Aluminum Increases Levels of β-Amyloid and Ubiquitin in Neuroblastoma But Not in Glioma Cells (44507)

A. CAMPBELL,* A. KUMAR,† F. G. LA ROSA,† K. N. PRASAD,† AND S. C. BONDY*,[1]

Department of Community & Environmental Medicine, Center for Occupational and Environmental Health, University of California, Irvine, California 92697–1820; and †Center for Vitamins and Cancer Research, Department of Radiology, University of Colorado Health Sciences Center, Denver, Colorado 80262

Abstract. **Several epidemiological studies suggest the involvement of aluminum (Al) in the pathogenesis of Alzheimer's disease (AD). There is an increase in the levels of Aβ and ubiquitin in the pathological lesions of AD. Therefore, we have investigated whether aluminum (Al) treatment alters the levels of Aβ and ubiquitin in murine neuroblastoma (NBP2) and rat glioma (C-6) cell cultures. At a low concentration (10 μ*M*), aluminum sulfate stimulated the level of immunoreactive Aβ and ubiquitin in NBP2 cells without changing the levels of the amyloid precursor protein (APP). However, at higher concentrations (100 and 500 μ*M*), aluminum failed to elicit any significant effect on β-amyloid, whereas ubiquitin levels continued to increase. No changes in the Aβ and ubiquitin content were found in the C-6 glioma cells following treatment with Al at any of the concentrations tested. Exposure of cells to aluminum salts did not alter the rate of proliferation in either of the two cell lines. These data suggest that one of the mechanisms by which Al may play a role in AD is by promoting the formation of Aβ and ubiquitin in neurons.** [P.S.E.B.M. 2000, Vol 223:397–402]

It has been proposed that aluminum plays a role in the etiology of Alzheimer's disease (AD) (1). This hypothesis is based on epidemiological studies (2–4) and the observation of aluminum (Al) deposits in neuritic plaques (5). However, the issue is very controversial, and the involvement of this metal in neurodegenerative disease is as yet unproven (6, 7).

Amyloid precursor protein (APP) is a transmembrane glycoprotein composed of a large extracellular domain, a short transmembrane domain, and a cytoplasmic tail (8). There are several isoforms, which are generated by tissue-specific splicing of a gene located on the human chromosome 21. β-Amyloid (Aβ) peptide, generated by splicing APP, is one of the major components of senile plaques that are one of the hallmarks of Alzheimer's disease (9, 10). in addition to Aβ, an increase in the level of ubiquitin has been implicated in AD (11, 12). In view of the possibility that aluminum may contribute to the pathogenesis of AD, the study of the role of this metal in regulating levels of Aβ and ubiquitin was studied. The availability of murine neuroblastoma (NBP2) and rat glioma (C-6) cell lines provides a unique opportunity to investigate whether Al is able to modulate the levels of Aβ and ubiquitin and to inquire what cell types may respond in this manner.

Materials and Methods

Cell Culture. Murine neuroblastoma cells (NBP2), developed in our laboratory (13), containing both tyrosine hydroxylase and choline acetyltransferase, were used in this study. These cells were grown in F-12 medium containing 10% γ-globulin-free newborn bovine serum (Gibco, heated at 60°C 1 hr prior to use), penicillin (100 units/ml), and streptomycin (100 units/ml). They were maintained at 37°C in a humidified atmosphere of 5% CO_2. Rat glioma (C-6) cells of astrocytic origin (14) were grown in RPMI containing 10% fetal calf serum with the same amounts of antibiotics described for NBP2 cells. In all experiments, mycoplasma-free cultures were used.

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[1]To whom requests for reprints should be addressed at Department of Community & Environmental Medicine, University of California, Irvine, CA 92697–1820. E-mail: scbondy@uci.edu

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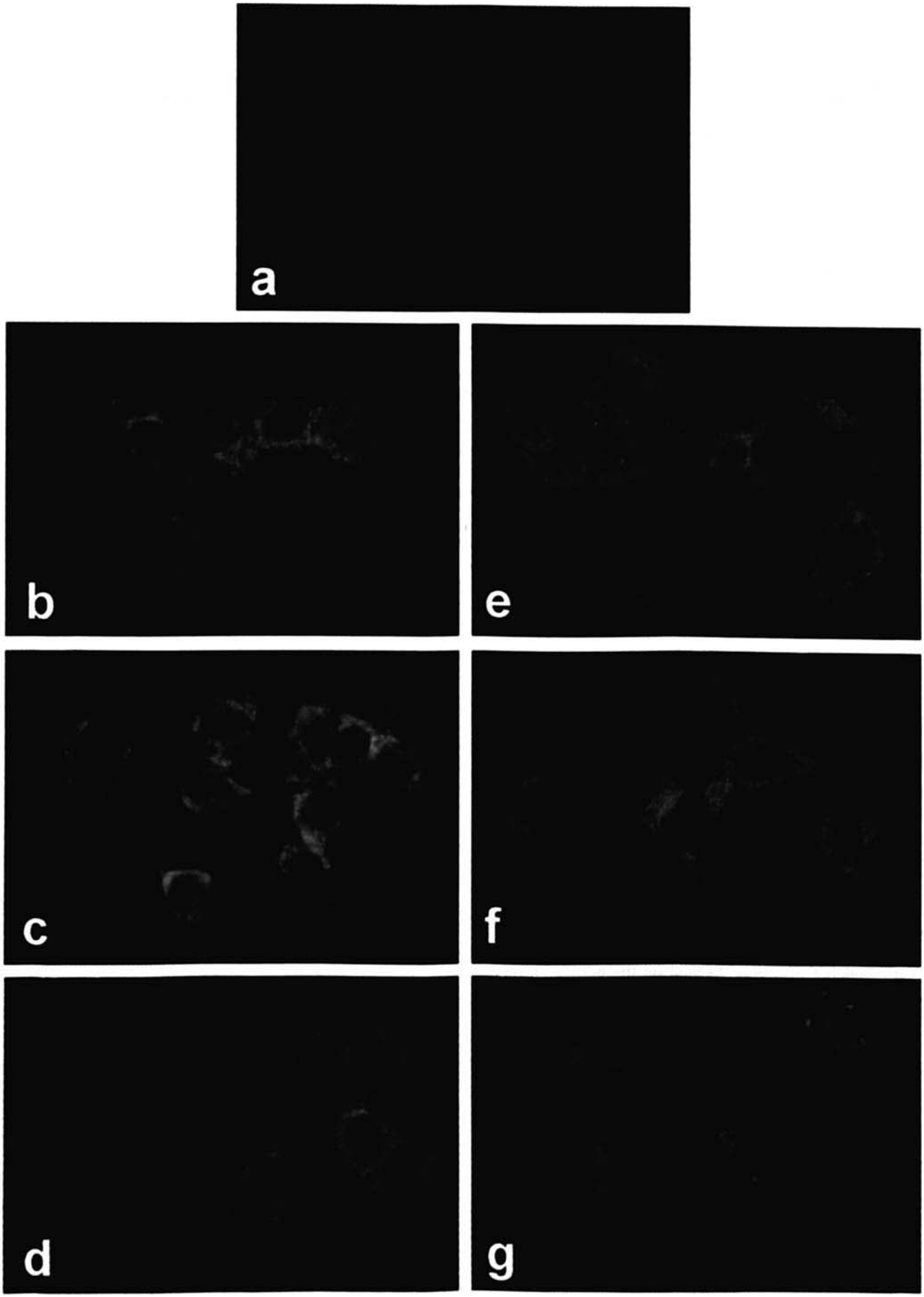

Figure 1. Immunofluorescent staining of neuroblastoma cells with the primary antibody to Ab_{1-14} and ubiquitin, 2 days after treatment with $Al_2(SO_4)_3$. (a) Neuroblastoma cells (NBP2) without the primary antibody did not stain. The cytoplasm of untreated control NBP2 cells was stained with both (b) Ab_{1-14} and (e) ubiquitin antibodies. Treatment of cells with 10 μM $Al_2(SO_4)_3$ increased the intensity of both (c) Ab_{1-14} and (f) ubiquitin staining. (d) Staining of NBP2 cells, treated with 100 μM $Al_2(SO_4)_3$, was not significantly increased above the basal level, but (g) there was a further increase in ubiquitin staining. Magnification: x400

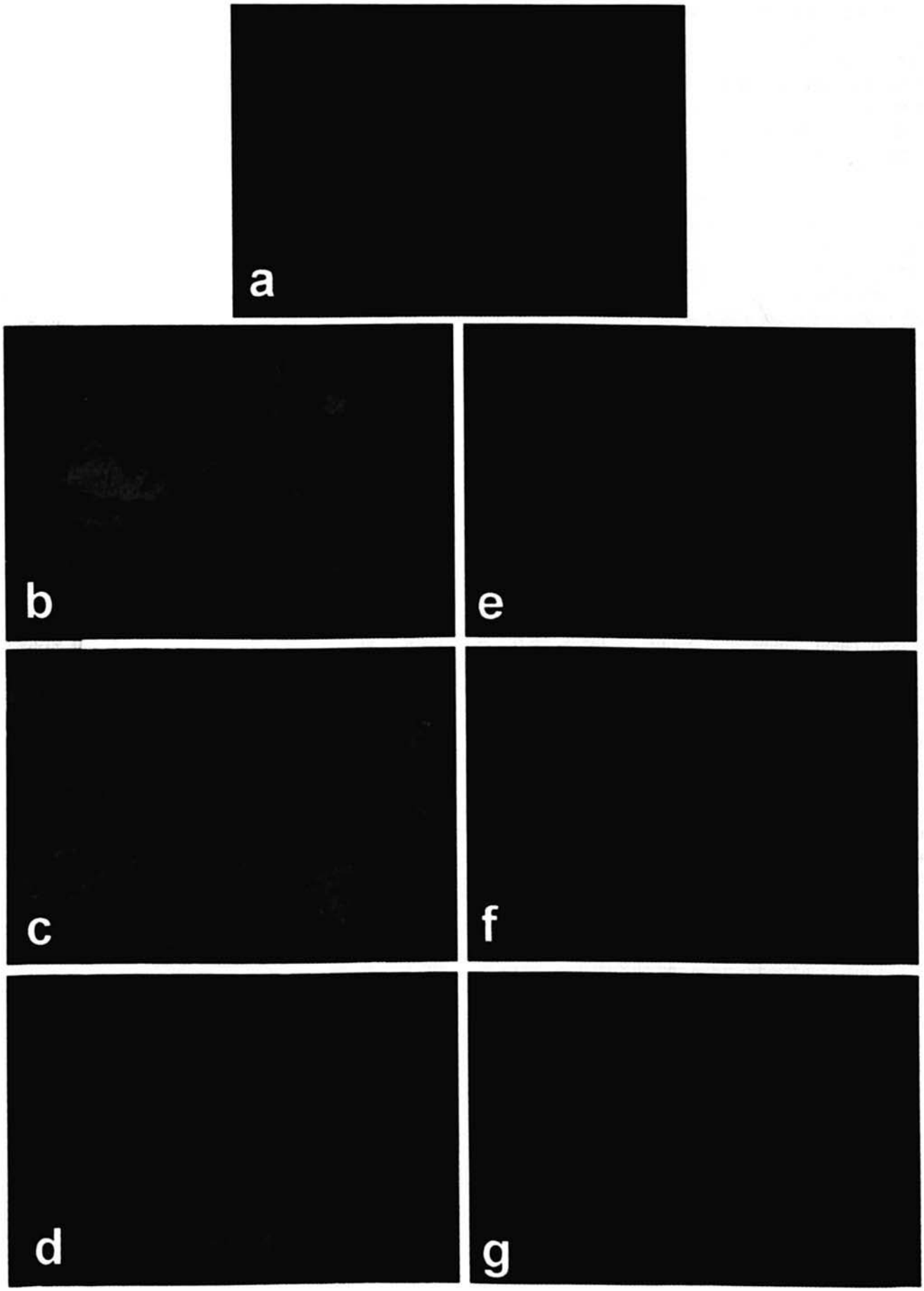

Figure 2. Immunofluorescent staining of C-6 glioma cells with the primary antibody to Ab_{1-14} and ubiquitin, 2 days after treatment with $Al_2(SO_4)_3$. (a) Glioma cells without the primary antibody did not stain. (b) The cytoplasm of untreated control glioma cells was stained with both Ab_{1-14} and (e) ubiquitin antibodies. (c) Treatment of cells with 10 μM $Al_2(SO_4)_3$ did not alter the intensity of staining with Ab_{1-14} or (f) ubiquitin antibodies. (d) A higher concentration of $Al_2(SO_4)_3$ (100 μM) did not alter the levels of Ab_{1-14} or (g) ubiquitin. Magnification: x400.

Cell Growth Assay. Growth was measured as the sum of the changes in the rate of cell proliferation and cell death. Aluminum sulfate was dissolved in double-distilled water. To study the effect of aluminum on growth, cells were plated in tissue culture dishes (60 mm). Aluminum sulfate at concentrations of 10, 100, and 500 μM was added to these cultures 1 day after plating the cells. The growth medium containing aluminum was replaced with fresh Al-free medium 2 days after treatment. The number of cells per dish was determined after a further 24-hr incubation using a Coulter counter. Cell number in experimental groups was expressed as percentage of control values.

Immunostaining. Neuroblastoma cells and glioma cells (5000 cells) were plated in each chamber of a 4-chamber glass slide, and aluminum sulfate was added at concentrations of 0, 10, and 100 μM 1 day later. The growth medium containing Al was changed to Al-free medium 2 days after treatment. Cells were immunostained with the primary antibody to $A\beta_{1-14}$ (rabbit antiβ-amyloid, prepared from a synthetic peptide corresponding to the first 14 amino acids of the N-terminal sequence of β-amyloid). This recognizes only Aβ fragments, although it cannot distinguish between $A\beta_{1-40}$ and $A\beta_{1-42}$. Cells were also stained with APP_{44-63}, the epitope of which lies within the C-terminal amino acids 44–63 of full-length APP (Chemicon International, Inc., Temecula, CA). This antibody recognizes full-length APP. The primary antibody to ubiquitin (polyvalent antibody rabbit antiubiquitin, Sigma, St. Louis, MO) was also used to assess changes in the level of the protein after Al treatment. The immunostaining was performed as described previously (15). The subcellular distribution and extent of staining were documented by photomicrographs. Each experiment was repeated four times.

Image Analysis of Immunofluorescence. The quantitative analysis of immunofluorescence was performed using a digital image analysis system (Imaging Research, Inc., St. Catharines, Ontario, Canada). This system is composed of a computer and image analysis software interfaced with a charge coupled device digital (CCD) video camera. The software enables a variety of image analysis and quantitation procedures to be performed on several image formats including autoradiographs and photographs. Analysis of immunofluorescence was conducted by digitizing the images under identical conditions of illumination, lens aperture setting, and magnification. Relevant areas on the image were selected either manually or by using an autoscan feature of the software. At least three representative areas from the images were analyzed for each photograph. Integrated optical density (IOD) was obtained in numerical form. Mean IOD values between control and treatment groups were compared by one-way Analysis of Variance followed by Fisher's Least Significant Difference Test. The acceptance level of significance was $P < 0.05$ using a two-tailed distribution.

Results

Effect of Al on the Growth of Neuroblastoma and Glioma Cells. Aluminum, at concentrations of 10 and 100 μM, did not significantly alter the growth of either the NBP2 or the C-6 glioma cells.

Effect of Al on β-Amyloid Levels in Neuroblastoma and Glioma Cells in Culture. To ascertain the specificity of fluorescence, NBP2 cells, which were not treated with the primary antibody to $A\beta_{1-14}$, were examined. These cells showed no fluorescent staining (Fig. 1a). Control NBP2 cells expressed basal levels of cytoplasmic staining with the primary antibody to $A\beta_{1-14}$ (Fig. 1b). NBP2 cells treated with 10 μM $Al_2(SO_4)_3$ exhibited a pronounced increase in staining with the antibody to $A\beta_{1-14}$ (Fig. 1c; Table I) when compared with the untreated controls. On the other hand, neuroblastoma cells treated with 100 μM $Al_2(SO_4)_3$ did not reveal any significant increase in β-amyloid (Fig. 1d). The quantitative image analysis of immunofluorescence revealed that the level of staining found after exposure to Al increased only at an Al concentration of 10 μM (Table I). However, the level of staining with APP_{44-63} did not show any significant changes after treatment with $Al_2(SO_4)_3$ (unpublished data).

Rat C-6 glioma cells without the primary antibody for $A\beta_{1-14}$ did not show any fluorescence (Fig. 2a). Untreated glioma cells exhibited cytoplasmic staining with both $A\beta_{1-14}$ (Fig. 2b) and APP_{44-63} (unpublished data). Treatment of glioma cells with either 10 or 100 μM $Al_2(SO_4)_3$

Table I. Levels of Immunoreactivity of β-Amyloid and Ubiquitin in Neuroblastoma (NBP2) and Glioma Cells (C-6) in Culture Following Exposure to Various Concentrations of Aluminum Sulfate

Cell type	$Al_2(SO_4)_3$ conc. (μM)	Antibody type	Integrated relative optical density
Neuroblastoma	0	β-amyloid	147 ± 10
Neuroblastoma	10	β-amyloid	174 ± 2[a]
Neuroblastoma	100	β-amyloid	141 ± 8
Neuroblastoma	0	Ubiquitin	126 ± 14
Neuroblastoma	10	Ubiquitin	143 ± 1
Neuroblastoma	100	Ubiquitin	158 ± 11[a]
Glioma	0	β-amyloid	100 ± 6
Glioma	10	β-amyloid	105 ± 4
Glioma	100	β-amyloid	97 ± 4
Glioma	0	Ubiquitin	105 ± 7
Glioma	10	Ubiquitin	103 ± 7
Glioma	100	Ubiquitin	111 ± 13

Note. Cells were immunostained with the primary antibody to β-amyloid peptide. ($A\beta_{1-14}$) or ubiquitin, 2 days after treatment with $Al_2(SO_4)_3$. Digital image analysis (microcomputer imaging device program, MCID) was performed by measuring integrated relative optical density of individual cells. Each value represents an average of 10 cells ± SEM, derived from three separate experiments.
[a] Value differs significantly from the control value $P < 0.05$, one-way Analysis of Variance followed by Fisher's least Significant Difference Test).

did not markedly alter the cytoplasmic staining pattern observed for these two antibodies (Figs. 2c & 2d, respectively; Table I).

Effect of Al on Ubiquitin Levels in Both NBP2 and C-6 Glioma Cells. Control NBP2 cells not treated with the primary antibody to ubiquitin did not show any fluorescence whereas in the presence of the primary antibody, control cells had basal levels of cytoplasmic staining (Fig. 1e). When compared with these cells, the intensity of staining with the ubiquitin antibody was increased after treatment with 10 μM and 100 μM of $Al_2(SO_4)_3$ (Figs. 1f & 1g, respectively). This increase was dose-dependent and only significant at the higher aluminum concentration (Table I).

The cytoplasm of rat C-6 glioma cells was also stained in the presence of the primary antibody to ubiquitin (Fig. 2e). Glioma cells treated with low (10 μM) or higher concentrations (100 μM) of $Al_2(SO_4)_3$ did not show any pronounced change in the intensity of staining with the ubiquitin antibody (Figs. 2f & 2g, respectively; Table I).

Discussion

Although NBP2 and C-6 glioma cells are of tumor origin, they represent separate homogenous populations of cells of neuronal or glial origin, retaining many of the biochemical features of the original untransformed parent cells. A 2-day treatment of NBP2 cells with 10 μM $Al_2(SO_4)_3$ increased the level of immunoreactive Aβ. Although $A\beta_{1-14}$ cannot distinguish between $A\beta_{1-40}$ and $A\beta_{1-42}$ fragments, it is specific for the Aβ peptide. Western blotting would be able to clarify which isoform increased. The change observed in Aβ content is probably due to increased conversion of APP to Aβ, since under the experimental conditions used, the level of immunoreactive APP did not change. At 100 μM, Al was unable to alter the level of immunoreactive Aβ or APP in NBP2 cells. This may be because Al at high concentrations increases the degradation of Aβ. Indeed, a study has reported that treatment of animals with high concentrations of Al increases the rate of degradation of Aβ in the peripheral circulation (16). Further evidence for this phenomenon is provided by the increase in ubiquitin, a protein involved in the proteolytic degradation of proteins, which is increased after treatment with 100 μM of aluminum sulfate.

According to a study by Neill *et al.* (17), Al treatment does not effect either the expression or the processing of immunoreactive APP in human neuroblastoma cells. This observation is in agreement with our current results since in neither the NBP2 nor the glioma cells were we able to demonstrate any significant changes in immunoreactive APP content. However, our results demonstrated that exposure of the neuroblastoma cell line to low levels of Al increases Aβ levels whereas the former study did not measure this parameter. Our laboratory and two other groups have shown that aluminum causes aggregation of physiological concentrations of Aβ (18–20).

Ubiquitin is a highly conserved protein composed of 76 amino acids. One of its major functions is to tag proteins, preparatory to proteolytic destruction. The main mechanisms for the control of many cellular responses such as embryogenesis, metabolic control, and signal transduction pathway, require protein degradation (21). In addition to the increase in Aβ level, a 2-day treatment of NBP2 cells with aluminum also increased the level of immunoreactive ubiquitin in a concentration-dependent manner. Since aluminum treatment did not significantly alter the growth of NBP2 cells in culture, the increased levels of Aβ and ubiquitin were not associated with an increase in the rate of cell proliferation. The function of Aβ in undifferentiated proliferating NBP2 cells may be different from that in nondividing differentiated neuronal cells. We have reported earlier that the induction of increased levels of Aβ and ubiquitin in differentiated cells occurs before degeneration and death (22). It would be of interest to repeat the current study with nonproliferating differentiated NBP2 cells.

Aluminum treatment of glioma cells at any concentrations examined did not affect the intensity of staining of immunoreactive Aβ or ubiquitin. We have shown in a previous study that exposure to aluminum increased oxidative events in glioma but not NBP2 cells (23). The current finding that Al increased the level of Aβ and ubiquitin only in NBP2 cells, implies that the mechanism by which the metal exerts its effect may be different in the two cell lines.

One of the pathological features of Alzheimer's disease is neurofibrillary tangles (NFT) that are composed of paired helical filaments (PHF) formed by abnormally phosphorylated human tau (PHFτ or A68) protein (24). The presence of aluminum has been demonstrated in NFT of AD patients and elderly controls whereas healthy neurons are shown not to contain the metal (5). To establish the role Al plays in the formation of these tangles, Shin *et al.* injected τ into rodent brains either with or without coinjection of aluminum chloride (25). They found that coinjection of aluminum with PHFτ results in aggregates that lasted longer than the deposits, which are usually formed by the injection of PHFτ alone. These aggregates consisted of codeposits of β-amyloid, ubiquitin, α_1-antichymotrypsin (ACT), and apolipoprotein E (ApoE), all of which are thought to play a role in the pathogenesis of AD. Our results suggested that aluminum exposure can lead to increased levels of Aβ and ubiquitin in cells of neuronal origin. This action of Al may form the basis for the suspected involvement of the metal in AD.

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