

Differential Toxicity of Aluminum Salts in Human Cell Lines of Neural Origin: Implications for Neurodegeneration

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Abstract

Aluminum is highly oxophilic and its minerals are usually found surrounded by six oxygen atoms. A role for the metal has been established in dialysis encephalopathy and Al-induced osteomalacia. The metal has been implicated in Alzheimer's disease but the issue is at present controversial. Human cell lines of neural origin were utilized to study the effect of lipophilic aluminum acetylacetonate and non-lipophilic aluminum sulfate on cell proliferation and viability. Although analysis of Al species in the cell culture media demonstrated that there are positively charged Al species present in solutions prepared with both Al salts, only the aluminum acetylacetonate salt caused changes in cell proliferation and viability. Therefore, the lipophilic nature of the organic Al salt is a critical determinant of toxicity. The effect of aluminum acetylacetonate was dose-dependent and time-dependent. Neuroblastoma (SK-N-SH) cells were more susceptible to decreased cell proliferation although the lipophilic Al salt was more toxic to the glioblastoma (T98G) cells. While the toxicity of aluminum acetylacetonate was inhibited in the T98G cells by the addition of phosphate, the same treatment did not reverse cell death in the SK-N-SH cells. Thus, the mechanism of Al toxicity appears to be different in the two cell lines. It is possible that the principal neurotoxic target of the metal is glial and when these cells are in a compromised state, this may secondarily impact the neuronal population and thus eventually lead to neurodegeneration. © 2001 Elsevier Science Inc. All rights reserved.

Keywords: Aluminum; Neuroblastoma; Glioblastoma; Cytotoxicity

INTRODUCTION

Approximately 8% of the earth's crust is composed of aluminum by weight. In biological systems, the trivalent cation is rarely present as an ion because it forms extensive complexes with biologically available ligands. Since Al has a high charge to radius ratio, it is predicted that the metal prefers ionic rather than covalent bonding (Berthon, 1996). At neutral pH, the salt undergoes extensive hydrolysis and $\text{Al}(\text{OH})_3$ is produced. As the solution ages, $\text{Al}(\text{OH})_4^-$ is also present and this leads to precipitation of Al in solutions (Corain et al., 1996).

Fresh human brain tissue, from subjects who were healthy based on autopsy findings and review of medical history, has been collected and analyzed for aluminum content. The brain contains approximately $0.399 \pm 0.27 \mu\text{g/g}$ dry weight of Al in the gray matter and $0.339 \pm 0.3 \mu\text{g/g}$ dry weight of Al in the white matter (Bush et al., 1995). Until recently, it was generally believed that this burden of aluminum was harmless. However, a direct causal role for the metal has been established in dialysis dementia (Alfrey et al., 1976) and microcytic anemia without iron deficiency (Touam et al., 1983). Aluminum has also repeatedly been implicated in the etiology of neurodegenerative disorders although the issue is controversial (Crapper et al., 1973; Good et al., 1992; Bjertness et al., 1996).

The neurotoxicity of aluminum is directly linked to its bioavailability. Since ingestion of Al from both the

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diet and drinking water is the most common form of human exposure, the main route of Al absorption is through the gastrointestinal tract (Exley et al., 1996). Studies in rats estimate that the total aluminum absorbed is approximately 0.1% of the total aluminum ingested (Jouhanneau et al., 1997). In a recent study, two human volunteers were given a single dose of ^{26}Al in tap water. The rate of absorption was found to be 0.22% (Priest et al., 1998). The bioavailability of Al is dependent on the metal's speciation in an aqueous environment and this in turn is dependent on the anion species to which the metal is complexed with (Smith, 1996). We had previously shown that although a 48 h treatment with aluminum sulfate does not change cell viability, it increases oxidative events in glial but not neuronal rodent cell lines. Since Al is thought to be complexed to lipophilic moieties in biological systems, the current study looks at the effect of aluminum acetylacetonate in a human cell line model. It was found that while aluminum sulfate did not have an effect, aluminum acetylacetonate caused a dose-dependent and time-dependent decrease in the cell viability of the two cell lines tested. It was also established that the lipophilic Al salt is more toxic to the glioblastoma (T98G) cells compared to the neuronal SK-N-SH cells, which suggests that glial cells may be the principal targets of Al-induced neurotoxicity.

MATERIALS AND METHODS

Materials

Human glioblastoma (T98G) cells and human neuroblastoma (SK-N-SH) cells were purchased from the American Type Culture Collection (ATCC). Aluminum acetylacetonate was from Aldrich Chemical Company, Inc. (Milwaukee, WI). The Tris buffer salt was from Boehringer Mannheim (Indianapolis, IN). The cell proliferation and cell viability/cytotoxicity kits were from Molecular Probes, Inc. (Eugene, OR). All other chemicals used were from Sigma Co. (St. Louis, MO). All tissue culture supplies were obtained from GIBCO Co. (Grand Island, NY).

Methods

Cell Maintenance and Growth

Human cell lines were grown in minimum essential medium (MEM) with α modification containing 10% fetal bovine serum. The cells were incubated at 37°C in a humidified atmosphere of 5% CO_2 . They were grown

in 96 well plates coated with poly-lysine (MW: 70,000–150,000). The aluminum and sodium solutions were prepared and sterile filtered prior to dosing and the salts were added at a final concentration of 0, 2, 10, 100 and 500 μM . After dosing, the media was removed and the cells were rinsed twice with sterile 50 mM Tris-HCl buffer at pH 7.4.

Morin Assay

Aluminum and sodium solutions at 10, 100, and 500 μM were prepared and sterile filtered prior to analysis. The levels of positively charged aluminum species were assayed using the morin dye. Morin, which is a negatively charged compound, can selectively bind aluminum and upon binding to the metal, form a fluorescent complex that can be quantitated by a fluorometer (Browne et al., 1990). For the determination of Al concentration in cells, pellets were washed four times with 50 mM Tris buffer at pH 7.4 to remove any traces of the media. The membranes were dissolved by diluting the cells in 2 ml of Tris buffer containing 1% triton. The morin dye (20 μM) was added to each sample. The samples were incubated for 1 h at room temperature. The response was measured using a FL600 microplate fluorescence reader. The wavelength was set at 420 nm excitation and 515 nm emission.

Cell Proliferation Assay

Cells were gently washed with sterile Tris-HCl buffer at pH 7.4 and then frozen overnight at -70°C . The number of attached cells was determined using the CyQuant cell proliferation assay kit (Molecular Probes, OR). Briefly, cells were thawed at room temperature and treated with 200 μl of the dye diluted in cell lysis buffer. The dye rapidly binds to nucleic acids and exhibits strong fluorescence when bound. The plate was incubated at room temperature for 5 min and the fluorescence was measured. Wavelengths were set at excitation 480 nm and emission 520 nm. A standard of cells counted with the hemacytometer was used to determine the actual cell number.

Cell Viability Assay

The viability/cytotoxicity kit (Molecular Probes, OR) was used to measure cell survival after treatment. This assay is based on the simultaneous measurement of the fluorescence of two dyes. Live cells are determined by the retention of the calcein AM dye, which is non-fluorescent and upon enzymatic conversion by ubiquitous intracellular esterase activity, becomes intensely fluorescent. The EthD-1 dye can only enter

cells with damaged membranes and there it binds to nucleic acids and produces a strong red fluorescence. A final concentration of 1 μM calcein AM and 2 μM EthD-1 was used in the assay.

Since 500 μM of aluminum acetylacetonate completely detached cells from the plate, the cells in the supernatant were collected and analyzed for percent viability. Upon addition of the dye, samples were incubated at room temperature for 45 min. The fluorescence due to calcein was measured at excitation 485 and emission 530 while that of EthD-1 was measured at excitation 530 and emission 645. The percentage of live and dead cells was calculated based on the intensity of fluorescence and a standard of live and dead cells. The dead cells were prepared by treatment with 0.25% digitonin for 10 min. For the effect of phosphate on cell attachment and subsequent viability, only the attached cells were analyzed.

Atomic Absorption Spectroscopy

Cells were grown in 30 mm $\times$ 100 mm plates. When the cells reached confluency, they were treated with 100 μM of aluminum acetylacetonate or aluminum sulfate for 48 h. The cells were then washed four times with 5 ml of Tris-HCl-7% NaCl (pH 7.4). After the last wash, buffer was added and the cells were gently scraped off the plate. The samples were then centrifuged for 4 min at 1000 g and the supernatant was removed. The resulting pellets were frozen overnight at -70°C . The samples were then shipped on

dry ice to Desert Analytics (Tucson, AZ) for evaluation of aluminum content by atomic absorption spectroscopy.

Statistical Analysis

The difference among groups was assessed using one-way Analysis of Variance followed by the Student's t -test.

RESULTS

Aluminum Speciation in Cell Culture Media

Extensive hydroxylation of Al in solution has lead researchers to believe that at physiologic pH, the metal is scarcely soluble and has formed aggregates (Corain et al., 1996). The level of positively charged Al complexes was determined in fresh medium (Table 1) and also in medium that was incubated for 48 h at 37°C in a humidified atmosphere of 5% CO_2 (Table 2). The assay demonstrated that there was a dose-dependent, non-linear change in the amount of positively charged Al species in the medium. When the morin dye was added to 500 μM of aluminum sulfate, the increase in fluorescence was greater than when the dye was added to 500 μM of aluminum acetylacetonate. This may be because the latter compound is more metastable and does not dissociate and speciate as easily as aluminum sulfate. When morin was added to the sodium salts

Table 1
Al-morin complex formation in cell incubation medium^a

Sample	Al concentration (μM)	Morin-Al complex formation (arbitrary units of fluorescence); value $\pm$ S.E.
Medium alone	0	-0.04 ± 0.26
Medium + morin	0	6.88 ± 0.45
Medium + aluminum acetylacetonate	10	7.63 ± 0.57
	100	$16.12 \pm 2.30^*$
	500	$35.98 \pm 5.12^*$
Medium + sodium acetylacetonate	10	6.33 ± 1.10
	100	5.62 ± 0.61
	500	5.63 ± 0.86
Medium + aluminum sulfate	10	7.68 ± 0.68
	100	$15.90 \pm 1.98^*$
	500	$51.32 \pm 5.76^*$
Medium + sodium sulfate	10	6.63 ± 0.93
	100	4.68 ± 0.76
	500	5.90 ± 0.78

^a Values are mean $\pm$ S.E. ($n = 4-5$). Each sample was assayed in duplicates on separate days. An amount of 20 μM morin was present in all Al-containing medium.

Table 2

Al-morin complex formation in cell incubation medium after a 48 h incubation at 37°C in a humidified atmosphere of 5% CO₂^a

Sample	Al concentration (μM)	Morin-Al complex formation (arbitrary units of fluorescence); value ± S.E.
Medium alone	0	−0.06 ± 0.08
Medium + morin	0	2.93 ± 0.56
Medium + aluminum acetylacetonate	10	8.35 ± 1.09
	100	18.42 ± 1.65*
	500	22.53 ± 2.82*
Medium + sodium acetylacetonate	10	3.73 ± 0.11
	100	3.42 ± 0.44
	500	2.60 ± 0.90
Medium + aluminum sulfate	10	11.30 ± 0.98*
	100	14.90 ± 1.34*
	500	26.83 ± 4.56*
Medium + sodium sulfate	10	3.50 ± 0.55
	100	3.84 ± 0.43
	500	4.48 ± 0.56

^a Values are mean ± S.E. (*n* = 3–5). Each sample was assayed in duplicates on separate days. An amount of 20 μM morin was present in all Al-containing medium.

* Value is significantly different (*P* < 0.01) than the corresponding value using the morin dye in cell culture medium.

fluorescence was comparable to the control values (Table 1).

After a 48 h incubation at 37°C in a humidified atmosphere of 5% CO₂, the media composition of the 500 μM Al solutions changed in such a way that the fluorescence of the Al-morin complex decreased compared to the values obtained with freshly prepared Al-containing solutions (Tables 1 and 2). This effect was not apparent when lower concentrations of aluminum were used. The formation of polymeric and colloidal Al complexes may be responsible for the decrease in the amount of the positively charged Al species. Again, the level of fluorescence did not change significantly upon addition of sodium sulfate or sodium acetylacetonate (Table 2). The non-linear changes seen were probably due to the formation of colloidal Al species, which occur as the solution ages (Corain et al., 1996).

Effect of Aluminum on Cell Proliferation

Aluminum sulfate, at concentrations of 2–500 μM, did not significantly affect the rate of cell proliferation or cell attachment in either the neuroblastoma or the glioblastoma cells (Figs. 1 and 2). On the other hand, aluminum acetylacetonate detached cells and decreased the rate of cell growth, both dose-dependently and time-dependently (Figs. 3 and 4). As early as 4 h after exposure to 500 μM Aluminum acetylacetonate, the cells begin to separate from the growth

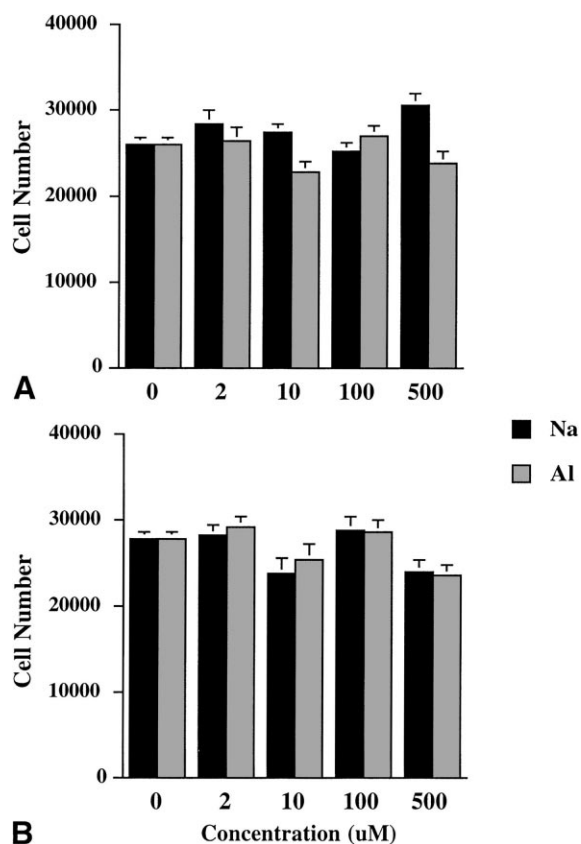


Fig. 1. Cell proliferation in human SK-N-SH cells treated with different concentrations of aluminum and sodium sulfate. (A) Cells treated for 4 h. (B) Cells treated for 48 h. Values are mean ± S.E. (*n* = 12). Two to three experiments were conducted on separate days with freshly prepared samples (some error bars are too small to visualize).

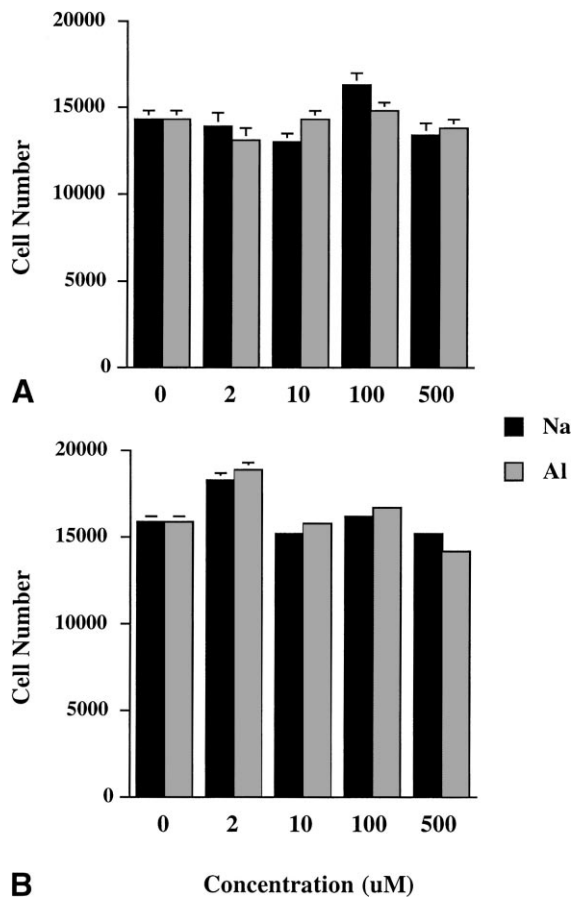


Fig. 2. Cell proliferation in human T98G cells treated with different concentrations of aluminum and sodium sulfate. (A) Cells treated for 4 h. (B) Cells treated for 48 h. Values are mean $\pm$ S.E. ($n = 12$). Two to three experiments were conducted on separate days with freshly prepared samples (some error bars are too small to visualize).

surface. Once detached, the ability of the cells to grow is compromised. SK-N-SH cells exposed to the same concentration of sodium acetylacetonate for 48 h showed a lesser decline in the number of attached cells (Fig. 3). The T98G cells were more resistant to the effect of aluminum acetylacetonate and sodium acetylacetonate on cell detachment, in that a 48 h exposure to 100 μ M of the salts did not significantly alter the number of the attached cells (Fig. 4).

Effect of Aluminum on Cell Viability

After 48 h of treatment with 500 μ M of aluminum acetylacetonate, few cells remained attached. Among the detached cells, 55% of the SK-N-SH cells were viable while 28% of the T98G cells were alive (Fig. 5). Thus, although cell proliferation was reduced to a greater extent in the SK-N-SH cells and these cells

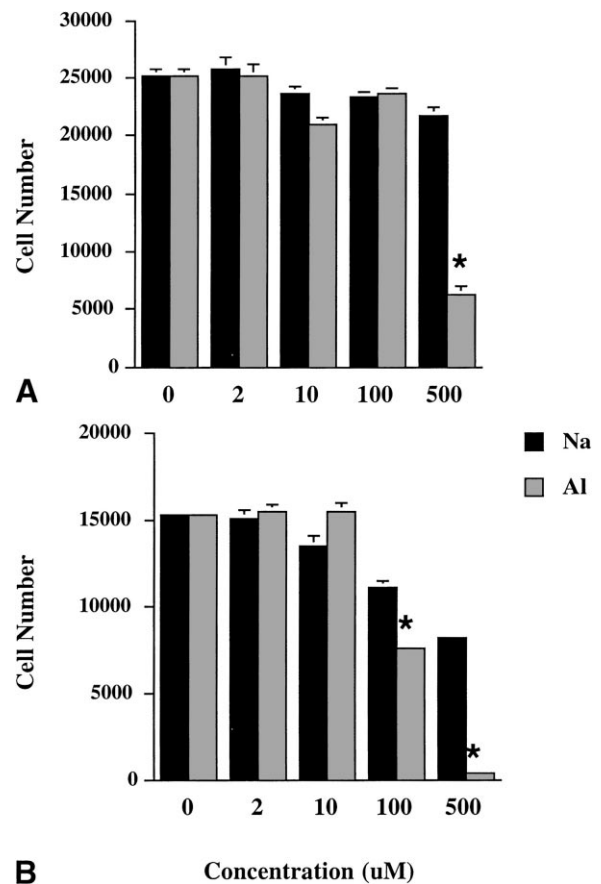


Fig. 3. Cell proliferation in human SK-N-SH cells treated with different concentrations of aluminum and sodium acetylacetonate. (A) Cells treated for 4 h. (B) Cells treated for 48 h. Values are mean $\pm$ S.E. ($n = 12$). Two to three experiments were conducted on separate days with freshly prepared samples. (*) Value is significantly different ($P < 0.001$) than the corresponding value using the sodium salt (some error bars are too small to visualize).

were more prone to becoming separated from the growth surface following exposure to aluminum acetylacetonate, once detached, the T98G cells were more sensitive to the toxic effect of the aluminum salt. Aluminum sulfate had no significant toxicity at the same concentration and time points assayed. Therefore, the acetylacetonate anion was necessary for the ability of aluminum to elicit an effect on the human cell lines.

Effect of the Addition of Phosphate on Cell Attachment

To determine whether phosphate would protect cells against the detachment and subsequent decrease in viability caused by aluminum acetylacetonate, 3 mM of sodium phosphate was added concomitantly with 500 μ M of the Al salt. The addition of phosphate fully

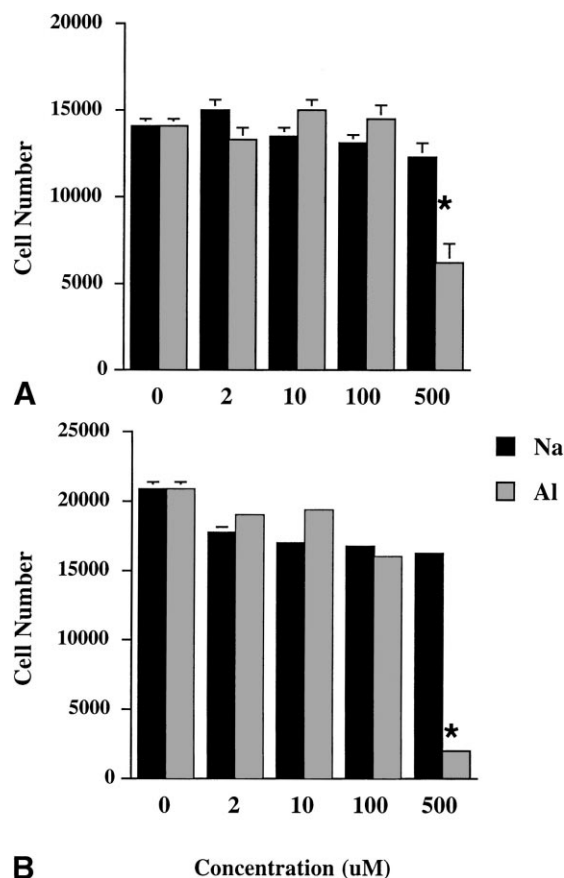


Fig. 4. Cell proliferation in human T98G cells treated with different concentrations of aluminum and sodium acetylacetonate. (A) Cells treated for 4 h. (B) Cells treated for 48 h. Values are mean $\pm$ S.E. ($n = 12$). Two to three experiments were conducted on separate days with freshly prepared samples. (*) Value is significantly different ($P < 0.001$) than the corresponding value using the sodium salt (some error bars are too small to visualize).

protected the T98G cells against aluminum acetylacetonate-induced cell detachment and subsequent death, but did not protect, even partially, the SK-N-SH cells (Fig. 6). Since only the attached cells were studied, and all of the SK-N-SH cells were detached from the plate, the viability is indicated as zero. This meant that there were no cells remaining in the well.

Intracellular Aluminum Content

Aluminum acetylacetonate, at high concentrations, was toxic to cell lines of neural origin. Since aluminum sulfate was not toxic to the cells at any of the concentrations tested, it was postulated that the lipophilic nature of aluminum acetylacetonate increased the rate of absorption of the metal into cells. To determine cellular uptake of Al, 20 μ M of the morin dye was

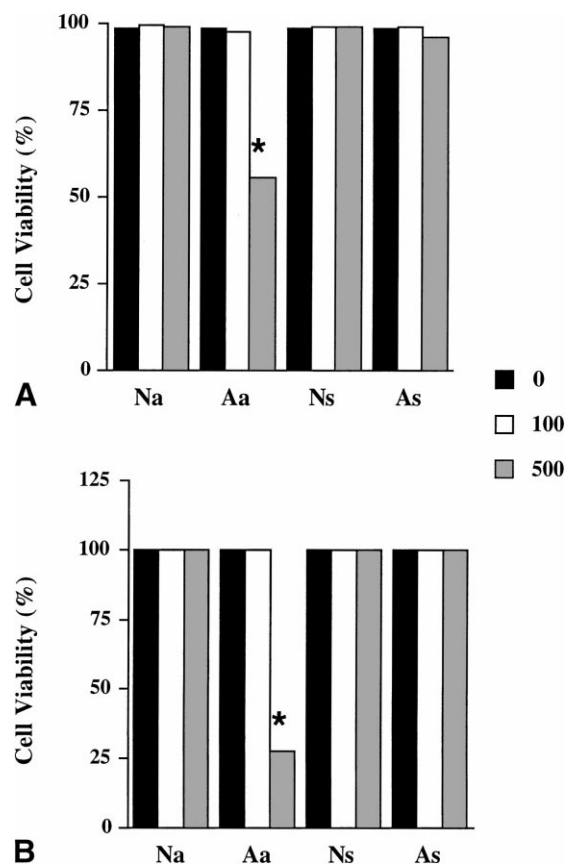


Fig. 5. Viability of human neuroblastoma SK-N-SH (A) and human glioblastoma T98G (B) cells treated with different concentrations of aluminum and sodium salts for 48 h (Aa: aluminum acetylacetonate; Na: sodium acetylacetonate; As: aluminum sulfate; Ns: sodium sulfate) (error bars are too small to visualize).

incubated with washed cell lysates and the fluorescence was monitored. The addition of the morin dye to the treated samples did not produce significant fluorescence (Table 3).

Table 3
Al-morin complex formation in Al-treated cells^a

	Al-morin complex formation after 1 h (arbitrary units of fluorescence) $\pm$ S.E.
(A) T98G cells	
Control	1.27 $\pm$ 0.52
Al(acac) ₃	1.88 $\pm$ 0.32
Na(acac) ₃	0.02 $\pm$ 0.30
(B) SK-N-SH cells	
Control	0.65 $\pm$ 0.54
Al(acac) ₃	0.68 $\pm$ 0.59
Na(acac) ₃	0.63 $\pm$ 0.40

^a Al-morin binding in Al-treated cell lines. Cells were treated for 48 h with 100 μ M of aluminum or sodium acetylacetonate. (A) Human glioblastoma (T98G) cells. (B) Human neuroblastoma (SK-N-SH) cells. Values are mean $\pm$ S.E. Each group represents results from three separate experiments ($n = 4-6$).

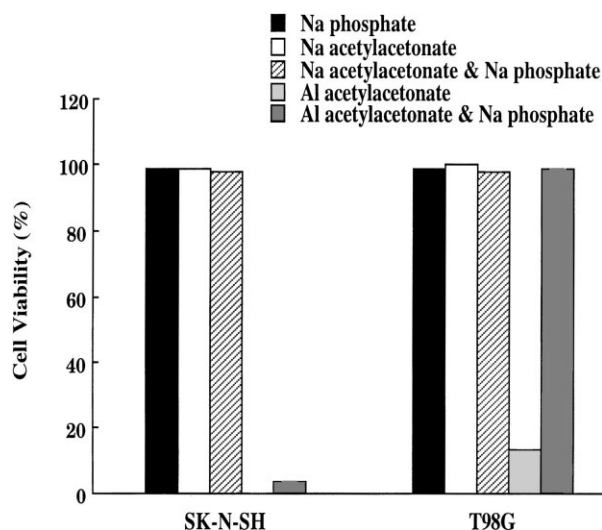


Fig. 6. The effect of the addition of 3 mM sodium phosphate simultaneously with 500 μ M of either sodium or aluminum acetylacetonate on the viability of human neuroblastoma SK-N-SH and human glioblastoma T98G cells. Cells were treated for 48 h (error bars are too small to visualize). In this figure, only the attached cells are analyzed. The viability of SK-N-SH cells is indicated as zero because all of the cells were detached and none remained in the well.

Since morin-Al complex formation depends on the presence of positively charged aluminum species, it was postulated that the aluminum may be bound to cellular components and thus cannot associate with the morin dye. Therefore, samples of the treated cells were sent to a commercial laboratory, Desert Analytics (Tucson, AZ), for the determination of Al content by atomic absorption spectroscopy. However, there was high variability in the results and there was no significant change apparent in Al content in the control and treated cells (data not shown).

DISCUSSION

Human cell lines constitute a useful model for determining the mechanisms responsible for the toxicity of exogenous compounds. This system provides a homogenous population of cells where the effect of a compound on a specific cell type can be assessed. In the present study, two biologically relevant aluminum salts were studied to determine neurotoxicity. Aluminum sulfate is found in drinking water and antiperspirants while aluminum acetylacetonate is an analog of a neutral Al complex formed with isomaltol, a starch byproduct, in the GI tract (Powell and Heath, 1996). Treatment of cells with inorganic aluminum sulfate did not change proliferation or viability in either of the two

human cell lines. This finding was paralleled by earlier results using rodent cell cultures (Campbell et al., 1999). However, aluminum acetylacetonate showed time and dose-dependent toxicity. At high concentrations, both aluminum sulfate and aluminum acetylacetonate form positively charged Al complexes. It is possible that these charged Al species interact with negatively charged proteins on the surface of the cell membrane or bind to phosphate groups in the phospholipid bilayer and by doing so compromise the integrity and function of the cell membrane. However, since aluminum sulfate did not cause adverse effects in the cells, the lipophilic nature of the salt may be necessary for this interaction.

The binding of Al to membrane phospholipids was reported in a study, which showed that membranes containing phosphatidyl serine and dipalmitoylphosphatidylcholine showed significant rigidification after treatment with aluminum (Deleers et al., 1986). It has also been demonstrated that aluminum can cause the formation of negatively charged phospholipid clusters which then decrease the mobility of fatty acids (Verstraeten et al., 1997) and this may underlie the rigidity seen in Al-treated membranes. The exposure of human erythrocytic membranes to aluminum acetylacetonate changed the conformation of membrane proteins (Zatta et al., 1997) and modified the discoid shape of erythrocytes by interacting with both the inner and outer membrane of the cells (Suwalsky et al., 1999). It is possible that the difference in the sensitivity of the two different cell lines to the organic aluminum salt is due to the dissimilarity between their membrane composition. The capability of Al to bind strongly to phosphate groups may underlie its potential to bind to the cellular phospholipid bilayer and disrupt the integrity of the membrane. The addition of 3 mM sodium phosphate was able to completely reverse the effect of aluminum acetylacetonate on the glioblastoma cells. However, it did not alter the response of neuroblastoma cells to the aluminum salt. This difference suggests distinct mechanisms of Al toxicity in the two cell lines.

Neurofibrillary tangles (NFT) are composed of paired helical filaments (PHF) formed by abnormally phosphorylated human tau (PHF τ or A68) protein (Shin et al., 1995). The presence of aluminum has been demonstrated in NFT present in cases of AD and elderly controls while healthy neurons were reported not to contain the metal (Perl and Brody, 1980). Circular dichroism and NMR spectroscopy studies have shown that aluminum does indeed bind to tau and by doing so induces the aggregation of the protein (Madhav et al., 1996). Co-injection of aluminum with

PHF τ results in aggregates that last longer than the deposits that are usually formed by the injection of PHF τ alone (Shin et al., 1994). Al-induced aggregation of tau protein increases 3.5 fold upon phosphorylation of the protein and the aggregates formed in the presence of the metal are resistant to proteases and phosphatases (Li et al., 1998). Since aluminum may strongly bind to neuronal cell components, this may explain why the addition of phosphate does not have a protective effect on aluminum acetylacetonate induced cell detachment. Since the SK-N-SH cells were not differentiated before addition of aluminum and the metal is known to interact with neurofilament proteins and tau, it would be interesting to study the effect of the metal on differentiated, post-mitotic neuronal cells.

Interactions of Al with membrane components seem to be an important factor in Al-induced toxicity, and glial cells appear to be more susceptible to the toxic effect of the salt. The increased susceptibility of glial cells rather than neurons is in accord with a report that primary glial cells are more vulnerable than neurons to long term exposure to aluminum (Suárez-Fernández et al., 1999). Al induces apoptosis only in cultured astrocytes and not neurons, and this provides yet another clue that neurodegeneration may indeed initially be due to the compromised state of the astroglial cells leading to the loss of viability and function of neuronal cells (Suárez-Fernández et al., 1999). It is possible that a stressor, such as increased levels of extracellular aluminum, may trigger the activation of glial cells. During aging, the cerebral microvasculature becomes more prone to damage and this may result in the compromise of the blood brain barrier (BBB) (Kemper, 1984). Since this barrier is the major mechanism by which the brain keeps out foreign antigens, jeopardizing the BBB could lead to compounds, such as aluminum, which are generally confined to the systemic circulation to enter the brain. Cerebral levels of aluminum have in fact been found to increase with age (McDermott et al., 1979). Once Al is accumulated in the brain, it can activate microglia which then play an important role in neurodegeneration by initiating inflammation and secreting complement proteins and oxygen radicals which can then kill healthy neurons and ultimately lead to dementia (Campbell and Bondy, 2000).

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