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Possible Factors in the Etiology of Alzheimer's Disease

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Abstract

Inherited cases of Alzheimer's disease (AD) comprise only a very small proportion of the total. The remainder are of unknown etiopathogenesis, but they are very probably multifactorial in origin. This article describes studies on four possible factors: aluminum; viruses—in particular, herpes simplex type I virus (HSV1); defective DNA repair; and head trauma. Specific problems associated with aluminum, such as inadvertent contamination and its insolubility, have led to some controversy over its usage. Nonetheless, the effects of aluminum on animals and neuronal cells in culture have been studied intensively. Changes in protein structure and location in the cell are described, including the finding in this laboratory of a change in τ resembling that in AD neurofibrillary tangles, and also the lack of appreciable binding of aluminum to DNA. As for HSV1, there has previously been uncertainty about whether HSV1 DNA is present in human brain. Work in this laboratory using polymerase chain reaction has shown that HSV1 DNA is present in many normal aged brains and AD brains, but is absent in brains from younger people. Studies on DNA damage and repair in AD and normal cells are described, and finally, the possible involvement of head trauma is discussed.

Index Entries: Alzheimer's disease etiology; aluminum; herpes simplex type 1 virus; defective DNA repair; head injury.

Introduction

Many recent studies on Alzheimer's disease have been concerned with genetic linkage in various inherited cases, with mutations in the gene for the amyloid precursor protein (APP), and with abnormalities in the processing of β-amyloid the major component of senile plaques. However, inherited cases of AD comprise only a very small proportion of the total number of cases. The causes of the disease in the noninherited cases remain a mystery, but presumably, environmental factors are involved. The only risk factors known at present for AD are Down's syndrome (DS) and aging. Thus, the old adage, "If you wish to be long-lived, choose long-lived parents" should perhaps be amplified thus: "If you wish to be long-lived and of sound mind, choose long-lived parents and an identical environment."

The possible environmental agent that has been investigated most often is aluminum; other possible agents or factors include herpes simplex virus type 1 (HSV1), defective DNA repair, and head trauma. Conjectures about the role particularly of aluminum or viruses in AD pathogenesis arouse extraordinarily strong passions in those hostile to the concept, yet arguments for or against are more or less equally balanced; indeed, it is difficult on technical and theoretical grounds to devise definitive experiments with any one of these putative agents.

Aluminum as a Possible Factor

In the case of aluminum, the reasons for the technical difficulties include:

1. The high prevalence of this element: It is present in dust—and hence can easily contaminate the specimens under investigation, in drinking water, in many common foods such as cheese, in toiletries, such as deodorants, and in medicines, such as antacids;
2. There is little consensus of opinion about the sensitivity—or even accuracy—of the various techniques that have been used for examining levels of aluminum in brain, or more specifically, levels in plaques and neurofibrillary tangles; a related source of contention is the possibility of aluminum contamination;
3. Aluminum salts, such as aluminum chloride or aluminum phosphate, are very insoluble at physiological pH; therefore in the many studies that have been made on animals or cells injected with these salts (usually dissolved necessarily in acid), there is uncertainty as to the amount of aluminum actually in solution within the animal or the culture medium;
4. No suitable radioactive isotope has been available until recently for basic studies on aluminum metabolism; and
5. Animals vary in their susceptibility to aluminum, rabbits and cats being far more affected than rats, macaques, and mice. Further, the mode of delivery to the animal of aluminum that is most relevant to AD (or to another disease, amyotrophic lateral sclerosis, in which aluminum might be involved) is unknown; this is because it is quite uncertain whether aluminum enters the brain through the olfactory route or after passage through the gastrointestinal tract.

Aluminum is well known to be neurotoxic, although the molecular basis of its toxicity is unknown. The possible involvement of aluminum in AD pathogenesis was proposed because of its neurotoxicity and because rabbits injected intracerebrally with aluminum salts were found to display neurofibrillary degeneration, impaired memory, and lower capacity for learning *(1–6)*. Soon after, studies on aluminum levels in human brain suggested that levels were higher in AD than in normal brain *(7–9)*. Also, aluminum was implicated in the dialysis dementia afflicting patients with renal failure *(10)*. More recently, cognitive defects were detected in miners who had previously been given an aluminum compound as a prophylactic against silicotic lung disease, the impairment increasing with duration of exposure to the compound *(11)*. Another study from the same group found that treatment of AD patients with the chelating agent, desferrioxamine, apparently retarded the progression of the disease *(12)*. However, some of these findings—or their relevance to AD—have subsequently been contested, as described below, and others await confirmation.

Aluminum Treatment of Animals and Cells in Culture

Most studies involving injection of animals with aluminum salts have necessarily been short-term (usually not more than about 2 wk) because of the highly toxic effect of the treatment, which leads eventually to convulsions and death. In general, aluminum chloride or aluminum phosphate has been used, and therefore, much of the salt is likely to have precipitated at the site of the injection. However, Uemura *(13)* and Takeda et al. *(14)* used the complex aluminum tartrate, which is soluble at physiological pH, and this enabled studies to be made for up to several months. Other long-term studies on animals were achieved by usage of another soluble complex, aluminum-maltol *(15–18)*. Although the insoluble as well as the soluble compounds were found to induce perinuclear tangles, as revealed by silver staining, the soluble complexes caused far more numerous and more widespread lesions. The tangles consisted of single, 10-nm filaments; in contrast, AD neurofibrillary tangles (NFT) are composed of paired helical filaments (PHF). Immunochemical examination of AD NFT shows that they consist of an abnormally phosphorylated form of τ, a microtubule-associated protein (MAP), and probably ubiquitin and MAP2, also. Similar techniques, as well as electrophoretic analysis, applied to aluminum-induced tangles revealed that they contained neurofilament proteins *(19)*, specifically, the triplet 68-, 160-, and 200-kDa proteins *(20)*. These proteins are normally phosphorylated to some extent in the perikaryon, but further phosphorylation occurs when they enter axons; however, in aluminum-treated rabbits, abnormally phosphorylated neurofilament proteins were found in the perikaryal tangles—and also in some neurons without well-formed fibrillary structures *(21)*. Other studies showed that the 200-kDa protein was immunoreactive with antibodies to both phospho-

rylated and nonphosphorylated neurofilament proteins (22–24). Another approach, measuring the incorporation of ^{32}P into proteins (25), indicated that increased phosphorylation of rat brain cytoskeletal proteins occurred after oral administration of aluminum.

In several immunological studies, aluminum-induced tangles in treated rabbits were found not to be reactive with antibodies to MAP2, τ, or class III b-tubulin isotype (16,17,26). However, Takeda et al. (14) detected increased staining by anti-MAP2 antibody in a subset of pyramidal neurons, although staining was reduced in dendrites of hippocampal neurons; also, positive staining with antiubiquitin antibody was detected in the lower brain stem nuclei.

In AD brain, the levels of several neurotransmitters—in particular acetylcholine—and their associated enzymes are known to change. Various studies have been made to find if any parallel changes occur in aluminum-treated animals. Yates and coworkers (27,28) found that in $AlCl_3$- treated rabbits, there was a slight but not very consistent decrease in choline acetyl transferase (CAT) level in spinal cord gray matter, and in CAT and acetylcholinesterase in the hypoglossal nucleus. The authors pointed out that these brain regions were those in which aluminum-induced tangles occurred, but that in AD, there was loss of these enzymes in nontangle-bearing regions also. Similarly, Pendlebury et al. (24) and Beal et al. (29) found in $AlCl_3$-treated rabbits that CAT activity was reduced, especially in the entorhinal cortex and hippocampus—the regions in which they detected most neurofibrillary degradation; no decrease was detected in somatostatin or in neuropeptide Y. Pendlebury et al. (24) commented that this was in contrast to AD brain, but could be related to the absence of senile plaques in aluminum-treated animals—which, in turn, could be the result of the limited survival period of the animals.

Many of the studies that have been made on aluminum-treated cells in culture suffer from the same complication as the animal research: usage of aluminum salts that are insoluble at physiological pH. However, more recently the soluble complexes, aluminum-lactate or aluminum-maltol, have been utilized. In most cases, neuroblastoma or fetal rabbit or rat brain cells have been examined. The first publication was that of Miller and Levine (30), who found that treated cells showed an abundance of 10-nm neurofilaments. Cole et al. (31), Langui et al. (32), and Shea and coworkers (33,34) also found protein aggregates, most of which were perikaryal in treated cells, although only in a small proportion of the cells. This was attributed to a greater susceptibility of a small subpopulation to aluminum or to heterogeneity in the extent of maturation of the cells. Subsequent attempts to elucidate this produced conflicting results: Roll et al. (35) found that undifferentiated cells were less susceptible than differentiated cells, whereas Shea et al. (33) found no difference in their response to aluminum.

As to different cell types, cerebral neurons were much less susceptible than spinal cord and brain stem neurons (32), cerebral cortical explants less than midbrain explants (36), and hippocampal neurons less than motor-neuron enriched cultures (37). Most of the studies used immunological techniques that revealed the presence of phosphorylated neurofilaments, but not of PHF, τ, MAP2, or different β-tubulin isotypes (18,32,36). However, in the author's laboratory, human neuroblastoma cells treated with aluminum-EDTA for periods of up to 8 wk were found to stain (Fig. 1) with an antibody to phosphorylated τ, which reacts specifically with AD NFT (38). One-dimensional electrophoresis of whole-cell proteins revealed no gross changes after aluminum treatment (39). Our immunocytochemical results and similar studies—using Western blotting also—in the same year by Mesco et al. (40) were the first to find immunoreactivity of aluminum-treated cells to an AD-specific antibody.

As far as the author is aware, only one publication has appeared on neurotransmitter-associated effects of aluminum treatment. Singer et al. (41) found that in a neuroblastoma-glioma hybrid cell line treated with the soluble complex aluminum lactate, CAT activity increased at lower doses; at a higher dose of aluminum, which suppressed cell growth, CAT levels decreased, as did muscarinic receptors, but no change was found in acetylcholinesterase or glutamate decarboxylase activity. In the author's laboratory, preliminary results on human neuroblastoma cells indicate that CAT activity decreases after treatment with aluminum-EDTA (Dobson and Itzhaki, unpublished).

Sites of Aluminum Binding in Brain and in Cells in Culture

The NFT of AD occur in specific neurons in the hippocampus, cerebral regions, and basal forebrain. Aluminum-induced tangles occur mainly in neu-

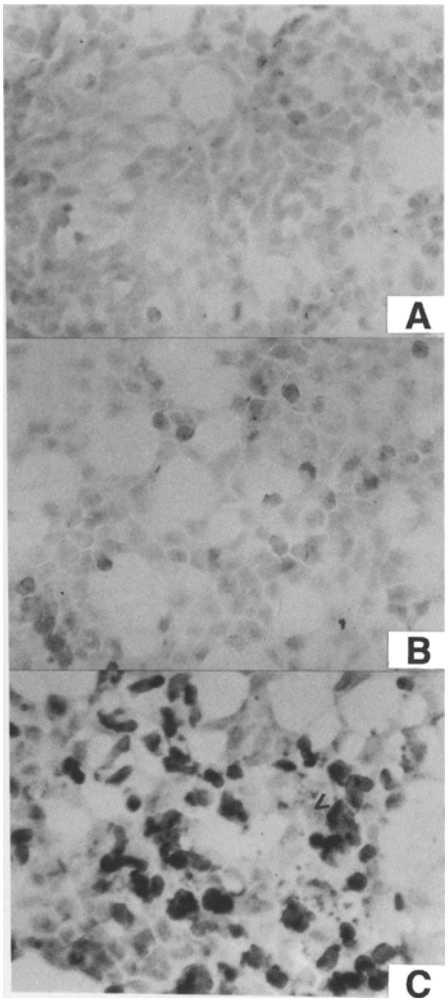

Fig. 1. Human neuroblastoma cell cultures treated for 16 d. **(A)** Control medium; **(B)** EDTA alone; **(C)** Al-EDTA. Immunoperoxidase-hematoxylin. Reproduced from Fig. 1 in Guy et al. (1991) *Neurosci. Lett.* **121,** 166–168, with permission of the publishers.

rons of the spinal cord, cerebellum, and brain stem; however, in the longer-term studies using soluble complexes of aluminum, tangles were detected also in cortical regions and in the hippocampus *(13)*, and in certain projection neurons in which AD NFT occur *(17,26)*. These longer-term treatments seem more likely to resemble the situation in humans, who presumably are subjected to a lifetime of exposure to aluminum, even if at a very low level.

There is no consensus of opinion about sites of binding of aluminum within cells. Sites as diverse as nuclei, endoplasmic reticulum, and lysosomes have been proposed (*see* summary table by Schuurmans Stekhoven, et al. *[42]*). Those who support the nuclear location generally assume that the aluminum is bound to DNA, but there appears to be no experimental evidence for this.* Indeed, only in two studies has the actual uptake of aluminum into cells been measured *(43,44)*. Shi and Haug *(43)* assayed uptake by atomic absorption spectrophotometry, but only after very brief treatment. They found no uptake after 1 h, but there was an appreciable amount when apotransferrin or fatty acids were added, or when the medium was adjusted to pH 6.5.

In the author's laboratory, uptake was measured by atomic absorption spectroscopy, after treatment of human neuroblastoma cells with various soluble complexes of aluminum for 7 d. Uptake was found to be far greater using aluminum-EDTA than aluminum complexes with maltol or citrate *(45)*; addition of transferrin did not appear to affect the uptake appreciably. The intracellular location of aluminum in treated cells is now being investigated by use of the radioisotope ^{26}Al. In separating subcellular components, we use at least two different techniques in order to check that no artifactual displacement of aluminum occurs during the procedure; in fact, agreement between different techniques has been good in each case. The main finding is that vastly more aluminum is associated with the nucleus than with the cytoplasm. Surprisingly, very little is bound to DNA, and consistent with this, admixture of pure DNA with aluminum results in a very low level of binding—less than one aluminum ion per thousand DNA-phosphates *(44)*.

Levels of Aluminum in Brain and Uptake of Aluminum

Techniques used for examining aluminum levels in human brain include graphite furnace atomic absorption spectrometry, neutron activation analysis, electron probe X-ray microanalysis (EPXMA), and laser microprobe mass analysis (LAMMA). Comparison of the results of different laboratories is not straightforward, not merely because of disputes over sensitivity or contamination, but also because of differences in type of specimen. Perl *(46)* has commented that neutron activation analyses were carried out on samples containing appreciable white matter, whereas the atomic absorption studies

*However, Lukiw et al. *(9)* have reported its presence in dinucleosomes, at higher levels in AD than in age-matched normals.

of Crapper-McLachlan and colleagues *(7–9)* used only gray matter; thus, the former might be less able to reveal small differences in aluminum level.

The presence of aluminum in senile plaques was claimed by Candy et al. *(47)*, but a number of subsequent studies have contested this *(48–51)*. As for NFT, Perl and Brody *(52)* detected aluminum in these structures and later concluded that the site of deposition was in the tangle itself *(53)*. In contrast, Lovell et al. *(54)* found no significant difference between mean levels in corresponding regions of NFT-bearing and non-NFT-bearing neurons and control neurons; however, AD neurons did exhibit a greater number of aluminum values significantly above the control mean than did control neurons.

The uncertainty about the level of aluminum in AD brain—or, if it is higher than in normals, whether it is involved in formation of lesions rather than a secondary effect—has not been elucidated by examination of the brain of dialysis dementia patients. Although aluminum is heavily implicated as a causal agent, the brain of such patients displays only infrequent NFT and immature plaques *(55)*.

Epidemiological surveys have shown a weak correlation between levels of aluminum in drinking water and prevalence of AD, in different geographical regions *(56–58)*. Flaten *(59)* revealed a correlation between aluminum level and dementia (not specifically AD), but pointed out that the results might have been affected by a less frequent reporting of dementia in rural areas compared to urban areas. As to absorption of aluminum from water or food, Lote and Saunders *(60)* and van der Voet *(61)* have pointed out that, unfortunately, far too little is known about the gastrointestinal absorption and renal excretion of aluminum. Even less, however, is known about transport into the human brain.

Possible Mechanisms of Action of Aluminum on Neuronal Cells

It appears that aluminum treatment results in a selective perturbation of neurofilament and/or related cytoskeletal proteins in treated animals, as proposed by Sayre et al. *(62)*, in treated cells in culture, and in abnormal phosphorylation of cytoskeletal proteins. The abnormal phosphorylation could lead to an accumulation of neurofilaments in the perikaryon. Alternatively, if, as suggested by Troncosco et al. *(63)* and Bizzi et al. *(64)*, aluminum causes impaired transport of neurofilament proteins, their consequent eventual accumulation in the perikaryon could result in their abnormal phosphorylation. Accumulation of these proteins is apparently not the result of increased transcription of the relevant genes: Muma et al. *(65)* and Parhad et al. *(66)* found that the expression of these genes was reduced after aluminum treatment. Another possibility is that aluminum causes a decreased rate of degradation, and Nixon et al. *(67)* found that in vitro, calpein-mediated proteolysis is in fact inhibited by aluminum. Other possible relevant findings in vitro include the promotion by division of tubulin assembly *(68,69)* and the aggregation of cytoskeletal proteins *(70)*.

Interestingly, isolated 200-kDa human and bovine neurofilament proteins were found by Pierson and Evenson *(71)* to contain at least 1 mol of aluminum (as well as copper and zinc). As to other possibly relevant interactions of aluminum, Clauberg and Joshi *(72)* have suggested, on the basis of its effect in vitro on serine proteases, that it might accelerate the proteolytic processing of APP by suppressing the inhibitor domain of the latter. Another way in which aluminum might act is through substitution for magnesium in magnesium-dependent enzymes *(73)*; a further possibility is via effects on calcium, such as perturbation of phosphoinositide-mediated calcium signaling *(74,75)* or blockage of β-amyloid-derived calcium channels (if they exist in vivo) in bilayer membranes *(76)*. Aluminum has been found to act directly on membranes *(77)* and, specifically, to affect brain lipid peroxidation *(78,79)*. Jope and Johnson *(80)* have reviewed these effects (1992) and have stressed the major increase in cAMP level that they detected after aluminum treatment of rats.

Viruses as a Possible Factor

Introduction

There are several arguments in favor of a role for a virus, in particular HSV1, in AD:

1. A number of neurological diseases are caused by viruses; for example, subacute sclerosing panencephalitis (SSPE), which is caused by measles virus, and progressive multifocal leucoencephalopathy (PML), which is caused by the polyomavirus, JC;
2. The regions of the brain that are the most affected in AD are the same as those affected in HSV encephalitis; and
3. HSV1 has a predilection for producing latent infection in neuronal cells; for example, it resides in the trigeminal ganglia (TGG) of most adult

humans—from which it can be reactivated by a variety of stimuli, resulting in some cases in cold sores.

One counterargument—that AD is not an infectious disease—is not wholly convincing, since if the virus were latent, it would not be infectious until reactivated, and would then need a susceptible host species *(81)*. Indeed, SSPE is not a transmissible disease, despite its viral origin, probably because virus assembly is faulty because of defects in the matrix (and possibly some other viral) protein.

The route by which the virus could reach the brain is unknown, but like aluminum, it might be via the olfactory pathway. Alternatively, it could migrate from the TGG when reactivated by various circumstances, such as stress. Ball *(82,83)* has proposed that on repeated reactivation of the virus, it could spread to the mesial temporal lobe, then to other limbic regions, and later to neocortical regions; this could account for the severe damage to the hippocampus and to the loss of memory observed in AD patients. Entry into the brain would be more probable in the elderly, since the immune system declines with age. Certainly the immune system appears to be involved in the establishment and maintenance of latency *(84,85)*. In the author's previous laboratory, it was found that HSV1 DNA was present in the CNS of immunosuppressed patients who had serological evidence of past HSV infection; it was absent in the CNS of the nonimmunosuppressed and in those people who had never been infected *(86)*. It was therefore suggested that immunosuppression, and perhaps stress also, could lead to entry of virus into the CNS or to reactivation of latent HSV1 already in the CNS.

Immunological Studies on Viruses in Relation to AD

A number of workers have sought antibodies to HSV—and in some cases, other viruses too—in serum or cerebrospinal fluid (CSF) of AD patients *(87,88)*, or viral antigens in brain *(89–91)*. Only in the study of Libikova et al. *(87)* was a greater proportion of positive results obtained in AD patients than in age-matched normals. Renvoize et al. *(88)* found no statistical difference between the two groups with respect to serum antibodies to adenovirus, cytomegalovirus, influenza A and B, measles virus, and HSV. Similarly, negative results were obtained by Friedland et al. *(92)* who sought crossreactive antibodies to three lentiviruses, in serum and CSF.

Search for HSV DNA in Brain by Hybridization

Studies on mice by Cabrera et al. *(93)* and Kastrukoff et al. *(94)* showed that latent infections could be established experimentally in the CNS as well as in the TGG; in the latter case, massive immunosuppression led to expression of infectious virus in the CNS. At about the same time, two groups claimed to have detected HSV1 in the brain of some humans *(95,96)*. However, four other studies obtained totally negative results with human brain *(97–100)*, and one of these studies *(99)* was unable to detect HSV2, cytomegalovirus 2, SV40, or measles, also.

All the hybridization techniques used in the above studies—solution, Southern blotting, and *in situ*—are relatively insensitive; in most cases, HSV1 DNA would have been detectable only if present at a level of at least 1 genome/cell genome. In contrast, polymerase chain reaction (PCR) is extraordinarily sensitive, being capable of detecting sequences at as low a level as $1/10^5$ cells. However, because of this sensitivity, it is essential to preclude even the slightest degree of crosscontamination. In the author's laboratory, PCR has been used for seeking evidence of HSV1 in brain samples, taking extreme care to prevent false positives and false negatives. A sequence within the viral thymidine kinase (TK) gene and another within the human hypoxanthine phosphoribosyl transferase (HPRT) gene are amplified in the same tube; a positive HPRT signal signifies that no contaminant is present that might interfere with amplification of the TK DNA. Also, HSV1-infected and uninfected, Vero cell DNAs are routinely amplified as positive and negative controls, as well as reagent "blanks." Using brain specimens from two independent brain banks, we have found viral TK sequences in 17/25 (68%) AD patients and 14/22 (64%) age-matched normals *(101,102)*. Figure 2 shows typical examples. In some cases, primers for the viral ICPO gene were used also, and these were consistent with the TK primer results. The temporal and frontal cortex and the hippocampus—the regions most affected in AD—were usually virus-positive. In contrast, the occipital cortex was always virus-negative (in 9/9 AD cases and 5/5 aged normals); interestingly, this region is relatively spared in AD. The specificity of amplification was confirmed using an internal oligonucleotide

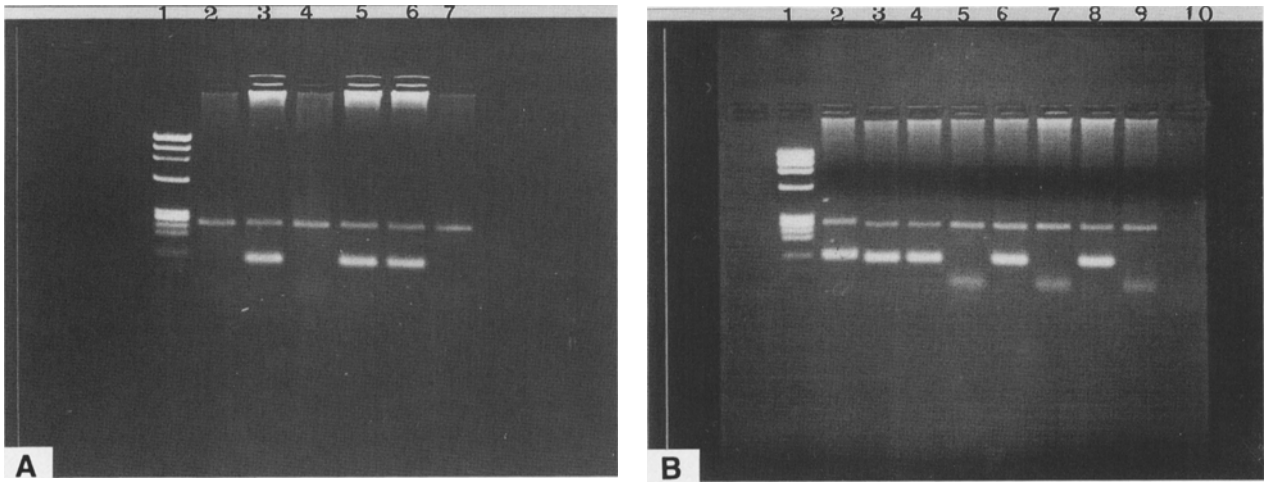

Fig. 2. Agarose gel electrophoresis of amplified DNA sequences, stained with ethidium bromide, from old normal and AD brain specimens; upper band amplified HPRT sequence (267 bp); lower band, amplified TK sequence (110 bp). **(A)** Lane 1, marker ϕX174 DNA, *Hae*III-digested; Lanes 2, 3, 4, 5, AD; lane 6, HSV1-infected Vero cells; lane 7, uninfected Vero cells. **(B)** Lane 1, marker ϕX174 DNA, *Hae*III-digested; lanes 2, 3, 6, 7, old normals; lanes 4, 5, 8, 9, AD; lane 10, reagent blank.

probe. No viral DNA was detected in lymphocytes from AD patients or aged normals, indicating that our findings in brain were not attributable to lymphocytes within the brain–blood vessels *(103)*.

We found no HSV1 DNA in brain specimens from middle-aged people or infants *(102)*. Thus, our results indicate that the presence of the virus is age-related, brain-region-related, and possibly tissue-specific. As to expression of the viral genome, we have examined cDNA from some of the virus-positive brain specimens by PCR; we have detected the latency-associated transcript, but not the TK transcript, showing that the virus is present in latent form in these elderly brains.

Possible Mechanism of HSV1 Involvement in AD

It seems likely that repeated reactivation of latent virus in brain—perhaps through immunosuppression or stress—could cause localized damage. Certainly cases of mild HSV1 encephalitis have been described (in middle-aged people) from which a mild degree of neurological damage results *(104,105)*. We have proposed that there may be differences in host or virus characteristics between those elderly normals and AD patients who are virus-positive which could lead to significant reactivation only in the latter group. (In our study, the fact that not all AD patients are virus-positive could reflect the likelihood that AD is multifactorial in origin.) A possible interesting parallelism is the recent finding of JC and BK virus in non-PML-affected brain, detected by *in situ* hybridization *(106)* and by PCR *(107,108)*; it is suggested that PML could result from reactivation of previously unapparent JC virus in the CNS, for example, during severe immunosuppression.

DNA Repair as a Possible Factor

Introduction

A defect in DNA repair has been detected in a number of neurological diseases. For example, patients suffering from xeroderma pigmentosum are highly sun-sensitive, because of a defect in repair of UV-induced damage in DNA. The consequences of such a defect are likely to be especially serious in nondividing cells, such as neurons. More directly relevant to AD is the known repair defect found in the cells of Down's syndrome patients. Further, since AD patients could be regarded in some respects as cases of accelerated aging and damage in DNA appears to accumulate with age (although it is uncertain whether repair declines concomitantly), they might be more susceptible than age-matched normals to DNA-damaging agents. Of course, a greater susceptibility would then signify that such damage is a factor in, or perhaps a consequence of, the disease rather than a cause.

Irradiation Studies

The first work on this topic examined lymphoblastoid cells from sporadic AD patients and indicated that after X-irradiation, cell viability was lower than that of cells from age-matched normals *(109,110)*. However, Robinson and Bradley *(111)* did not detect a greater than normal sensitivity of fibroblasts to UV-irradiation, using alkaline elution assays. In the author's laboratory, γ-irradiated lymphocytes have been examined by unscheduled DNA synthesis (UDS), extent of replication (after stimulation), single-strand breaks (ssb), chromosome aberrations, double-strand breaks (dsb) using pulsed-field gel electrophoresis, and RNA synthesis *(112–115)*. We found a difference between cells from AD patients and age-matched normals only in chromosome aberrations; the number of dicentrics was significantly higher in each of six AD cases compared to six normals, the mean being about 25% higher. A higher (but much more variable) level of aberrations was detected by Lavin et al. *(116)* in γ-irradiated AD lymphoblastoid cells, mean values being two to three times normal values; cells were, however, irradiated in exponential phase and would therefore have varied in their radiosensitivity.

The increased number of dicentrics in AD lymphocytes found in this laboratory *(114)* was almost identical to that found in lymphocytes from DS patients (*see*, e.g., Takeshita et al. *[117]*). Because of the involvement of chromosome 21 in Down's syndrome and in some inherited cases of AD, we decided to look for possible localization of chromosome aberrations. However, preliminary experiments examining p or q arms of chromosomes 1,2,3 or the Denver groups B-G have revealed no difference in the distribution either for normal or AD cells *(118)*.

Chemical Agents

Most studies on AD cells have used fibroblast cells from familial patients. The most frequently used chemicals have been the alkylating agents, *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG), methyl nitrosourea (MNU), or methylmethane sulfonate (MMS); all are methylating agents, but MNU and MNNG, unlike MMS, produce in DNA the premutagenic adduct, O^6-methylguanine (O^6- MeG). The techniques used have generally been one or more of the following: cell survival, colony-forming ability, UDS, or alkaline elution. However, at least two of these techniques have certain disadvantages. Viability is an extremely variable parameter. Alkaline elution, as pointed out by Kinsella et al. *(119)*, measures only ssb and alkali-labile sites, and thus reveals very little of the damage produced by alkylating agents; also, these sites increase in number on prolonged storage of cells in alkali. Perhaps for these reasons, there has been little consensus of opinion about the relative sensitivity of AD and normal cells to DNA-damaging agents, even when different groups have used the same cell lines. However, there are some indications of a greater susceptibility of cells from familial cases.

The first studies, again by Robbins and coworkers, used fibroblast lines mainly from sporadic cases. They found that after treatment with MNNG *(120–122)* but not after MMS *(123,124)*, AD cells were more sensitive than cells from normals. Apparently consistent with these MNNG results, Li and Kaminskas *(125)* detected by alkaline elution a slower than normal rate of repair in MNNG-treated familial AD fibroblast cell lines; however, Robbins' group, when using the same technique on the cell lines of Li and Kaminskas, found no such difference *(119)*. Similar inconsistencies were reported after MMS treatment in that, unlike Robbins and coworkers' results, a greater sensitivity was detected in MMS-treated AD fibroblasts than in normals *(111,126)*. Later studies in the same laboratory using alkaline elution to examine MNNG-treated lymphoblastoid cells from dominantly inherited AD cases predicted—and detected—a repair defect in half the offspring *(127)*. In another alkaline elution study, Boerrigter et al. *(128)* found a repair defect in ethylnitrosourea-treated lymphocytes from familial, but not from sporadic AD cases.

Two groups have compared the activity of the enzyme O^6-MeG transferase (O^6-MeGT) (which removes O^6-MeG from DNA by direct demethylation) in AD and normal cells, by incubating cell homogenates with DNA methylated in vitro by MNU. A very large variation among individuals was revealed, but no significant difference in mean values between AD and normals was found either for lymphoblastoid cells from familial cases *(127)* or for lymphocytes from sporadic cases *(129)*.

In the author's laboratory, a small-scale exploratory investigation was carried out on lymphocytes from AD patients (sporadic cases) treated with MNU or MMS. A range of techniques was used in the hope of overcoming the disadvantages inherent in some of those mentioned above. We examined UDS, viability, sister chromatid exchange (SCE),

extent of methylation, and rate of repair of the main adducts in extracted DNA using high-performance liquid chromatography. We considered that direct measurement of methylated sites in DNA would be preferable to assaying O^6-MeGT: First, adducts other than O^6-MeG could be assayed, and second, repair would have taken place in the living cell within chromatin rather than in a mixture of cell homogenate with methylated pure DNA. We found no evidence by any criterion of a difference in response between AD and normal cells either to MNU or to MMS *(130)*. The only other investigation of chromosome damage was the recent large-scale study by Cherry et al. *(131)*. Stimulated lymphocytes treated with MMS or bleomycin revealed more chromosome breaks in lymphocytes from AD women patients than in those from age-matched normal women, but numbers of breaks were similar in AD men and age-matched normal men; this was attributed to the high level of breaks found in the latter case. Direct comparison with our study is difficult, since stimulated lymphocytes were used by Cherry et al. *(131)* whereas ours were treated before stimulation.

Head Injury as a Possible Factor

After repeated head injury, boxers have been known to develop clinical dementia. Further, numerous NFT have been found in their brains *(132,133)* as well as amyloid deposits *(133)*. Several surveys appear to have found an association between head trauma and AD *(134–138)*, although there has been some dispute about possible bias in the collection of data. Further support for the involvement of head trauma comes from a report *(139)* that deposits of β-amyloid were observable in postmortem brain from 6/16 patients who had suffered head injury; these were detectable even after the remarkably short time of 6–19 d. All the patients were aged <65 yr and so were unlikely to have been preclinical cases of AD.

Head trauma could lead to damage to the blood–brain barrier (BBB), which in turn could permit access of toxins and/or loss of immunological protection *(140)*. In fact, two studies have detected a dysfunction of the BBB in AD patients *(141,142)*.

An Inherited Risk Factor

An important recent finding is that there is an association between a particular allele of the gene coding for the blood protein, apolipoprotein E, and AD. It was discovered that in both familial and sporadic cases of late onset, possession of at least one copy of the E4 allele was far more common than in controls *(143–145)*. Thus, the risk of developing the disease is increased in those who are heterozygous for the apoE4 gene and very greatly increased in those who are homozygous. Nonetheless, by no means all persons possessing one or even two copies of the gene do develop the disease, and conversely, there are a number of AD patients who have no E4 allele; this indicates that other factors or causes must be involved also.

Summary

No firm conclusions can be drawn from any of the voluminous studies on the possible factors discussed above. Aluminum, without any doubt, is a neurotoxin, but presumably the disputes concerning levels in human brain will continue until different groups pool their resources or collaborate more fully. However, in any case, it would be difficult to show whether any accumulation of aluminum in the CNS is causative or merely a consequence of the disease. Studies on animals suffer from the disadvantage of probable species differences in response and, as with studies on cells in culture, they are necessarily short-term compared to the very long period during which humans might be exposed to an environmental agent. Epidemiological surveys on AD and aluminum in water (and in toiletries, and so forth) appear to show weak correlations, but like the surveys on head trauma, are subject to criticisms of possible methodological bias.

Viral involvement in AD is equally uncertain. The detection of viral genomes in human brain does not necessarily implicate viruses as agents in a disease; as with aluminum, their presence might be a consequence—or it might be wholly irrelevant. As to the response of AD cells to DNA-damaging agents, and in particular, to ionizing radiation, their susceptibility does appear to be greater than that of normal cells (despite the convoluted findings of various investigations), but again, this could merely be a result of the disease.

It is obviously very difficult to prove that an agent is the "cause" of any noninfectious human disease, especially a disease like AD, which can take many years to become apparent. Nonetheless, progress is clearly being made in elucidating some features of this devastating disease; in the last few

years, a vast amount of information has been obtained about inherited cases, about the nature and origin of senile plaques and NFT, and about some aspects of the interaction of the putative agents with the CNS. The next few years may well be even more fruitful.

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Note Added in Proof

We have very recently found that the probability of occurrence of AD is far greater in persons in whose brain HSV1 is present *and* who possess one or more apoE4 alleles than in those with either characteristic alone (Lin and Itzhaki, to be published). This indicates that HSV1 is indeed a risk factor for AD.

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