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An Aluminum-Induced Increase in GFAP Is Attenuated by Some Chelators

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YOKEL, R. A. AND J. P. O'CALLAGHAN. *An aluminum-induced increase in GFAP is attenuated by some chelators.* NEUROTOXICOL TERATOL **20**(1) 55–60, 1998.—Enhanced expression of glial fibrillary acidic protein (GFAP) has been shown to be associated with gliosis, a generic response of the CNS to neural injury. The effects of aluminum (Al) on regional GFAP concentrations were evaluated to determine potential sites of Al-induced neural injury. Rabbits received 20 Al (100 μmol/kg) or sodium lactate injections over 1 month. Frontal cortical GFAP increased (≈twofold above control) in Al-loaded rabbits, whereas hippocampal and cerebellar GFAP concentrations were not affected. Frontal cortical synaptophysin, neurofilament 68, and myelin basic protein concentrations were then examined in an attempt to determine cell-specific targets of Al neurotoxicity. These proteins were not affected by Al. The ability of chelators to influence brain Al concentrations and the Al effect on GFAP were assessed. Desferrioxamine (DFO) and six 3-hydroxypyridin-4-ones (CPs) were given 12 times, over 1 month, to Al-loaded rabbits. CP24 significantly reduced brain Al. CP93, CP52, and CP24 significantly reduced frontal cortical GFAP. The data suggest an Al-induced gliosis consequent to subtle damage in the frontal cortex and a protective role of some chelators against this CNS injury. © 1998 Elsevier Science Inc.

Aluminum Desferrioxamine Glial fibrillary acidic protein 3-Hydroxypyridin-4-ones Myelin basic protein Neurofilament 68 Rabbit Synaptophysin

CHEMICAL-INDUCED damage of the CNS is known to be revealed by enhanced expression of the astrocytic intermediate filament protein, glial fibrillary acidic protein (GFAP). This response is indicative of toxicant-induced reactive gliosis (20,22,24). In this study we used GFAP as a biochemical indicator of the potential sites of Al-induced neurotoxicity. Aluminum has been implicated as the cause of a neurodegenerative syndrome first described in hemodialysis patients (dialysis encephalopathy, dialysis dementia) (10), and has been suggested to contribute to the etiology of Alzheimer's disease (18).

When our initial measurements revealed an increase in GFAP in the frontal cortex after systemic Al loading, we attempted to elucidate the cellular targets of Al-induced neurotoxicity in this region by measuring proteins specific to selected elements of neurons and oligodendroglia. Thus, we measured the concentration of the neurotypic proteins synaptophysin (SP) and neurofilament 68 (NF-68) and the gliotypic protein, myelin basic protein (MBP). Synaptophysin is a synaptic vesicle localized protein that serves as a marker of nerve terminal damage (3). Neurofilament-68, the 68 kDa (light, low-MW) subunit of neurofilaments, serves as a marker of damage to the axonal cytoskeleton (3,31). Myelin basic protein is a cytoplasmic component of the myelin sheath (30) which, in the adult CNS, can be used as a marker of demyelination.

Aluminum is avidly chelated by desferrioxamine (DFO), which has been reported to reverse Al-induced neurotoxicity in dialysis patients [reviewed in (34)], retard the progression of cognitive impairment in AD patients (6), and partially reverse Al-induced neuropathology in the rabbit (15,29). However, it is unclear if DFO enters the brain (7), whereas the Al chelating 3-hydroxypyridin-4-ones (CPs) (38,40,41) can enter the brain (11). Therefore, we examined whether Al chelators could reduce the Al-induced increase in GFAP. Six CPs, having a wide range of lipophilicity (Fig. 1), were studied to as-

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certain the influence of this property on their ability to reduce brain Al and Al-induced neurotoxicity. The results suggest that systemic Al administration to the rabbit results in cortical gliosis, which can be reduced by some Al chelators.

METHOD

Materials

Aluminum lactate was obtained from Pfaltz and Bauer (Waterbury, CT). Sodium lactate was prepared in our laboratory. The CPs were synthesized as HCl salts in the laboratory of Robert C. Hider (Kings College London, UK) and in our laboratory, as described (9). Their purity was >99.5%, confirmed by HPLC, NMR, and elemental analysis. DFO was a gift of Ciba-Geigy.

Antibodies. Anti-SP was the generous gift of Dr. Paul Greengard, The Rockefeller University, New York, NY; polyclonal anti-GFAP was from Dako (Carpenteria, CA); and monoclonal anti-GFAP, anti-NF-68, and anti-MBP were from Boehringer–Mannheim (Indianapolis, IN). Remaining reagents and their sources have been described (21).

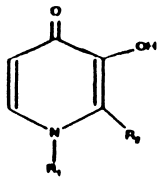
Subjects

The subjects were male New Zealand white rabbits, weighing 2.4 ± 0.2 (mean ± SD) kg at the initiation of the study. Fifty-seven rabbits were Al loaded by 20 IV injections of 100 μmol Al/kg (as the lactate), given into an ear vein 5 days weekly (Monday–Friday) for 4 weeks. This regimen produces tissue Al concentrations and distribution that approximate those seen in the human with dialysis encephalopathy (40) and that moderately decrease in some tissues from 1 to 5 weeks after completion of the injections (36,40). A control group of eight rabbits received equimolar lactate as the sodium salt. Beginning 10 days after the last of the 20 injections, the Al-loaded rabbits were given DFO, one of the six CPs or vehicle (saline) three times weekly for 4 weeks. The DFO was given IV, because it is not absorbed after oral administration, in a dose of 150 μmol/kg. The CPs were given orally, because they have the clinical advantage of being effective by this route, at a dose equal to 450 μmol/kg divided by the oral bioavailability of the CP (39). These doses should produce CP blood concentrations with comparable Al chelation potential to that produced by DFO. The vehicle was given orally. The sodium lactate-injected rabbits were given PO vehicle and serve as controls in this study. Treatments (DFO, CP, or vehicle) were given 24 h after removal of food and placement of an Elizabethan collar on the rabbits to prevent coprophagy. Food was returned 4 h later. All rabbits were euthanatized 24 h after the last chelator or vehicle dosing by pentobarbital anesthesia and carbon dioxide asphyxiation. The brain was removed and stored frozen (−10°C) for later dissection to obtain samples (≈50 mg) of frontal cortex, hippocampus, and cerebellum for determination of neurotypic and gliotypic proteins. A frontal cortical sample (≈400 mg) was obtained for Al determination.

The present results are part of a larger study in which there were initially seven to nine subjects/group. Nine rabbits died during treatment. Details, including chelator-induced Al mobilization and excretion and other assessments of toxicity in these rabbits, have been reported (41).

GFAP Immunoassay

The frontal cortical, hippocampal, and cerebellar samples were weighed and then homogenized in hot (90–95°C) sodium dodecyl sulfate (SDS). GFAP was assayed by minor modifications of a sandwich ELISA (21). Briefly, a rabbit polyclonal



CP code	R_1	R_2	$D_{o/a}$
CP40	$-(CH_2)_2OH$	$-CH_3$	<0.002
CP20	$-CH_3$	$-CH_3$	0.094
CP93	$-CH_3$	$-CH_2CH_3$	0.28
CP52	$-(CH_2)_3OCH_2CH_3$	$-CH_3$	0.7
CP24	$-(CH_2)_3CH_3$	$-CH_3$	1.8
CP94	$-CH_2CH_3$	$-CH_2CH_3$	2.1

FIG. 1. Structures of the 3-hydroxypyridin-4-one chelators studied and their lipophilicities, as the equilibrium distribution coefficient ($D_{o/a}$) between *n*-octanol and an aqueous phase at pH 7.4 [from (38)].

antibody to GFAP was coated on the wells of 96-well microtiter plates. Aliquots of the SDS homogenates and GFAP standards, diluted in sample buffer, were then added to the wells of the plate. A mouse monoclonal antibody to GFAP was then added to "sandwich" GFAP between the two antibodies. After addition of an enzyme-linked antibody directed against mouse IgG, substrate was added and the colored reaction product was quantified by spectrometry at 405 nm using a UV Max microplate reader running on a Soft Max program (Molecular Devices, Menlo Park, CA). Total protein in the SDS homogenates was determined as described (32). GFAP values were expressed as μg per mg total homogenate protein. The only modifications to the original protocol were: 1) microplate wells were coated with the rabbit polyclonal antibody to GFAP for 1 h rather than overnight, and 2) dilutions of the GFAP standards and unknowns were performed robotically using a sample processor (Model 5052, Tecan, U.S., Research Triangle Park, NC).

Quantification of Cortical Synaptophysin, Neurofilament 68, and Myelin Basic Protein by PhosphorImager®

Cortex samples (35 μg total protein each) were resolved on SDS-polyacrylamide gels and then electrophoretically transferred to nitrocellulose sheets. The blots were then probed with antibodies directed against SP (1:4000), NF-68 (1:300), and MBP (1:500) followed by sequential incubation with rabbit antimouse IgG and ^{125}I Protein A (4). The exact protocol for these incubations is described (21) under Slot-Immunobinding Protocol. The single bands corresponding to the molecular weights of native SP, NF-68, and MBP were visualized and subsequently quantified by PhosphorImager® analysis of the band volume corresponding to each protein using ImageQuant® software (Molecular Dynamics, Sunnyvale, CA). Arbitrary units of band volume were normalized to percent of corresponding control group.

Aluminum Analysis

Aluminum was determined by electrothermal atomic absorption spectroscopy. The samples were acid digested in a 70:30 HNO_3:H_2O_2 mixture and analyzed for Al by comparison to aqueous standards, using a Perkin–Elmer 4100ZL spectrophotometer. The general procedures have been described (35).

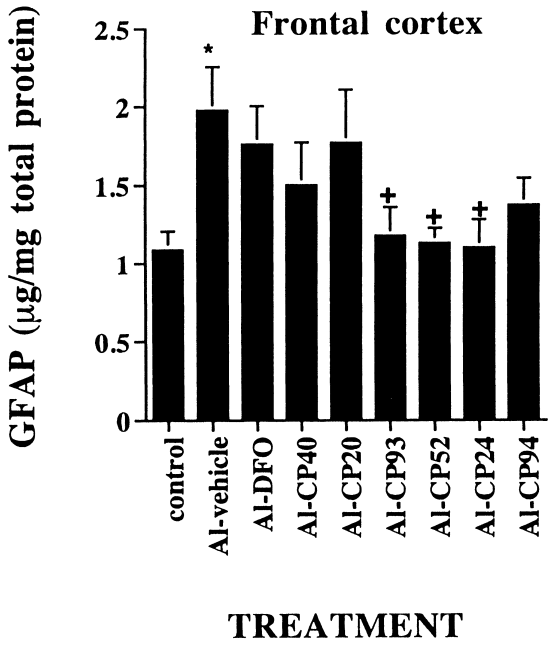


FIG. 2. Concentration of GFAP in the frontal cortex of rabbits after various treatments. Results are mean ± SE from n = 7, 8, 9, 6, 7, 7, 8, 3, and 8 rabbits for the control, Al-vehicle, Al-DFO, Al-CP40, Al-CP20, Al-CP93, Al-CP52, Al-CP24, and Al-CP94 groups, respectively. *Significantly different from control. + Significantly different from Al-vehicle group.

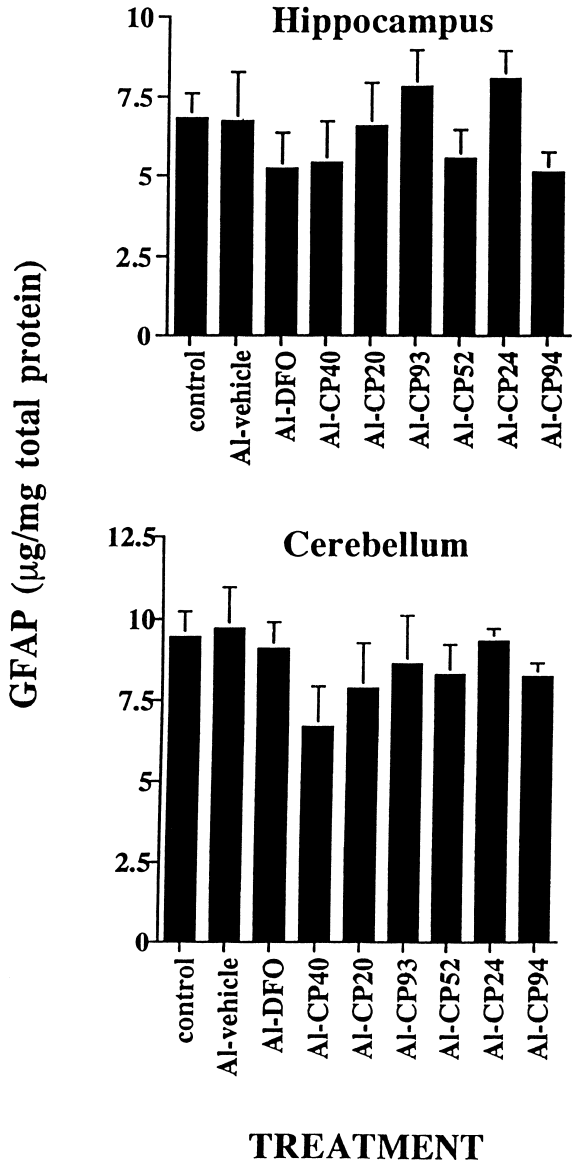


FIG. 3. Concentration of GFAP in the hippocampus and cerebellum of the rabbits shown in Fig. 2. Results are mean ± SE from n = 8 and 8, 7 and 8, 9 and 9, 6 and 6, 6 and 7, 6 and 7, 8 and 8, 3 and 3, and 6 and 8 for the hippocampus and cerebellum for the control, Al-vehicle, Al-DFO, Al-CP40, Al-CP20, Al-CP93, Al-CP52, Al-CP24, and Al-CP94 groups, respectively.

Data Analysis and Statistics

Significant differences in GFAP and Al concentrations among the treatment groups were determined by one-way ANOVAs and a post hoc Duncan's multiple range test after a significant ANOVA. The presence of significant relationships between frontal cortical GFAP and CP lipophilicity, between frontal cortical GFAP and Al concentration, and between Al concentration and CP lipophilicity were determined by correlation analyses. Significance was accepted at $p < 0.05$ for all tests.

RESULTS

The repeated Al injections, in the absence of subsequent chelator treatments, significantly increased frontal cortical GFAP ≈80% (Fig. 2) without affecting hippocampal or cerebellar GFAP concentrations (Fig. 3) approximately 40 days after completion of the Al injections. Repeated treatment with three of the four most lipophilic 3-hydroxypyridin-4-ones (CP93, CP52, and CP24) significantly reduced frontal cortical GFAP (Fig. 2). The ability of the CP chelators to reduce GFAP did not correlate with their lipophilicity. There were no significant effects of desferrioxamine or 3-hydroxypyridin-4-one treatment on hippocampal or cerebellar GFAP (Fig. 3). Aluminum loading did not significantly influence the neurotypic (synaptophysin and neurofilament 68) or other gliotypic (myelin basic protein) proteins in the frontal cortex (Fig. 4). The more lipophilic CPs (CP93, CP52, CP24, and CP94) reduced frontal cortical Al concentration, although only CP24 produced a significant decrease (Fig. 5). The correlation between lipophilicity and frontal cortical Al concentration was not statistically significant. Frontal cortical GFAP and Al concentration significantly correlated among the Al-loaded groups ($r = 0.75$, $t = 2.77$).

DISCUSSION

Aluminum has been implicated in the etiology of Alzheimer's disease. A prominent feature of Alzheimer's disease is marked cortical gliosis, evidenced by an increase in GFAP immunoreactivity (2), GFAP content (8,27), and GFAP mRNA (28). Elevation in these biomarkers of gliosis is also a common response at sites of brain damage caused by neurotoxic chemicals (16,20,22). The present results demonstrate that increased brain levels of GFAP are a feature of systemic Al intoxication. These findings extend a previous report (14) showing an increase in GFAP immunoreactivity after direct application of metallic Al powder to the guinea pig cortex.

The lack of a significant increase in cortical Al approximately 40 days after completion of Al injections is not surprising in light of the rapid decrease in rabbit brain extracellular Al after IV Al lactate injection (37). This decrease is probably due to the active transport of unbound, extracellular Al out of the brain (1).

The Al-induced elevation in GFAP was restricted to the cortex, suggesting damage was limited to this region. This may be due to the higher Al concentrations in the frontal cortex than the hippocampus after Al lactate injection (37). The possibility exists that Al has a direct effect on astrocytes that results in an increase in GFAP. However, astrocyte cultures exposed to 10–5000 μM Al show either a decrease (5,19) or no

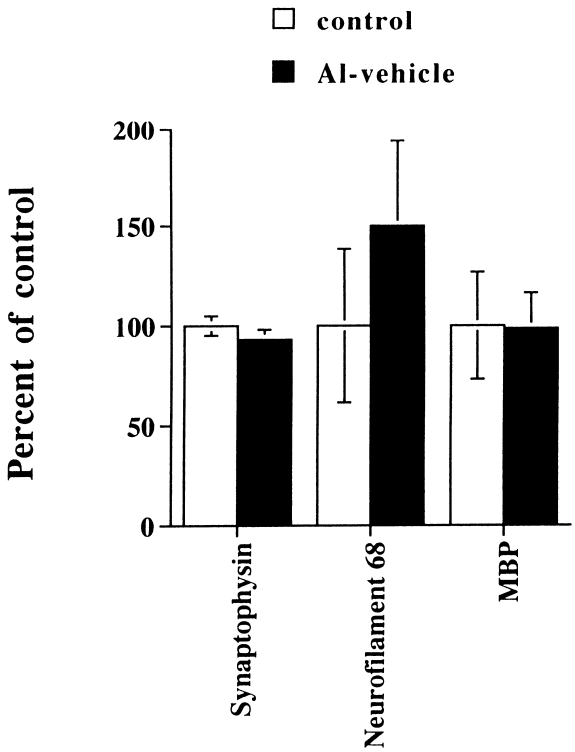


FIG. 4. Concentration of neurotypic (synaptophysin and neurofilament 68) and gliotypic (myelin basic protein [MBP]) proteins in frontal cortex of the control and Al-loaded rabbits shown in Fig. 2. Concentrations (per mg total protein) are expressed compared to the control group mean, which was set at 100%. Results are mean ± SE from seven rabbits in each condition except for MBP in the Al-vehicle group, for which $n = 6$.

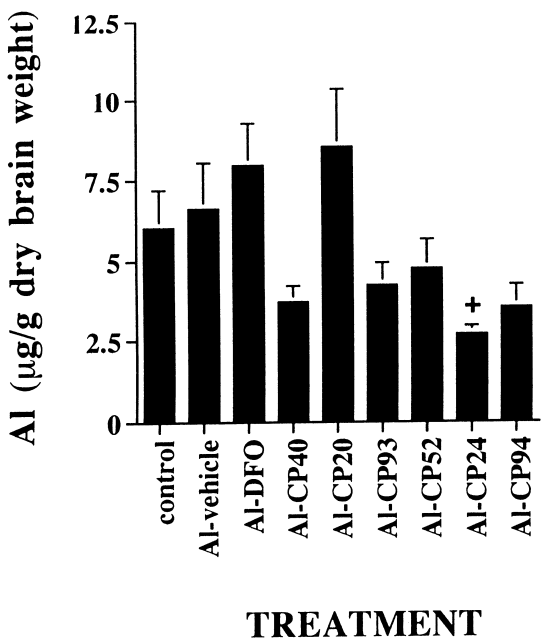


FIG. 5. Aluminum concentration in frontal cortex of the rabbits shown in Fig. 2. Results are mean ± SE from $n = 8, 8, 9, 6, 7, 8, 8, 3$, and 8 rabbits for the control, Al-vehicle, Al-DFO, Al-CP40, Al-CP20, Al-CP93, Al-CP52, Al-CP24, and Al-CP94 groups, respectively. + Significantly different from Al-vehicle group.

change (33) in GFAP, suggesting that the Al effect observed in the present study was due to reactive gliosis rather than a direct effect on astrocytes. Neuronal damage or death resulting from exposure to a toxicant, such as TMT, or from a neurological disease such as Alzheimer's, results in a loss of synaptic proteins such as SP (3,13) and axonal cytoskeletal proteins such as NF 68 (3). Not surprisingly, such generalized evidence of neuronal damage is associated with an ensuing reactive gliosis and a large (11–60-fold) increase in GFAP (3,8). Because the increase in GFAP observed in the present study was modest in comparison, our failure to detect decreases in neuronal (SP and NF 68) and myelin (MBP) proteins was not unexpected. Thus, as was observed for subtle damage resulting from exposure to other neurotoxicants (12,25), enhanced expression of GFAP appears to be a more sensitive indicator of underlying neural damage than are changes in widely distributed neuron- and myelin-localized proteins. As was also the case in previous studies of other neurotoxicants (17,23,24), future investigations of Al-induced neurotoxicity should benefit from the combined use of GFAP analysis and a silver degeneration stain to further define the regions and cell types affected by a given exposure regimen.

The lack of ability of DFO to decrease the Al-induced elevation of frontal cortical GFAP and to decrease Al in the present study compared to the effectiveness of some of the CPs suggests a greater potential for the CPs to reverse brain Al accumulation and its resultant neurotoxicity. These results are consistent with the ability of the CPs and CP·Al complexes to penetrate the blood–brain barrier (BBB) (1,11). The lack of a significant increase of brain Al and GFAP after CP treatments suggests that these chelators do not promote Al redistribution into the brain or enhance Al-induced neurotoxicity, which have been concerns expressed about Al chelators and Al-chelator complexes that cross the BBB.

CP24 was the only chelator tested in the present study that significantly decreased cortical Al, and was one of three CPs that attenuated the Al-induced increase in GFAP. The lack of concordance between reduction of brain metal and attenuation of metal-induced CNS injury (i.e., a decrease in GFAP) has been described. Cadmium chelation with diethyldithiocarbamate abolished the neurotoxic effects of cadmium (i.e., necrosis and increase in GFAP) yet increased brain cadmium (26). Thus, chelation therapy can effectively reduce neurotoxicity without significantly reducing brain metal. The lack of significant correlation between CP lipophilicity and the CP-induced decrease in GFAP and frontal cortical Al concentration suggests that selection of the CPs for Al chelation should be based on their efficacy and toxicity profile rather than their lipophilicity.

In summary, results of this study suggest that the frontal cortex warrants more attention as a site of Al neurotoxicity. The ability of 3-hydroxypyridin-4-ones to reduce Al-induced neurotoxicity after their oral administration encourages further investigation of their ability to treat the complications of Al accumulation disorders.

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