aβ aggregation in saturated solutions of peptide (Kawahara et al., 1994; Kuroda & Kawahara, 1994) and this increase in the rate of peptide aggregation may be an indicator of increased stability in the final Al-induced β-sheet structure. There has not been a comprehensive study of the nature of Aβ fibrils formed in the absence and presence of Al. However, it has been shown that fibrils formed in the presence of a physiologically significant concentration of Al (10^{-5} M) were very

much different to those formed in the absence of Al (Exley et al., 1995). Amyloid fibrils formed in the presence of Al were slightly thicker, significantly longer and spirally wound around each other. We speculated that this arrangement might have reflected a number of binding sites for Al with one or more of these sites contributing to the observed periodicity. Subsequent studies have used circular dichroism (cd) and nuclear magnetic resonance (NMR) to confirm that Aβ(1–40) will bind up to 4 Al atoms and that binding increased the β-sheet content of the peptide (Vyas & Duffy, 1995a, 1995b).

It is not known if Al, or indeed any other metal, is bound by Aβ in vivo. Their co-localisation in amyloid deposited in the brain may or may not be the result of the prior formation of Aβ–Al (Aβ–metal) complexes. The physiological significance of the in vivo binding of Al by Aβ is, similarly, unknown. However, if it is proven that the precipitation of Aβ is a seminal event in the aetiology of AD (Hardy & Allsop, 1991) then the instigation or acceleration of this event by Al would suggest a possible mechanism of Al-induced disease. Aβ is neurotoxic (Yankner et al., 1989) and Al has been shown to potentiate the toxicity of Aβ(1–40) in both cultured rat hippocampal neurones (Kuroda et al., 1995) and in rats which received infusions of the peptide into the lateral cerebroventricle of the brain (Oka et al., 1999). In both cases the greater neurotoxicity was suggested to be the result of the increased aggregation of Aβ by Al.

Whilst the toxic form or fragment of Aβ remains unknown (Roher et al., 1996; Walsh et al., 1999; Howlett et al., 1999) it is difficult to speculate upon Al's role in potentiating the toxicity of Aβ. For example, the neurotoxicity of Aβ (in whatever form) may involve the formation of reactive oxygen species (ROS) (Schubert et al., 1995). The formation of ROS in the presence of both Cu (Bondy et al., 1998; Huang et al., 1999) and Fe (Bondy et al., 1998) is promoted by Aβ. The mechanisms underlying these events are believed to involve the reduction of Cu(II) to Cu(I) by Aβ (Huang et al.,1999) and the stabilisation by Aβ of the ROS-promoting ferrous form of Fe (Yang et al., 1999). Al is a prooxidant (Gutteridge et al., 1985; Kong et al., 1992) and is known to promote the oxidant activity of Aβ in the presence of Fe (Bondy et al., 1998). Like Aβ, Al can also stabilise the ROS-promoting ferrous form of Fe (Yang et al., 1999). These observations of the ROS-promoting activities of Aβ and metals seem to be contrary to both the observation that Aβ attenuated the prooxidant activity of Al (Van Rensburg et al., 1997) and the suggestion that Aβ is itself an antioxidant the properties of which could be compromised by binding hard metal ions such as Al (Berthon, 2000).

Al may influence the neurotoxicity of Aβ by an indirect effect upon its enzymatic degradation in peripheral tissues (Banks et al., 1996). In this way Al could affect the presentation of blood-borne Aβ at the blood brain barrier and its subsequent uptake into brain interstitial fluid (Banks et al., 1997). Whether this could result in an abnormal accumulation of Aβ in the brain is unknown. Al has also been linked to Aβ production through the immune response (Armstrong et al., 1995). Al has been shown to be antigenic (Levy et al., 1998) and to activate complement (Ramanathan et al., 1979) which, in turn, has been linked to the enhanced aggregation of Aβ (Webster et al., 1994).

Whilst there is evidence that Al will influence the neurotoxicity of Aβ there is, as yet, no direct link with the known aetiology of AD. For example, whilst we can be certain that Aβ will bind Al (Exley et al., 1993) we cannot be as certain of a role for this interaction in the deposition of Aβ in brain. Anti-parallel β sheet amyloid fibrils are one

of the precursors to the formation of senile plaques in AD brain. The formation of such fibrils can be identified experimentally using the fluor thioflavin T (thT) (Levine, 1993; 1997). Whilst it was shown that Al was bound by Aβ the result of this interaction was not always an increase in thT-reactive amyloid fibrils (Exley & Birchall, 1996; Exley, 1997; Bondy & Truong, 1999; Exley & Korchazhkina, 2001). We have shown that the binding of Al by Aβ either increased (for Aβ(1–40)) or decreased (for Aβ(1–42)) or had no effect (for Aβ(25–35)) upon the formation of thT-reactive amyloid (Exley & Korchazhkina, 2001). The lack of any influence of Al on the formation of thT-reactive Aβ(25–35) confirmed earlier research with this peptide although an increase in thT-reactive amyloid was measured in assays where either ATP (Exley & Birchall, 1996; Exley, 1997; Bondy & Truong, 1999) or inorganic phosphate (Bondy & Truong, 1999) were present in addition to Al. It was shown that it was the complex of AlATP, as opposed to either MgATP or Al, that was particularly potent in promoting the formation of thT-reactive amyloid and we have now shown that AlATP will also promote the formation of fibrils of Aβ(1–40), Aβ(1–42) and an unrelated amyloidogenic peptide, amylin (Exley & Korchazhkina, 2001). We have previously suggested a link between AlATP and the aetiology of AD (Korchazhkina et al., 1998; Exley, 1999) and we shall expand upon this suggestion in the second half of this chapter.

Informed Opinion

The amyloid cascade hypothesis (Hardy & Allsop, 1991) was the stimulus for an enormous volume of research into all aspects of the biology of Aβ. Whilst there is a general consensus that Aβ is involved in the aetiology of AD there is very little agreement as to its exact role. Al is only one of many possible influences upon the biology of Aβ. For example, it is not the only metal to either be found associated with senile plaques (Beauchemin & Kisilevsky, 1998; Cherny et al.,1999) or to influence the processing of APP or to induce or promote the aggregation of Aβ (Clements et al., 1996; Esler et al., 1996; Brown et al., 1997; Davey & Breen, 1998; Huang et al., 2000). However, a closer examination of the latter, that metal ions promote the aggregation of Aβ, whilst confirming the formation of metal–Aβ complexes of varying stoichiometry (Exley et al., 1993; Garzon-Rodriguez et al., 1999; Miura et al., 2000) does not provide an unequivocal demonstration that the formation of such complexes will either induce or promote the aggregation of Aβ into deposits which are either structurally or biochemically similar to those deposited in AD. Certainly there have been many examples of metal-induced precipitation of Aβ though many of these have not made any attempt to identify the nature of the aggregates formed or their relation to Aβ fibrils implicated in the aetiology of AD. Many so-called aggregation assays have used metal concentrations which exceed the solubility product of the metal hydroxide in the pH range of their minimum solubility. In these studies it is almost impossible to know whether the stimulus for aggregation was a peptide–peptide interaction or the hydrolysis chemistry of the bound metal. These are important distinctions and they may underlie much of the controversy that surrounds the aggregation of Aβ by Al, Cu and Zn (e.g. Mantyh et al., 1993; Bush et al., 1994; Esler et al., 1996; Huang et al., 1997; Brown et al., 1997; Atwood et al., 1998). The

application of thT (Levine, 1993; 1997) to the identification of the formation of β-pleated conformations of Aβ peptides is altogether less ambiguous and it is a mystery as to why this technique has not been more widely applied in metal–Aβ aggregation research. It has been used to show that Al does not induce or promote the formation of thT-reactive aggregates of Aβ(25–35) (Exley & Birchall, 1996; Exley, 1997; Bondy & Truong, 1999; Exley & Korchazhkina, 2001) or Aβ(1–42) (Exley & Korchazhkina, 2001). Although Al does promote the formation of thT-reactive aggregates of Aβ(1–40), though only at pH 7.5 (Exley & Korchazhkina, 2001). It has also been used in combination with reverse phase high performance liquid chromatography (RP–HPLC) to show that Al will promote aggregation of all Aβ peptides if either ATP (Exley & Birchall, 1996; Exley, 1997; Bondy & Truong, 1999; Exley & Korchazhkina, 2001) or 100 mM phosphate (Bondy & Truong, 1999) are also present in the assay. The possible significance of these latter findings will be discussed later in the chapter.

Al is unlike most of the other metals that have been shown to be both present in high concentrations in senile plaques and complexed by Aβ in vitro in that it is a known neurotoxin without any biological function. For these reasons alone its presence in the brain should be a cause for concern.There are three significant pools (extracellular, surface-associated and intracellular) of biologically available Al in the brain (Exley, 1999). There are a number of ways in which the brain burden of Al could impact upon the brain biology of Aβ:

(i) neurotoxicity which does not involve any direct interaction between either APP or Aβ and Al (or Al complex);
(ii) neurotoxicity through the disruption of the (as yet unknown) transmembrane function of APP by an interaction with Al (or Al complex) through either the extracellular or intracellular binding domains of the protein;
(iii) neurotoxicity as the result of an affect of Al (or Al complex) on either the transcription or trafficking or processing of APP either prior to or after its incorporation into the neuronal membrane;
(iv) neurotoxicity as a consequence of the binding of Al (or Al complex) by one or more of the cleavage products (e.g. Aβ and sAPP) of the proteolytic processing of APP;
(v) neurotoxicity due to combinations of two or more of these possibilities.

Which, then, of these possible interactions will either occur first or will stimulate the most significant response in the brain of someone experiencing a slow but sure increase in their brain burden of aluminium? A major consideration will be whether this interaction was with Al (as Al^{3+}) or a complex of Al? The concentration of Al^{3+} in brain interstitial fluid or neuronal cytosol will be extremely low (10^{-9}–10^{-10} M) and, even allowing for kinetic constraints, will limit its complexation by both free (e.g. Aβ) and anchored (e.g. membrane associated APP) ligands. The concentration of Al^{3+} would not, by itself, preclude the formation of complexes with Aβ particularly if Al was chelated through 2 or more functional groups on the peptide. However, the likely combination of both a low concentration of available Aβ (10^{-9}–10^{-12} M) and a low concentration of readily available Al^{3+} could be critical in preventing the formation of such complexes in vivo.

However, if Aβ was to bind Al^{3+} in vivo then the resulting complex (AβAl) could be a precursor to the nucleation dependent precipitation of Aβ peptide. For example, if the solubility product of $(A\beta Al)_n$ was lower than that of $(A\beta)_n$ and the lower solubility of

$(A\beta Al)_n$ was also consistent with a greater propensity to react with itself (eqn. 2) then $A\beta Al$ would aggregate at a concentration of $A\beta$ which was lower than would be required for the spontaneous aggregation of $A\beta$ (eqn. 1) in the absence of Al. The presence of oligomers or polymers of $(A\beta Al)_n$ would then fuel further reactions with either itself or with $A\beta$. This concept of a seeded precipitation of $A\beta$ is central to much of the current thinking on its precipitation in vivo (Hasegawa et al., 1999).

$$(A\beta + A\beta)_n \xrightarrow{K_{sp}(A\beta)_n} (A\beta)_n + A\beta \rightarrow A\beta \text{ aggregation} \quad K_{sp}(A\beta)_n = X\ \mu M \tag{1}$$

$$(A\beta + Al)_n \xrightarrow{K_{sp}(A\beta Al)_n} (A\beta Al)_n + A\beta \rightarrow \text{Al-induced } A\beta \text{ aggregation}$$

$$K_{sp}(A\beta Al)_n = X/2\ \mu M \tag{2}$$

Whether or not such a reaction could occur in vivo would depend upon both the initial formation of $A\beta Al$ and the accumulation of this complex to a saturating concentration ($K_{sp}(A\beta Al)_n$). The latter would depend upon the metabolism of $A\beta$ in brain interstitial fluid (or possibly within the neuronal cytosol) and whether this process of $A\beta$ removal could be influenced by its interaction with Al. There is an immune response to $A\beta$ in vitro (Solomon et al., 1996) and in vivo (Schenk et al., 1999) and this is the basis of recent attempts to develop a vaccine to reverse or slow down the cognitive deficits seen in AD. The fact that injections of synthetic $A\beta$ induced anti-$A\beta$ antibodies must raise the question as to why endogenously produced $A\beta$ was not similarly effective? This in turn raises the intriguing possibility that $A\beta$ induced to aggregate by Al may not be antigenic? Support for this proposition can be found in the preferential use of Al-aggregated $A\beta$ to induce neurodegeneration in rat brains continuously infused via the lateral cerebroventricle with $A\beta(1–40)$ (Oka et al., 1999). As such, whilst one's first impression might be that the induction of $A\beta$ aggregation by Al in vivo is unlikely it is also not difficult to see how it could occur under a certain set of conditions.

There have been a number of reports of the affect of Al complexes, as distinct from Al^{3+}, on $A\beta$ fibril formation in vitro (Exley & Birchall, 1996; Exley, 1997; Bondy & Truong, 1999; Ramesh et al., 1999; Exley & Korchazhkina, 2001). Complexes of both Al–glutamate and Al–aspartate have been shown to induce conformational changes in $A\beta$ in a membrane-mimicking solvent (Ramesh et al., 1999) whilst AlATP was extremely effective in promoting the formation of anti-parallel β sheet amyloid fibrils (Exley & Korchazhkina, 2001). The concentration of these complexes in the brain will be orders of magnitude greater than Al^{3+} and this could make them prime candidates for reactions with either APP or $A\beta$. The nature of the interaction between $A\beta$ and these Al complexes has not yet been determined though it may be that AlATP, for example, may have acted in a chaperone-like manner in promoting $A\beta$ fibril formation (Bohrmann et al., 1999; Yang et al., 1999; Kuner et al., 2000; Exley & Korchazhkina, 2001).

Any interaction between Al and $A\beta$ in the brain will be determined by the biological availability of Al in the brain. The brain's response to biologically available Al will be governed by a number of competitive equilibria which could include reactions involving APP and $A\beta$. We have observed that ATP, and in particular, AlATP, promoted the fibrillisation of both $A\beta(1–40)$ and $A\beta(1–42)$. ATP is abundant in the brain both in its

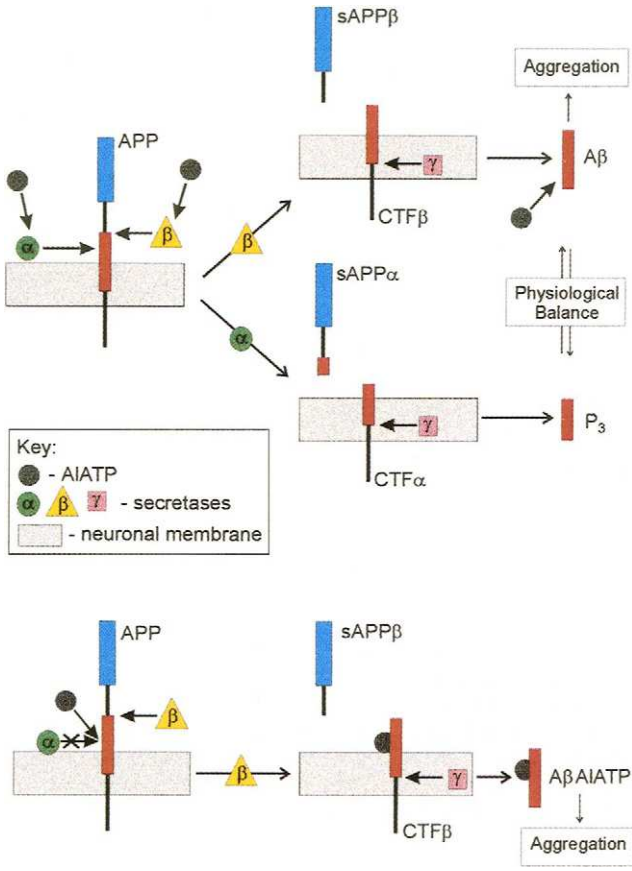

Fig. 1. Schematic identifying possible interactions of AlATP with the proteolytic processing of the amyloid precursor protein by α, β and γ secretases. Abbreviations: APP – amyloid precursor protein; sAPP – soluble APP; CTF – C-terminal fragment; Aβ – beta amyloid peptide fragment; P_3 – P_3 peptide fragment.

& Korchazhkina, 2001). Only a small proportion of the total Aβ released by proteolytic cleavage would need to be associated with AlATP (which would self aggregate to form the 'seed') to initiate an amyloidogenic event (Jarrett et al., 1993). A number of hypothetical interactions between APP, Aβ and AlATP are shown schematically in Fig. 1. The proteolytic cleavage of membrane bound APP and the production of P3 and Aβ is represented as a balance between the activities of α and β secretases respectively (Fig. 1, top). This balance might favour Aβ formation through either an inhibition of α-secretase activity or a stimulation of β-secretase activity by AlATP. Alternatively to, or in addition to, an influence of AlATP on the enzymatic cleavage of APP the Aβ produced might bind AlATP to increase its propensity to form aggregates. In a similar scenario (Fig. 1, bottom) the binding of AlATP by full length APP within the Aβ domain (perhaps involving nitrogen donor groups on lysine (amino acid no. 16 of the 1–40 sequence) and histone (amino acids nos. 13 and 14 of the 1–40 sequence) amino acids) is predicted to

capacity as an energy storage molecule and in its role in extracellular signal transduction (Burnstock, 1999). The intracellular or intraneuronal ATP pool is also a likely sink for intracellular Al (Panchalingam et al., 1991; Exley, 1999). It is surely inevitable that ATP (AlATP) and APP/Aβ will be found in the same compartment in vivo and that they may interact with one another. We have questioned the physiological significance of our observation of an interaction between Aβ and ATP and we have asked if it may have more to do with Aβ as part of APP than with Aβ alone? We hypothesised that APP in its role as a transmembrane protein may be a receptor or substrate for ATP. We have begun to test this hypothesis and we have found that the promotion of Aβ fibril formation by AlATP was blocked by the non-specific P2 ATP receptor antagonist suramin (Exley & Korchazhkina, 2001). Suramin was also found to promote the formation of Aβ fibrils and competed with AlATP to be bound by Aβ. Whilst the reaction between Aβ and AlATP was instantaneous it was also, apparently, relatively weak as we have not been able to demonstrate by RP–HPLC the formation of any AβAlATP complex. (This does not preclude the possibility that the reaction between Aβ and AlATP resulted in phosphoincorporation or phosphorylation of Aβ (see Abdel-Ghany et al., 1993) as this change would not have been as evident by RP–HPLC using UV detection.) The apparent weak nature of the interaction might be interpreted to support APP as a receptor as opposed to a substrate for ATP? However, it is probably not wise to extrapolate an association between Aβ and AlATP in vitro to an interaction between membrane bound APP and AlATP in vivo.

The possibility that membrane bound APP might function in vivo as either a receptor (Kang et al., 1987; Nishimoto et al., 1993) or as a substrate for phosphorylation by an ectoprotein kinase (Walter et al., 1997; Walter et al., 2000) has been raised previously as has a synergistic reaction between Aβ and ADP (Wolozin et al., 1998) and an Aβ-promoted increase in the activities of both casein kinase-I and casein kinase-II (Chauhan et al., 1993). If membrane bound APP was involved in signalling via extracellular ATP then we have already shown how the disruption of such a mechanism by AlATP (Korchazhkina et al., 1998) could initiate the cascade of cellular events responsible for the symptoms and pathologies of AD (Exley, 1999). On the other hand if the phosphorylation of APP at multiple sites on the protein is shown to be part of its in vivo function, for example, being involved in synaptogenesis and long term potentiation (Chen et al., 1996; Mattson, 1997) then the substitution of AlATP for Mg/CaATP in the enzyme/nucleotide/APP complex could also be an important factor in the aetiology of the disease. For example, AlATP is known to both stimulate and inhibit a number of kinases including glycerokinase, hexokinase, and the protein kinases P34, PKC and PKP (Furumo & Viola, 1989; El-Sebae et al., 1993; Exley et al., 1994) and it is known to stabilise the phosphorylated form of a number of proteins (Caspers et al., 1994; Li et al., 1998). AlATP has also been implicated in the non-enzymatic incorporation of phosphate into tau and other proteins (Abdel-Ghany et al., 1993).

The binding of AlATP by APP would not necessarily compromise any associated signalling function of the protein (Korchazhkina et al., 1998). However, if AlATP was to remain bound to Aβ(1–40) or Aβ(1–42) after their proteolytic cleavage from APP then the resultant AβAlATP fragments would be expected to form fibrils and seed precipitation of Aβ at concentrations of Aβ not normally prone to aggregation (Exley

prevent cleavage of APP by α-secretase. Proteolytic cleavage can now only proceed via β-secretase and the formation of Aβ. If AlATP remained bound to Aβ post-cleavage this could increase the likelihood of Aβ fibril formation and the precipitation of amyloid. We already know that Aβ will bind AlATP (Exley & Korchazhkina, 2001) it would be prudent to investigate if this interaction could interfere with the proteolytic cleavage of APP by α-secretase?

Abberant phosphorylation and phosphoincorporation of APP, the presenilins and tau may well be critical events in the pathogenesis of AD. Aluminium, particularly in concert with ATP, will initiate such events in vitro, it remains to be seen if this is also the case in vivo.

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List of Contributors

KENNETH ABREO — *Section of Nephrology and Hypertension, Department of Medicine, Louisiana State University Health Sciences Center, Shreveport, LA 71130, USA*

PAUL ALTMANN — *Oxford Kidney Unit, The Churchill, Oxford Radcliffe Hospitals, Oxford OX3 7LJ, UK*

STEPHEN C. BONDY — *Department of Community and Environmental Medicine, Center for Occupational and Environmental Health, University of California Irvine, Irvine, California 92697-1820, USA*

AREZOO CAMPBELL — *Department of Community and Environmental Medicine, Center for Occupational and Environmental Health, University of California Irvine, Irvine, California 92697-1820, USA*

DANIEL COMMENGES — *INSERM U 330, Université Victor-Segalen, 146 rue Léo Saignat, 33076 Bordeaux Cedex, France*

ROBERT R. CRICHTON — *Unité de Biochimie, Université Catholique de Louvain, 1348 Louvain-la-Neuve, Belgium*

PATRICK C. D'HAESE — *Department of Nephrology/Hypertension, University Hospital Antwerp, Wilrijkstraat 10, Antwerp, B-2650 Belgium*

MARC E. DE BROE — *Department of Nephrology/Hypertension, University Hospital Antwerp, Wilrijkstraat 10, Antwerp, B-2650 Belgium*

CHRISTOPHER EXLEY — *Birchall Centre for Inorganic Chemistry and Materials Science, School of Chemistry and Physics, Keele University, Keele, Staffordshire, ST5 5BG, UK*

VICENTE FELIPO — *Laboratory of Neurobiology, Instituto de Investigaciones Citologicas, FVIB, Amadeo de Saboya 4, 46010 Valencia, Spain*

RICHARD FLAREND — *Division of Mathematics and Natural Science, The Pennsylvania State University–Altoona College, 3000 Ivyside Park, Altoona, PA 16601, USA*

MICHAEL S. FORBES — *Department of Pathology, University of Virginia, Charlottesville, VA 22908, USA*

GRACIELA GARBOSSA — *Departamento de Química Biológica, Facultad de Ciencias Exactas y Naturales, Universidad de Buenos Aires, Ciudad Universitaria, (1428) Ciudad Autónoma de Buenos Aires, Argentina*

OTHMAN GHRIBI — *Department of Pathology, University of Virginia, Charlottesville, VA 22908, USA*

MARI S. GOLUB — *Department of Internal Medicine, University of California, Davis, Davis, CA 95616, USA*

CHARLES R. HARRINGTON — *Department of Mental Health, University Medical Buildings, University of Aberdeen, Foresterhill, Aberdeen, AB25 2ZD, UK*

MARY M. HERMAN — *IRP, NIMH, NIH, Bethesda, MD 20892, USA*

MIKLÓS HOLLÓSI — *Department of Organic Chemistry, Eötvös Loránd University, P.O. Box 32, H-1518 Budapest, Hungary*

TAMÁS KISS — *Department of Inorganic and Analytical Chemistry, University of Szeged, P.O. Box 440, H-6701 Szeged, Hungary*

OLGA KORCHAZHKINA — *Birchall Centre for Inorganic Chemistry and Materials Science, School of Chemistry and Physics, Keele University, Keele, Staffordshire, ST5 5BG, UK*

WALTER J. LUKIW — *LSU Neuroscience Center and Department of Ophthalmology, Louisiana State University School of Medicine Health Sciences Center, 2020 Gravier Street, Suite 8A8, New Orleans, LA 70112-2272, USA*

WILLIAM R. MUNDY — *Cellular and Molecular Toxicology Branch, National Health and Environmental Effects Research Laboratory, Office of Research and Development, USEPA, Research Triangle Park, North Carolina, 27711, USA*

ALCIRA NESSE — *Departamento de Química Biológica, Facultad de Ciencias Exactas y Naturales, Universidad de Buenos Aires, Ciudad Universitaria, (1428) Ciudad Autónoma de Buenos Aires, Argentina*

DANIEL P. PERL — *Professor of Pathology, Psychiatry and Neurobiology, Mount Sinai School of Medicine, New York, NY 10029, USA*

VIRGINIE RONDEAU — *INSERM U 330, Université Victor-Segalen, 146 rue Léo Saignat, 33076 Bordeaux Cedex, France*

JOHN SAVORY — *Departments of Pathology, Biochemistry and Molecular Genetics, University of Virginia, Charlottesville, VA 22908, USA*

TIMOTHY J. SHAFER — *Cellular and Molecular Toxicology Branch, National Health and Environmental Effects Research Laboratory, Office of Research and Development, USEPA, Research Triangle Park, North Carolina, 27711, USA*

RYONG-WOON SHIN — *Department of Neurological Science, Tohoku University School of Medicine, 2-1 Seiryo-machi, Sendai 980-8575, Japan*

BEKA SOLOMON — *Department of Molecular Microbiology and Biotechnology, George S. Wise Faculty of Life Sciences, Tel-Aviv University, Ramat Aviv 69978, Tel-Aviv, Israel*

MICHAEL J. STRONG — *Neurodegeneration Research Group, The John P. Robarts Research Institute, and the Department of Clinical Neurological Sciences, The University of Western Ontario, Room 7OF 10, UC-LHSC, 339 Windermere Road, London, N6A 5A5, Canada*

MARIO SUWALSKY — *Faculty of Chemical Sciences, University of Concepción, Casilla 160-C, Concepción, Chile*

ROBERTA J. WARD — *Unité de Biochimie, Université Catholique de Louvain, 1348 Louvain-la-Neuve, Belgium*

ROBERT A. YOKEL — *College of Pharmacy and Graduate Center for Toxicology, University of Kentucky Medical Center, Lexington, KY 40536-0082, USA*

PAOLO ZATTA — *Centro CNR per lo Studio della Biochimica e della Fisiologia delle Metalloproteine, Università di Padova, Dipartimento di Biologia, Viale G. Colombo 3, 35121 Padova, Italy*

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