Journal of Neuroscience Research 75:565–572 (2004)

Chronic Exposure to Aluminum in Drinking Water Increases Inflammatory Parameters Selectively in the Brain

A. Campbell,[1]* A. Becaria,[1] D.K. Lahiri,[2] K. Sharman,[1] and S.C. Bondy[1]

[1]Department of Community and Environmental Medicine, Center for Occupational and Environmental Health Sciences, Irvine, California

[2]Department of Psychiatry, Institute of Psychiatric Research, Indiana University School of Medicine, Indianapolis, Indiana

A link between aluminum (Al) exposure and age-related neurological disorders has long been proposed. Although the exact mechanism by which the metal may influence disease processes is unknown, there is evidence that exposure to Al causes an increase in both oxidative stress and inflammatory events. These processes have also been suggested to play a role in Alzheimer's disease (AD), and exposure to the metal may contribute to the disorder by potentiating these events. Al lactate (0.01, 0.1, and 1 mM) in drinking water for 10 weeks increased inflammatory processes in the brains of mice. The lowest of these levels is in the range found to increase the prevalence of AD in regions where the concentrations of the metal are elevated in residential drinking water (Flaten [2001] Brain Res. Bull. 55:187–196). Nuclear factor-κB as well as tumor necrosis factor-α (TNF-α) and interleukin 1α (IL-1α) levels were increased in the brains of treated animals. The mRNA for TNF-α was also up-regulated following treatment. Enhancement of glial fibrillary acidic protein levels and reactive microglia was seen in the striatum of Al-treated animals. The level of amyloid beta ($A\beta_{40}$) was not significantly altered in the brains of exposed animals. Insofar as no parallel changes were observed in the serum or liver of treated animals, the proinflammatory effects of the metal may be selective to the brain. Al exposure may not be sufficient to cause abnormal production of the principal component of senile plaques directly but does exacerbate underlying events associated with brain aging and thus could contribute to progression of neurodegeneration.

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Key words: aging; Alzheimer's disease; inflammatory cytokines; amyloid beta; microglial activation

A relationship between aluminum (Al) ingestion and several age-dependent neurological disorders, such as Alzheimer's disease (AD), has long been proposed. In 1976, Alfrey et al. implicated aluminum as the possible agent responsible for the outbreak of an encephalopathy in uremic patients on chronic hemodialysis who routinely received Al-containing phosphate-binding gels (Alfrey et al., 1976). Most of the intravenous feeding solutions used for preterm infants contain aluminum, and infants subjected to prolonged feeding with these mixtures show impaired neurologic development (Bishop et al., 1997). Al-induced encephalopathy has also been reported for renal-failure patients who have undergone bladder irrigation with 1% alum (Phelps et al., 1999). Abnormal neurological symptoms have been observed in several patients receiving intramuscular injections of Al-containing vaccines, and the WHO Vaccine Safety Advisory Committee has recognized that there may be a subset of predisposed individuals who are sensitive to Al-containing adjuvant (Authier et al., 2001).

Although there are no reported cases of acute aluminum toxicity in healthy individuals exposed to normal levels of the metal, several studies have been conducted to assess whether minimal chronic exposure may have long-term health effects. A study by McLachlan et al. (1996) correlates the risk of developing AD with residing in areas where aluminum concentrations in the municipal drinking water are 100 μg/liter or greater. The study further revealed a dose-response correlation between an increasing concentration of Al in the drinking water and a higher risk of developing AD. A more recent study, of elderly French populations exposed to Al in drinking water (100 μg/liter), also found a similar link between exposure and the prevalence of AD (Rondeau et al., 2000). A comprehensive review of 13 reports demonstrated that there is a significant association between residing in areas

Contract grant sponsor: National Institute of Health; Contract grant number: ES 7992; Contract grant number: AG-16794; Contract grant number: AG-018884.

*Correspondence to: Arezoo Campbell, Department of Community and Environmental Medicine, Faculty Research Facility, Irvine, CA 92697-1825. E-mail: aghadimi@uci.edu

Received 27 August 2003; Revised 23 October 2003; Accepted 25 October 2003

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where aluminum concentrations in the municipal drinking water are high and an increase in the incidence of AD (Flaten, 2001). Although the exact mechanism is unknown, the metal has been shown to promote events connected to brain aging.

Senescence of the brain is associated with increased levels of inflammation (David et al., 1997; Streit et al., 1999; Sharman et al., 2002). In age-related neurodegenerative disorders, such as AD, a further enhancement of inflammatory processes is thought to contribute significantly to pathogenic events. This neurodegenerative disease is also characterized by brain depositions of the toxic amyloid β-peptide (Aβ), which is generated from amyloid precursor protein (APP). In the brain of AD patients, reactive microglia producing proinflammatory cytokines and acute-phase proteins are associated with Aβ-containing neuritic plaques (Mrak et al., 1995; Styren et al., 1998). Al promotes Aβ aggregation in vitro (Bondy and Truong, 1999; Kawahara et al., 2001), and dietary exposure to the metal exacerbates oxidative stress, Aβ deposition, and plaque formation in the brains of transgenic mice overexpressing APP (Pratico et al., 2002).

The number of activated astrocytes is increased in AD, and these astrocytes are associated not only with the senile plaques but also witth cerebral microvessels (Cullen, 1997). In the hippocampus of AD patients, there is an up-regulation of proinflammatory genes (Colangelo et al., 2002), and levels of cytokines are elevated in the brain (Zhao et al., 2003) as well as cerebrospinal fluid and plasma (Sun et al., 2003). We have previously reported that Al increases cell proliferation, cytokine secretion, and nuclear factor-κB (NF-κB) activation in human glioblastoma cells (Campbell et al., 2002). To substantiate these in vitro findings, using a more biologically relevant model, the present study evaluated the effect of the metal on inflammatory markers in the brain of mice exposed for an extended period to various concentrations of Al lactate in the drinking water.

MATERIALS AND METHODS

Unless otherwise noted, all chemicals were purchased from Sigma (St. Louis, MO).

Animal Treatment

Two-month-old male B/6C3F1 mice (a hybrid between C57BL/6 and C3H mice) were obtained from Harlan Labs (Indianapolis, IN). They were housed four per cage and maintained on a 12-hr light/dark cycle in a temperature-controlled (20°C ± 1°C) room. There were four groups of animals (one control and three experimental), and each group contained 12 animals. Food and water were provided ad libitum. The drinking water was supplemented with aluminum lactate (0.01, 0.1, 1 mM). The amount of water consumed was recorded twice per week, and the weight of the animals was determined each week. The animals were sacrificed after a 10-week exposure.

Preparation of Samples

Blood was collected and centrifuged at 5,000 rpm for 10 min, and plasma was taken and stored at −80°C. The brain and liver of animals were removed and quickly frozen. Cytoplasmic fractions were prepared using the method of Lahiri and Ge (2000). Tissue from each animal was weighed and homogenized in 2 ml of an ice-cold buffer consisting of [10 mM HEPES (pH 7.9), 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM DTT, 0.5 mM PMSF, and 0.5% NP-40]. The suspension was incubated on ice for 10 min and centrifuged (1,500*g*) at 4°C for 1 min. The supernatant containing the cytoplasmic constituents was collected, and 200 μl of a 5× protein cocktail prepared following the method of Faure et al. (2001) was added. Samples were aliquoted and stored at −80°C.

Northern RNA Analysis

RNA was isolated using the Trizol reagent according to the protocol supplied by the provider (Life Technologies-Gibco BRL, Rockville, MD). Aliquots of total RNA (32 μg each, as determined from absorbance at 260 nm wavelength and verified by gel ethidium bromide fluorescence intensity) were denatured with formaldehyde and formamide, electrophoresed on 1.2% agarose gel containing 6% formaldehyde, and transferred onto nylon zeta-probe blotting membranes (Bio-Rad Laboratories, Hercules, CA). Membrane-bound RNA was then hybridized with the cDNA probe labeled with [^{32}P]dCTP using the RTS Radprime System (Life Technologies) to yield a specific activity of approximately 10^9 cpm/μg. Membranes were autoradiographed on X-ray film (X-Omat AR; Kodak, Rochester, NY). A densitometer (Eagle Eye image processor combined with DNA Scan signal analysis software; Stratagene, San Diego, CA) was used to quantify the signals as area-integrated optical density.

Competitive Enzyme Immunoassay

Levels of tumor necrosis factor-α (TNF-α) and interleukin-1α (IL-1α) were determined by using a competitive enzyme immunoassay kit from Neogen Corp. (Lexington, KY) for the detection of the total protein in the cytoplasmic tissue fractions. Briefly, 100 μl of the sample and murine TNF-α or IL-1α antibody were added to plates precoated with goat anti-rabbit antibody and incubated at room temperature for 3 hr. After the addition of murine TNF-α or IL-1α conjugate, the plate was incubated for a further 30 min. The plate was then washed and incubated for 30 min with streptavidin-alkaline phosphatase. After another wash, 200 μl of a color reagent solution was added to the plate, and the color generated was determined with a spectrophotometric plate reader set at 490 nm.

Immunostaining

Animals were anesthetized with pentobarbital (65 mg/kg, i.p.) and then perfused transcardially with saline, followed by 4% paraformaldehyde. The brains were excised and placed in 4% paraformaldehyde solution. They were treated with a 20% glycerol and 2% dimethylsulfoxide solution to prevent freezing artifacts (Rosene et al., 1986). Sixteen brains, arranged in a 4 × 4 array, were embedded in a gelatin matrix using MultiBrain Technology [Neuroscience Associates (NSA), Knoxville, TN] as previously described (Fix et al., 1996). This matrix encases the samples so that there is no interference with staining. Each block of embedded tissue was rapidly frozen by immersion in isopen-

TABLE I. Weight of Each Animal Before and After Exposure at Different Time Points*

Time	Control	Al (0.01 mM)	Al (0.1 mM)	Al (1 mM)
Before exposure	29.6 ± 0.35	29.2 ± 0.58	30.2 ± 0.40	29.8 ± 0.75
Week 2	31.8 ± 0.92	31.7 ± 0.29	32.5 ± 1.04	32.0 ± 0.92
Week 4	32.4 ± 0.58	32.6 ± 0.29	32.9 ± 1.64	32.7 ± 0.81
Week 6	33.9 ± 0.87	33.3 ± 0.12	34.1 ± 0.69	33.3 ± 1.20
Week 8	34.4 ± 1.10	34.1 ± 0.64	34.5 ± 0.69	33.7 ± 1.92
Week 10	33.9 ± 0.87	33.3 ± 0.12	34.1 ± 0.69	33.3 ± 1.21

*Values are in g ± SE. There were no significant differences among control and various experimental groups.

tane chilled to −70°C with crushed dry ice. Samples were freeze cut at 40 μm using an AO860 sliding microtome. All the sections were collected sequentially and placed in phosphate-buffered formaldehyde. A subset (one section every 960 μm) was used for staining.

For glial fibrillary acidic protein (GFAP) immunohistochemistry, the sections were stained freely floating. After hydrogen peroxide treatment and blocking in serum, the sections were immunostained with a 1:2,000 dilution of primary polyclonal rabbit anti-cow GFAP antibody (Dako, Carpinteria, CA) for 24 hr at 4°C. Samples were then treated for 30 min with a goat anti-rabbit secondary antibody at room temperature. They were conjugated with an avidin-biotin-horseradish peroxidase (HRP) complex for 1 hr at room temperature (ABC Kit; Vector, Burlingame, CA). Sections were treated with diaminobenzidine tetrahydrochloride (DAB) and mounted on gelatinized glass slides.

A silver stain, developed by NSA, was used to visualize the location of reactive microglia (modification from Campbell et al., 1987). This stain does not interact with quiescent microglia. NSA validated this fact by comparing these sections with ED-1- and lectin (ISOB4)-stained samples. Briefly, the sections were placed in freshly prepared 2% ammonium hydroxide for 5 min. They were then placed in a silver-pyridine-carbonate solution for 40 min, followed by incubation in 1% citric acid for 3 min, and were stored in a 0.5% acetic acid solution. The sections were developed (time was visually assessed) in physical developer ABC solution. This was stopped by briefly placing the sections in 0.5% acetic acid. The samples were then mounted on gelatinized glass slides.

All images were captured using a Nikon E5000 digital camera attached to a Nikon Fluophot microscope. To ensure that the entire section was captured in a single frame, a 1.3× objective was utilized. Images were transferred to an IBM computer, and Adobe photoshop was used for creating Figures 4 and 5.

Assay for $A\beta_{40}$ Levels

We used a sensitive ELISA test for measuring levels of $A\beta_{40}$ in the mouse brain samples as briefly described below by using modifications from the IBL kit similar to the previously described assay (Lahiri et al., 1998). We employed two kinds of highly specific antibodies to detect $A\beta_{40}$ species: 1) an affinity-purified anti-Aβ rabbit IgG as a capture antibody and 2) an affinity-purified HRP-conjugated anti-Aβ rabbit IgG Fab as a detection antibody. TMB was used as the coloring agent (Chromogen). The intensity of coloring was in proportion to the quantities of $A\beta_{40}$. Under our laboratory conditions, the detection range was from 15.6 to 1,000 pg/ml, which is equivalent to 3.6–230.9 pmol/liter. Levels of $A\beta_{40}$ that we detected in our samples ranged from 158 to 162 pg/mg protein (see Fig. 6).

Statistical Analysis

The difference among groups was assessed using one-way ANOVA, followed by the Tukey test. Results were considered significant at $P \leq .05$.

RESULTS

Three different exposure levels of Al in drinking water were used to study the dose-response effects on inflammatory parameters in mouse brains. The lowest concentration was similar to levels found in the drinking water of certain districts of Ontario, Canada. These levels (0.0037–0.0124 mM) have been correlated with an increase in the prevalence of AD (Flaten, 2001). Because epidemiological studies show that long-term exposure to the metal is necessary for adverse health effects, a 10-week exposure period was selected. In view of the longevity of mice compared with humans, this is approximately equivalent to an 8-year human exposure. The presence of Al in the drinking water did not affect consumption volume and, insofar as weight gain did not differ between groups, did not lead to overt systemic toxicity (Table I).

Increases in NF-κB activation were significant in the cortices of animals treated with the lowest dose of aluminum but not the higher levels (Fig. 1). In contrast, TNF-α mRNA was significantly increased only in the brains of animals exposed to the two highest concentrations of Al (Fig. 2). The cortical levels of the TNF-α and IL-1α proteins were also increased. However, this enhancement reached significance only at higher exposures (Fig. 3).

To localize the sites of the inflammatory response, mouse brains were sectioned and stained for both reactive microglia and astrocytes. Elevated GFAP is an index of astrocyte activation. An increase in the level of GFAP staining was seen in the striatum and globus pallidus (Fig. 4) but was not as obvious in the hippocampus of animals treated with Al lactate (data not shown). With a parallel stain specific for microglial activation, a similar pattern was observed (Fig. 5). There was a pronounced increase in both markers of gliosis in the corpus callosum of treated animals (Figs. 4, 5). Although specific inflammatory markers were increased in the brains of exposed animals, levels of $A\beta_{40}$ were not different between the groups (Fig. 6).

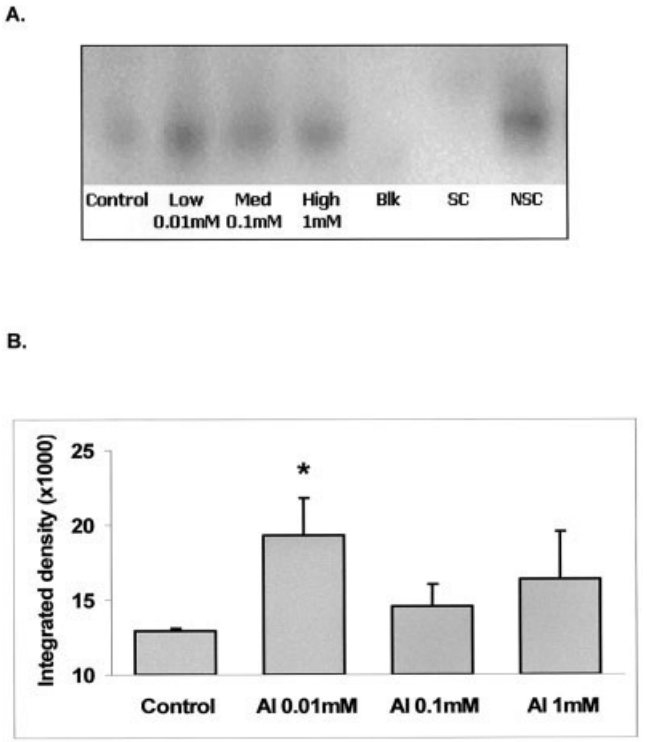

Fig. 1. NF-κB activation in the brain of mice exposed to Al lactate in drinking water for 10 weeks. **A:** Nuclear fractions were incubated with ^{32}P-labeled oligonucleotides containing the NF-κB consensus sequence, and the shifted band is shown. SC, specific competitor [unlabelled NF-κB consensus nucleotide and nuclear fraction from the brains of Al (1 mM)-exposed mice]; NSC, nonspecific competitor [unlabelled SP1 consensus nucleotide and nuclear fraction from the brains of Al (1 mM)-exposed mice]. **B:** The integrated density of the shifted band. ⋆Significantly different ($P < .05$) from the control. Bars represent mean of six individual determinations ± SE.

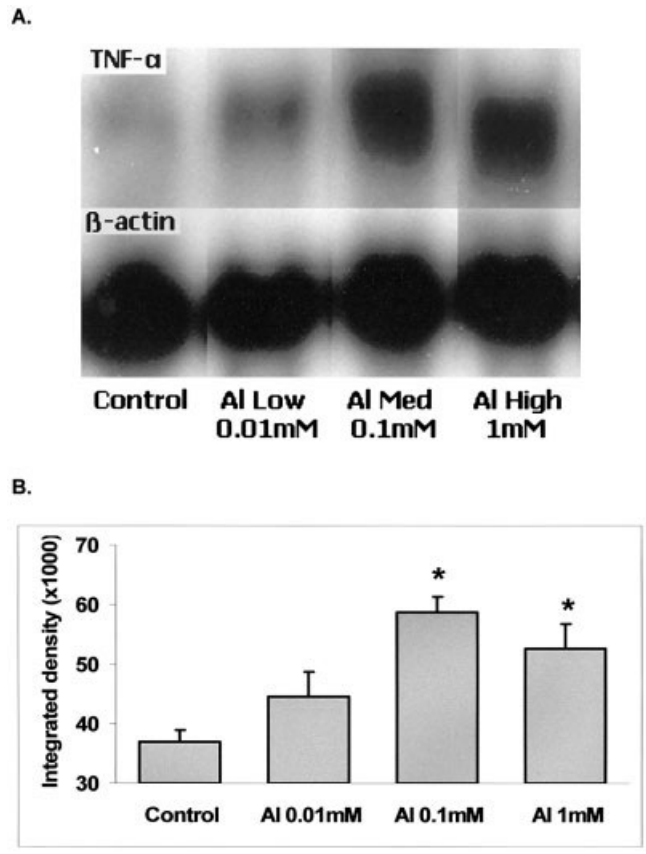

Fig. 2. **A:** Northern blot of TNF-α in the brains of Al-exposed mice. β-Actin was used to ensure equal loading of all samples. **B:** Integrated density of the blots. β-Actin intensity was used to adjust the values. ⋆Significantly different ($P < .05$) from the control. Bars represent mean of three individual determinations ± SE.

DISCUSSION

Al is used as a coagulant for purification of drinking water and as a food additive. Thus, the most common form of human exposure to Al is absorption through the gastrointestinal tract. The rate of absorption is approximately 0.22% (Priest et al, 1998), and, once in the blood, approximately 90% of the metal is bound to transferrin (Harris and Sheldon, 1990; Cabezuelo et al., 1997). Al can pass the blood–brain barrier by receptor-mediated endocytosis of the Fe-carrier protein (Yokel et al., 1999), and in rats approximately 0.005% of the metal enters the brain (Yokel et al., 2001). Al lactate exposure, in comparison with other Al salts, has a greater potential for increasing the metal content of the cerebral cortex (Testolin et al., 1996). In the present study, although there were dose-dependent changes in several inflammatory parameters, no changes in gross brain Al levels were observed (data not shown). This may be because the levels were too low to detect.

One of the initial events in the cascade leading to inflammatory responses is the activation of transcription factors. Levels of activated NF-κB, which is known to be immune related, were found to be significantly increased in the brains of mice treated with the lowest concentration of Al lactate. Once transcription factors are activated, they translocate to the nucleus and there bind to promoter regions of specific genes. NF-κB is known to accelerate the transcription of many immune-related genes, including proinflammatory cytokines such as TNF-α. A significant increase in TNF-α mRNA was found in the brain of animals exposed to the two highest concentrations of Al. This is in agreement with a prior study reporting that the level of the mRNA for this cytokine was increased in the brains of Al-exposed animals (Tsunoda and Sharma, 1999).

TNF-α and IL-1α content showed a modest increase in the brains of all experimental groups, but the values reached significance only at the higher exposures. This pattern of initial activation of transcription factors only in the brain of mice treated with the lowest concentration of Al salt and absent in the brain of experimental groups displaying significant levels of cytokines may be due to negative-feedback control at the level of gene promotion and may reflect the tight control over inflammatory cascades. Furthermore, it is possible that a thresh-

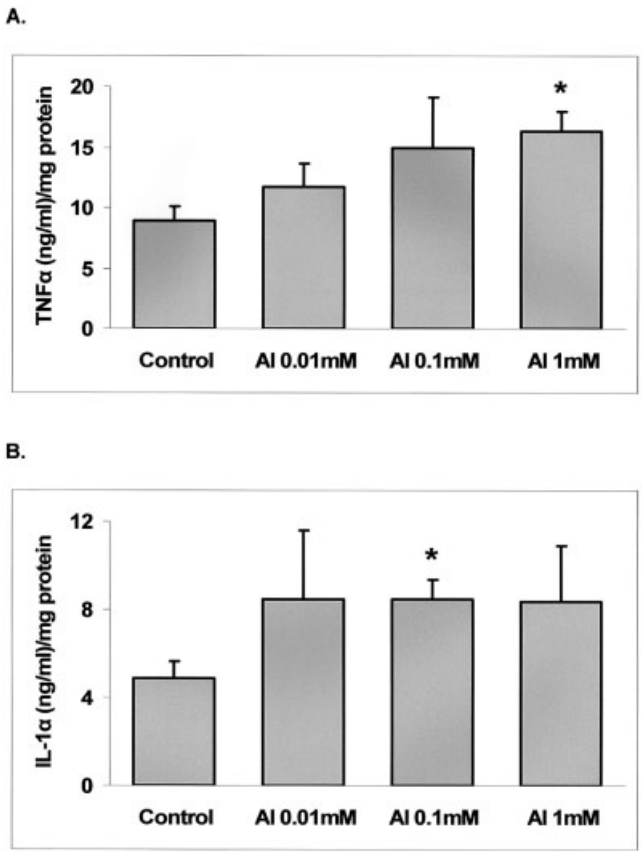

Fig. 3. Levels of TNF-α (**A**) and IL-1α (**B**) in the cytoplasmic fraction of the brain after treatment with Al. *Significantly different ($P < .05$) from the control. Bars represent mean of six individual determinations ± SE.

old concentration of the metal has to be accumulated in the brain before an inflammatory response is even initiated. Changes in cytokine levels were confined to the brain. In neither the liver nor the plasma were levels elevated (data not shown). Thus, the inflammatory effects observed may be mostly confined to nervous tissue. However, insofar as no direct increase in the level of Al was detected in the brain, the effects observed may be mediated by infilteration of proinflammatory mediators from the periphery. It is possible that, although systemic inflammation can be rapidly cleared, the brain is not as capable of adequately resolving an inflammatory foci and that this can lead to chronic inflammation.

A pronounced increase in both astrocyte proliferation and microglial activation was observed in the striatum and globus pallidus of mice exposed to 0.1 and 1 mM Al lactate in drinking water. This parallels a study of rats, in which Al was intracerebroventricularly injected daily for a period of 5 days, and the only region with substantial Al accumulation and gliosis was the striatum (Platt et al., 2001). A case study of a chronic-renal-failure patient, using phosphate-binding Al hydroxy gels for a prolonged period, also found increased proliferation of microglia and astrocytes in the same regions of the brain upon postmortem analysis (Shirabe et al., 2002). This patient had developed Al-induced encephalopathy 9 months prior to death. Although the levels of phosphorylated neurofilaments were increased in the brain, neurofibrillary tangles and amyloid plaques were absent. The regional specificity of the effects observed suggests that the Al-induced proinflammatory changes may play an important role in neurodegenerative diseases, such as Parkinson's disease, in which neuronal cell death occurs predominately in the brainstem as opposed to AD, in which the major degenerative changes are in the hippocampus.

In our experiments, we measured levels of shorter $A\beta_{40}$, which is the most predominant form among all Aβ species. Our results suggest that Al exposure did not lead to an increase in the production of $A\beta_{40}$. Therefore, Al exposure alone may not be sufficient for causing the pathological lesions characteristic of AD. Rather, the metal may contribute to disease progression by accelerating events associated with alterations in a number of biochemical steps. However, because we measured levels of soluble Aβ in brain cytoplasmic fractions, it is still possible that Al might have induced increases in Aβ levels, which were then translocated to the extracellular milieu and there diverted to insoluble Aβ forms. Our system would have missed this store of amyloid beta peptides. Thus, it will be interesting to compare levels of soluble vs. insoluble Aβ. Furthermore, insofar as the sequence of Aβ peptides is different in mice compared with humans, it is possible that the modulation of the peptide by inflammatory mediators might also be distinct in different species. We also attempted to measure levels of the more toxic and larger form of amyloid beta ($A\beta_{42}$), but their levels were too low to be detected in these samples.

Al has been shown to accumulate in lipid-rich white matter (Platt et al., 2001), and this may be due to its selective uptake by oligodendrocytes (Golub et al., 1999). In the present study, a dose-dependent increase in both GFAP and reactive microglia was documented in the corpus collasum, a brain region rich in myelin. This observation may explain the potential for Al to accelerate Fe-induced lipid peroxidation in brain tissue (Xie and Yokel, 1996) and promote prooxidant events (Bondy and Kirstein, 1996; Xie et al., 1996; Bondy et al., 1998).

In the United States, aluminum potassium sulfate is the only approved adjuvant used in human vaccines. Aluminum can enhance the systemic immune response to a concurrently presented antigen (Benjamini et al., 2000). Our results suggest that there may be a parallel between this and the potential for aluminum to promote ongoing inflammatory processes within the brain. We are reporting that Al levels far lower than those previously utilized and more relevant to human exposure can lead to induction of such proinflammatory events. Inflammatory processes are basally elevated within the aging brain, and this may provide the substrate upon which aluminum can act and thus accelerate the progression of age-related neurodegenerative disease.

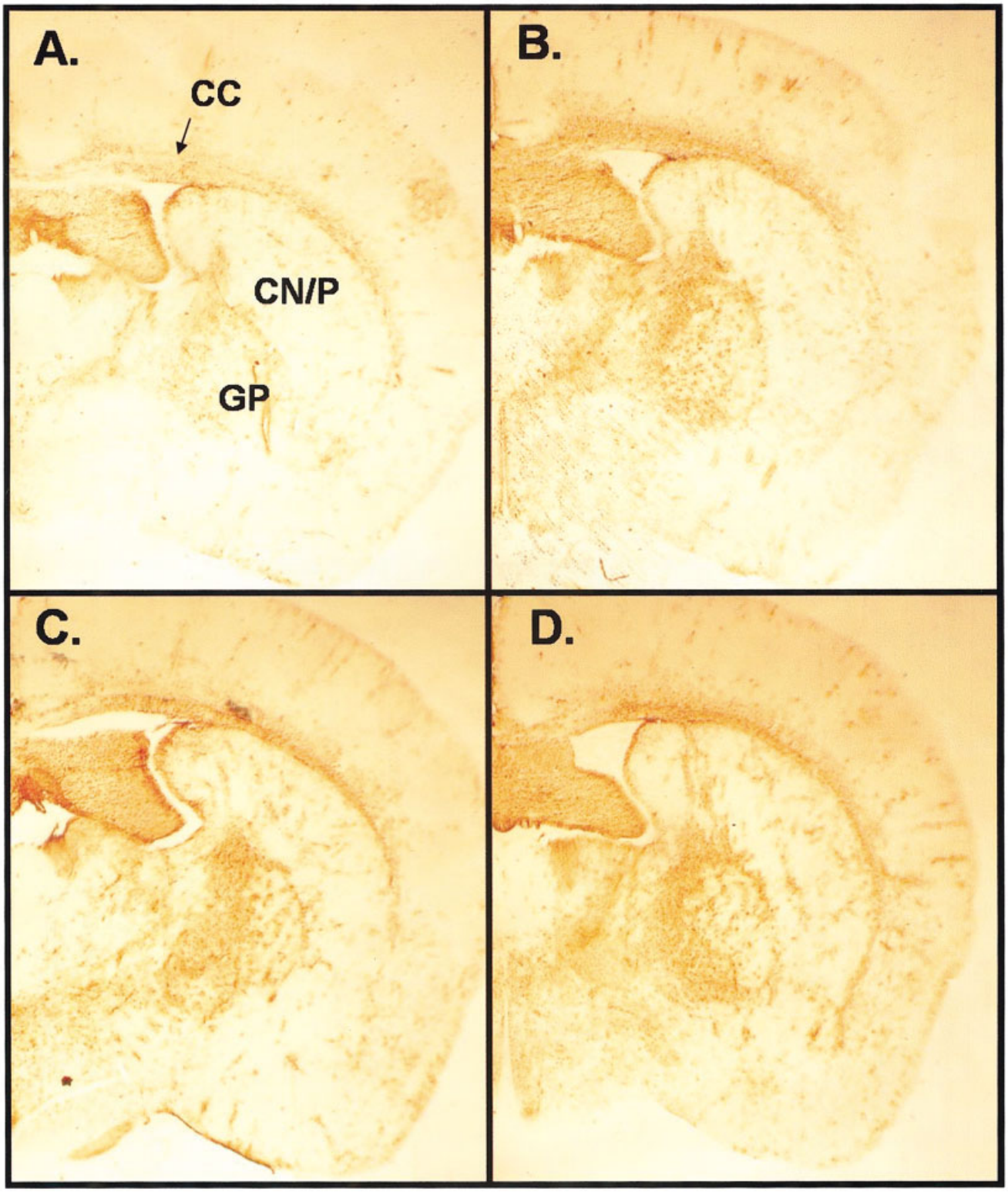

Fig. 4. GFAP staining in the striatum of Al-exposed animals. **A:** Control. **B:** Al (0.01 mM). **C:** Al (0.1 mM). **D:** Al (1 mM). CC, corpus callosum; CN/P, caudate nucleus/putamen; GP, globus pallidus. ×20.

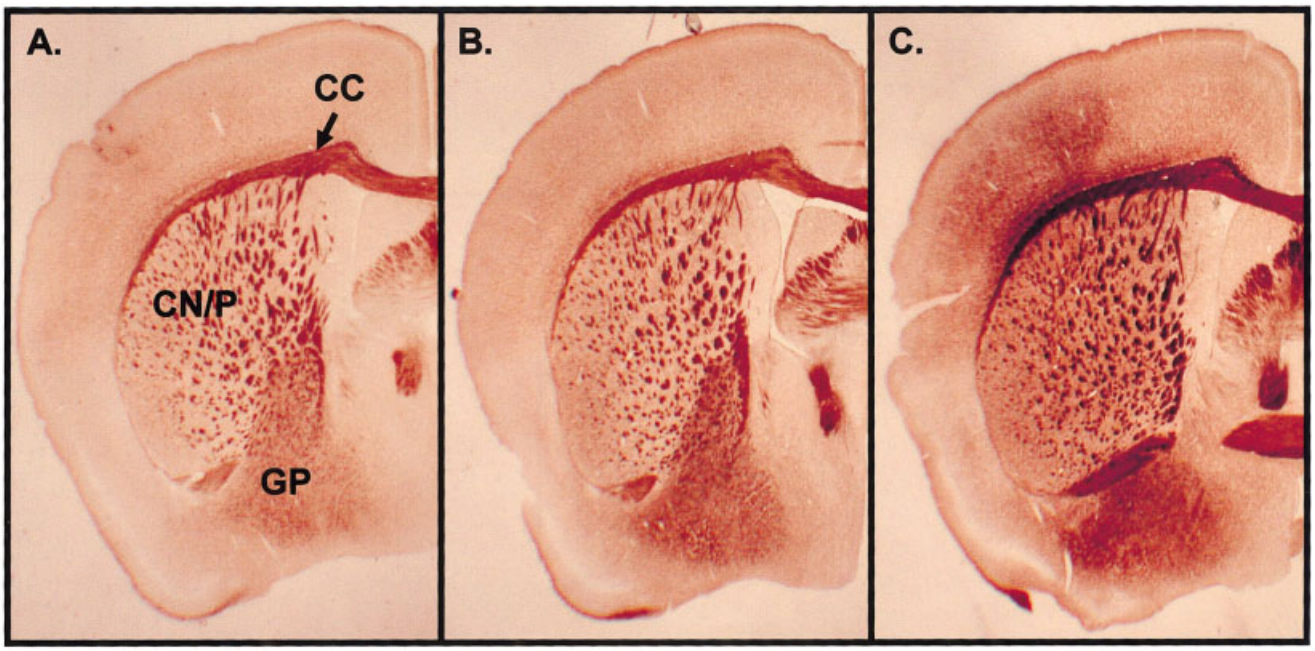

Fig. 5. Microglia activation in the striatum of Al-exposed animals. **A:** Control. **B:** Al (0.01 mM). **C:** Al (0.1 mM). CC, corpus callosum; CN/P, caudate nucleus/putamen; GP, globus pallidus. ×20.

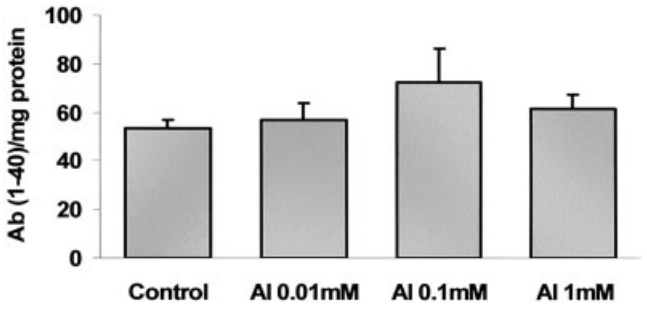

Fig. 6. Levels of $A\beta_{40}$ in the brain of mice exposed to Al lactate in drinking water for 10 weeks. Bars represent mean of six individual determinations ± SE.

ACKNOWLEDGMENTS

We sincerely thank DeMao Chen, Indiana University School of Medicine, Indianapolis, for excellent technical support.

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