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## ALUMINUM-INDUCED OXIDATIVE EVENTS IN CELL LINES: GLIOMA ARE MORE RESPONSIVE THAN NEUROBLASTOMA

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**Abstract**—Aluminum, a trivalent cation unable to undergo redox reactions, has been linked to many diseases such as dialysis dementia and microcytic anemia without iron deficiency. It has also been implicated in Alzheimer's disease although this is controversial. Because cell death due to oxidative injury is suspected to be a contributory factor in many neurological diseases and aluminum neurotoxicity, glioma (C-6) and neuroblastoma (NBP<sub>2</sub>) cells were utilized to assess early changes in oxidative parameters consequent to a 48-h exposure to aluminum sulfate. A 500- $\mu$ M concentration of this salt produced a significant increase in reactive oxygen species (ROS) production and a significant decrease in glutathione (GSH) content in glioma cells. However, the same concentration of the aluminum salt did not lead to any significant changes in the neuroblastoma cells. Mitochondrial respiratory activity in glioma cells was also found to be significantly higher in the aluminum treated cells. As judged by morin-metal complex formation, aluminum can enter glioma cells much more readily than neuroblastoma cells. Thus, it is possible that the cerebral target following an acute exposure to aluminum may be glial rather than neuronal. © 1999 Elsevier Science Inc.

**Keywords**—Glioma, Neuroblastoma, Aluminum, Reactive oxygen species, Glutathione, Mitochondrial activity, Free radicals

### INTRODUCTION

Aluminum is an abundant metal found in the earth's crust. Over decades, due to the advent of technology and the introduction of a variety of chemicals into the atmosphere, acid rains have led to the mobilization of this metal [1]. In the water supply, the presence of this metal has been shown to be toxic to both plants and animals [2,3]. Until recently, it was believed that the body burden of aluminum was innocuous to human health. However, a causative role has been established in dialysis dementia and microcytic anemia without iron deficiency [4,5]. Aluminum has also been implicated in Alzheimer's disease although a direct link between this metal and the disease has as yet not been established [6].

Aluminum is not known to be essential for any biological processes. However, it can bind to phosphates and other oxygen donating ligands and form stable com-

plexes. By doing so, Al may disrupt the enzyme activity in the mitochondria and thus effect the electron transport chain. This may lead to an increase in reactive oxygen species formation which in turn can lead to oxidative injury [7].

Aluminum sulfate is the most common aluminum-based coagulant used to purify water in North America. Typically, it is added at a final concentration of 1 and 5 mg/L of aluminum [8]. It has been reported that there is an increased relative risk of developing AD in districts where the aluminum concentrations in the municipal drinking water are 100  $\mu$ g/L or greater [9]. The exact mechanism by which aluminum might exert its effect on the nervous tissue is as yet unknown. However, there are several studies which show that aluminum has the ability to promote the pro-oxidant properties of iron and other transition metals [10–13].

In this study, isolated cell lines of neuronal and glial origin were utilized in order to assess which cell type would be more responsive to an acute exposure to aluminum sulfate without the further addition of iron. These

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cell lines provide a useful model to study the mechanisms underlying the toxicity of exogenous compounds. We have investigated changes in oxidative parameters in glioma (C-6) and murine neuroblastoma (NBP<sub>2</sub>) cells in order to assess early changes in oxidative parameters following the addition of aluminum sulfate to the cell culture medium. After a 48 h exposure, major changes in oxidative parameters were observed in glioma cells but not in neuroblastoma cells.

## MATERIALS AND METHODS

### Materials

Rat glioma (C-6) cells of astrocytic origin were purchased from American Type Culture Collection (ATCC), Rockville, MD, USA, and murine neuroblastoma (NBP<sub>2</sub>) cells have been studied and well characterized in our laboratory [14]. 2',7'-dichlorofluorescein diacetate (DCFH-DA) was purchased from Molecular Probes, Inc. (Eugene, OR, USA) while the DCF required for calibration was purchased from Polysciences, Inc. (Warrington, PA, USA). All tissue culture supplies were purchased from GIBCO Co., Grand Island, NY, USA. All other chemicals used were obtained from Sigma Co., St. Louis, MO, USA.

### Cell culture

Rat glioma (C-6) cells were grown in RPMI medium with 10% fetal bovine serum and penicillin-streptomycin (100 units/mL) while the neuroblastoma cells were grown in F12 medium with 10% gammaglobulin free newborn calf serum and penicillin-streptomycin (100 units/mL). Glioma and neuroblastoma cells were seeded at a density of 500,000 and 250,000 cells per dish (35 × 10 mm) respectively. Fewer neuroblastoma cells were seeded because their rate of replication is approximately twice as fast as the glioma cells and it was desired to keep the numbers of cells for each cell line approximately equal. All cells were maintained at 37°C in a humidified atmosphere of 5% CO<sub>2</sub>.

Forty-eight h after plating, the cells were treated with 500 μM aluminum sulfate for another 48 h. As it ages, aqueous aluminum sulfate undergoes extensive hydrolysis in water that leads to formation of colloidal complexes that can then effect the bioavailability of aluminum [2]. In this study, the aluminum sulfate solution was prepared fresh and sterile-filtered prior to its addition to the cells. Trypsin solution (0.25%) and pancreatin solution (0.25%) were utilized to detach glioma cells and neuroblastoma cells respectively. The cells were centrifuged at 1000 × *g* for 5 min. The resulting pellets were resuspended in 2 mL of 50 mM Tris-HCl buffer at pH 7.4 and aliquots were used for the biochemical assays.

### Cell viability

Cell viability among the attached cell population was assessed by the trypan blue exclusion technique. Trypan blue isotonic solution (0.5 mL of 0.2%) was added to each plate and the number of deeply stained cells (representing dead cells) per 100 cells was counted under an inverted microscope. The number of stained cells was subtracted from the total count in order to determine the percentage of viable cells.

### Protein assay

The amount of protein in 100 μL aliquots of cell samples was assayed using the method of Bradford [15].

### Reactive oxygen species (ROS) formation

ROS were measured using 2',7'-dichlorofluorescein diacetate (DCFH-DA), a lipophilic nonfluorescent dye which diffuses into the cells and is there hydrolyzed to 2',7'-dihydrodichlorofluorescein (DCFH). This is then oxidized by reactive oxygen to form the strongly fluorescent compound 2',7'-dichlorofluorescein (DCF) [16]. One hundred μL aliquots of the samples were diluted in 19 vol of 50 mM Tris-HCl buffer at pH 7.4 and then loaded with a final concentration in solution of 5 μM DCFH-DA. Samples were incubated at 37°C and fluorescence was monitored 15 and 60 min after the addition of DCFH-DA. The difference between these values reflected the velocity of ROS formation. Fluorescence was measured using a Perkin-Elmer LS-5 fluorescence spectrophotometer. Excitation wavelength was set at 488 nm (bandwidth 5 nm) while the emission wavelength was set at 525 nm (bandwidth 20 nm). The formation of reactive oxygen species was quantified using a 2',7'-dichlorofluorescein (DCF) standard curve and results were expressed as nmol DCF formed/mg protein/45 min.

### Intracellular glutathione (GSH) content

The intracellular concentration of glutathione (GSH) was assayed using the method of Shrieve et al. [17]. Utilizing the fact that GSH forms a fluorescent adduct with the non-fluorescent compound monochlorobimane (mBCl), the samples were incubated with 10 μM of the dye for 15 min at 37°C and the fluorescence was read at 395 nm excitation (bandwidth 5 nm) and 470 nm emission (bandwidth 20 nm). Glutathione concentration in the samples was calculated using a GSH standard curve. This determination was performed in the presence of 0.1 U/mL of liver GSH transferase in order to accelerate the formation of the fluorescent adduct derivative.

*Determination of mitochondrial activity by the 3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyltetrazoliumbromide (MTT) assay*

Active mitochondrial dehydrogenases of living cells cause cleavage and conversion of the soluble yellow MTT dye to the insoluble purple formazan [18]. Aliquots of cell samples were incubated at 37°C with 0.5 mg/mL of the dye for 3.5 h. The cells were then centrifuged at  $3000 \times g$  for 5 min. The pellets were then diluted in 2 mL of 100% DMSO and incubated at room temperature for a further 1 h. The absorption was spectrophotometrically determined at 570 nm.

*Intracellular aluminum*

The intracellular level of aluminum was assayed using morin dye which is a pentaprotic acid (2',3,4',5,7-pentahydroxyflavone). Cell pellets were washed twice with 50 mM Tris buffer at pH 7.4 to remove any traces of the media. The membranes were then dissolved by diluting the cells in 2 mL of the Tris buffer containing 1% triton. One  $\mu\text{M}$  of morin dye was then added to the solution and the samples were incubated for 1 h at 37°C. Fluorescence was read at 420 nm excitation (bandwidth 5 nm) and 515 nm emission (bandwidth 20 nm) [19].

*Statistical analysis*

The difference among groups was assessed using one-way analysis of variance followed by Fisher's least significant difference test. Values were accepted as different if the level of significance was  $p < .05$  using a two-tailed distribution.

**RESULTS**

*Cell viability and protein content*

The number of viable cells in both the glioma and neuroblastoma cell cultures was greater than 99% after a 48 h exposure to aluminum sulfate. The amount of protein in the control and aluminum treated cells did not change significantly in either the neuroblastoma or the glioma cells (data not shown). Therefore, subsequent indices were reported on a protein basis.

*Assessment of oxidative parameters (ROS and GSH)*

There were marked changes in several oxidative parameters in glioma cells following a 48 h exposure to aluminum sulfate. A 75% increase in ROS formation was observed in the treated glioma cells while in the neuroblastoma cell line, treatment had no significant effect

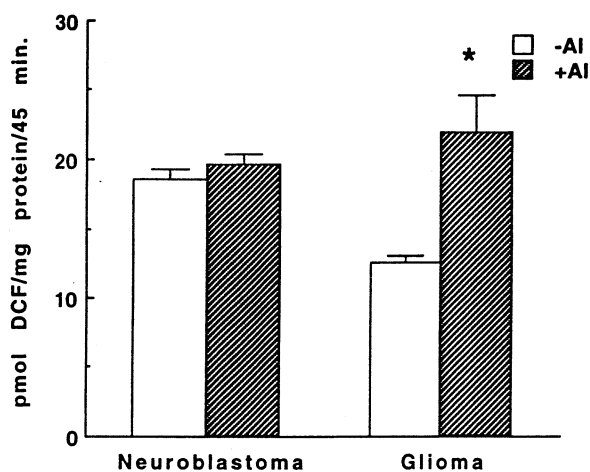


Fig. 1. Levels of ROS generated in neuroblastoma and glioma cells after a 48 h exposure to 500  $\mu\text{M}$  aluminum sulfate. Values are means  $\pm$  SE ( $n = 9$ ). Two trials were conducted on two separate days on newly prepared samples. \*Value is significantly different than the corresponding control.

(Fig. 1). Levels of GSH, a major intracellular defense against oxidant events, were significantly decreased in the treated glioma cells relative to untreated controls. However, the GSH content of aluminum treated neuroblastoma cells did not differ significantly from control values (Fig. 2).

*Mitochondrial activity*

In glioma cells, mitochondrial dehydrogenase activity was significantly increased following exposure to aluminum, as judged by an elevated production of formazan.

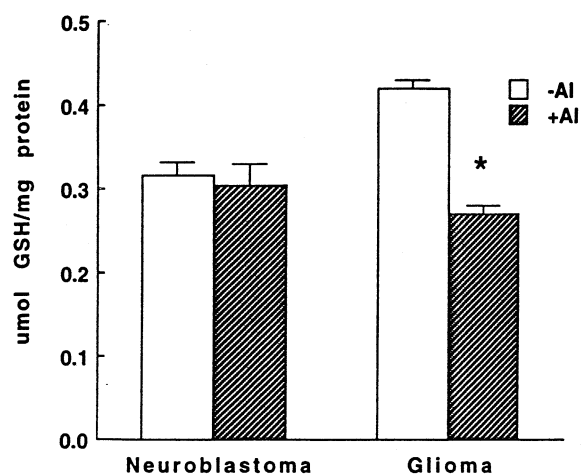


Fig. 2. Glutathione (GSH) content in neuroblastoma and glioma cells after a 48 h exposure to 500  $\mu\text{M}$  aluminum sulfate. Values are means  $\pm$  SE ( $n = 9$ ). Two trials were conducted on two separate days on newly prepared samples. \*Value is significantly different than the corresponding control.

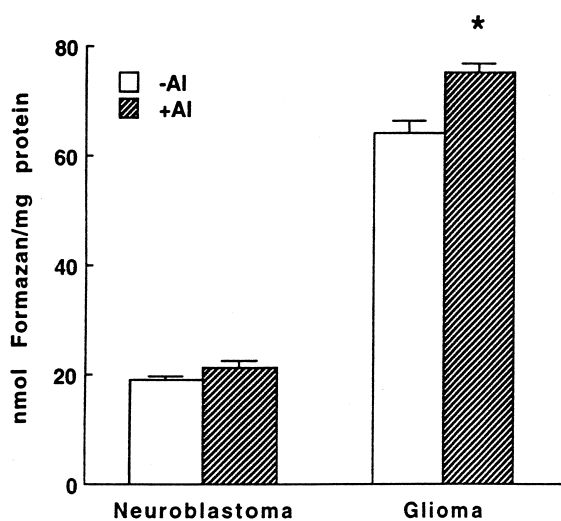


Fig. 3. Mitochondrial activity in neuroblastoma and glioma cells after a 48 h exposure to 500  $\mu\text{M}$  aluminum sulfate. Values are means  $\pm$  SE ( $n = 6-9$ ). Two trials were conducted on two separate days on freshly prepared samples. \*Value is significantly different than the corresponding control.

Neuroblastoma cells treated with aluminum showed a smaller insignificant increase in formazan production (Fig. 3).

#### Intracellular aluminum content

As judged by fluorescence of the morin-metal complex, the aluminum content of glioma cells was markedly increased following treatment. On the other hand, neuroblastoma cells showed no parallel change (Fig. 4).

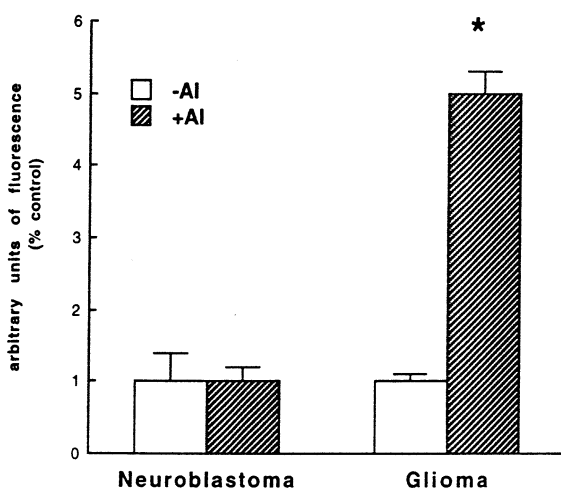


Fig. 4. Morin-metal complex formation in neuroblastoma and glioma cells after a 48 h exposure to 500  $\mu\text{M}$  aluminum sulfate. Values are means  $\pm$  SE ( $n = 6$ ). Two trials were conducted on two separate days on freshly prepared samples. \*Value is significantly different than the corresponding control.

## DISCUSSION

The role of aluminum in Alzheimer's disease (AD) is both controversial and unresolved. In the present work, although the acute exposure to aluminum sulfate was not toxic to either of the two cell lines, compared to neuroblastoma cells, glioma cells were found to be much more susceptible to aluminum-induced metabolic changes. ROS production is significantly increased in the glial cells. The observed reduction in the level of GSH, that is a major source of reducing power in the aqueous compartment of the cell, further suggests increased pro-oxidant status in these cells. This finding is similar to a study which demonstrated that chronic exposure of rats to aluminum leads to a decrease in glutathione peroxidase which parallels an increase in lipid peroxidation [20]. Although our previous work demonstrated that aluminum promotes iron-dependent oxidative processes in subcellular systems [10], in the intact animal, exogenous iron did not interact with aluminum [21]. Preliminary experiments in isolated cell lines also showed no interaction between aluminum and exogenous iron.

MTT results indicate that aluminum induces an increase in mitochondrial dehydrogenase activity which may be due to an increase in mitochondrial activity. Electron leakage from the respiratory chain can lead to the formation of excess reactive oxygen species [7]. Thus, increased mitochondrial respiratory activity may underlie the enhanced ROS generation found in aluminum-treated glioma cells. In order to substantiate whether the increased mitochondrial activity is indeed involved in ROS generation, more exhaustive evaluation of mitochondrial activity following aluminum exposure would be useful.

Although aluminum has been found to have the capacity to promote Fe-related oxidative events in isolated subcellular systems [11,13,22] the mechanism by which aluminum promotes production of ROS within intact cells is not known. It may be that a parallel interaction with endogenous iron takes place. A potential target site would be the mitochondrion where intracellular ROS are predominantly produced by leakage from the electron transport chain. The mitochondrion is rich in iron as a key constituent of cytochromes of the respiratory chain. While displacement of iron from ferritin is unlikely to account for Al-related pro-oxidant phenomena [10], the effects of aluminum on cytochrome-bound iron have not been examined in an analogous manner.

Aluminum accumulates in the brain by crossing the blood brain barrier [23]. The controversy concerning levels of aluminum in AD brain is complicated by a lack of consensus concerning levels of aluminum in normal brain which are reported to vary by as much as 10-fold [24,25]. In the present work, cell lines were exposed to



500  $\mu\text{M}$  of aluminum sulfate. This value was arrived at in our laboratory from a dose response study which showed that at 500  $\mu\text{M}$ , the oxidant effects of aluminum were maximal [10]. This concentration has also been used in previous reports [12,13] and in order to insure that this study would be comparable to these prior works, the same amount of the salt was utilized for our primary assessment of oxidative events.

The Morin analysis shows that unlike the glioma cells, neuroblastoma cells are unable to accumulate aluminum. This finding is supported by another report using NIE-115 neuroblastoma cells where it was observed that the cells were unable to accumulate intracellular aluminum [26]. In the current study, it appears that aluminum is capable of entering the glioma cells by a mechanism which is as yet undetermined. It is possible that extracellular aluminum is not harmful to the cells and the metal needs to be internalized before it would have any biochemical effects. Thus, the reason why the neuroblastoma cells are not responsive to aluminum treatment may simply be because of the fact that the aluminum cannot get into the cells. Since in the present study the response of murine neuroblastoma cells is compared to rat glioma cells, it is possible that the changes observed are due to species differences. At present, our laboratory is repeating the experiments on neuroblastoma and glioblastoma cell lines, both of human origin.

The involvement of glial cells in AD is becoming increasingly recognized. Anti-brain antibodies are present in the cerebrospinal fluid (CSF) of patients with Alzheimer's disease and it has been reported that these antibodies show selectivity for glioblasts and microglial cells [27]. It has also been discovered that neuritic plaques, which are hallmarks of AD, consist of an amyloid core which is surrounded by reactive glial cells [28]. These glial cells are probably present as a response to cell injury and plaque formation. However, it is possible that an insult such as an increase in CSF aluminum may also trigger the activation of glial cells.

It has been hypothesized that microglia play an active role in neurodegeneration by responding to inflammation and secreting complement proteins, oxygen radicals, cytokines, prostaglandins and adhesion molecules which can then kill healthy neurons and ultimately lead to dementia [29]. A recent study shows that lipopolysaccharide induced chronic neuroinflammation, which models chronic central inflammation following traumatic brain injury, causes extensive astrogliosis in the temporal lobe regions of rats. The activation of the astroglial cells was associated with an increase in the production of potentially cytotoxic IL- $1\beta$ , hippocampal cell loss, and impairment of spatial memory, all of which mirror changes seen in the Alzheimer's disease brain [30]. It is possible that in the brain, acute aluminum exposure may

activate glial cells which in turn may indirectly jeopardize the integrity of neuronal cells. One of the mechanisms by which aluminum may play a potential role in neurodegenerative disease could be by way of the activation and exacerbation of oxidant processes occurring within glia.

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