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Research report

Pro-inflammatory effects of aluminum in human glioblastoma cells

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Abstract

Inflammatory events have been associated with senile plaques, one of the pathological hallmarks of Alzheimer's disease (AD). It is believed that aggregated beta-amyloid (βA) proteins, which form the core of these plaques, may be responsible for triggering the inflammatory reaction. In the present study, the ability of aluminum (Al) to initiate similar inflammatory events was investigated in a human glioblastoma cell line. A 6-day exposure to either lipopolysaccharide (LPS) or aluminum sulfate caused a significant increase in the rate of proliferation of the glioblastoma cells. Both treatments also caused activation of the immune-responsive transcription factor NF-κB although there were time-related differences. The levels of secreted cytokines, interleukin-6 (IL-6) and tumor necrosis factor alpha (TNF-α) were both increased by the LPS treatment although exposure to Al decreased the secretion of the former while elevating the levels of the latter. These events may be due to the activation of glial cells and subsequent stress response to either Al complexes or LPS. Although exposure to either stress factor caused a stimulation of inflammatory markers, there were time-dependent differences in the response. This may reflect the ability of the cells to discern different stress factors and thus orchestrate an innate immune response profile distinct to each immunogen.   © 2002 Elsevier Science B.V. All rights reserved.

1. Introduction

A direct causal role for aluminum has been established in dialysis encephalopathy [1], osteomalacia [6,18] and microcytic anemia [34,16]. It has been proposed that the metal may play a role in the etiology of Alzheimer's disease since an increase in Al has been found in the brain of patients with this disorder [10,38]. However, other studies have contradicted this finding [24,4] and the issue remains unresolved. However, since the pathogenesis of this age-related disorder is multifactorial and the cerebral content of the metal has been shown to increase with age [31,24], Al may promote events connected with the disease process.

Systemically, Al has been shown to cause inflammation. The metal induced inflammatory nodules in Al-sensitized

adults revaccinated for hepatitis B [9] and there is a correlation between focal lung inflammation and total deposited Al in occupational workers [30]. In rats, low doses of aluminum, present in parenteral nutrition formula, produced marked portal inflammation that was correlated with the duration of exposure and the amount of Al accumulated in the liver [12]. A premise of the current study is that aluminum, once inside the brain, may elicit a similar inflammatory response.

In cells of glial origin, Al caused an increase in the rate of reactive oxygen species production, glutathione depletion and increased mitochondrial dehydrogenase activity while cells of neuronal origin were not responsive to the same treatment [7]. Al is prone to hydrolysis and will form insoluble precipitates within the physiological pH range [32]. It is possible that these extracellular particles may induce an innate immune response in cells of glial origin.

Human glioblastoma T98G cells were used to study the effect of 500 μM aluminum sulfate on several indices of inflammation. Al sulfate was chosen because of its bio-

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logical relevance. The salt is used for water purification as well as in clarifying fats and oils, antiperspirants, and agricultural pesticides. This dose was selected based on previous results in our laboratory [7,8]. Lipopolysaccharide (LPS), in a concentration of 1 μg/ml, was used as a positive control. This component of the cell wall of gram-negative bacteria has been used by several groups to demonstrate increases in proinflammatory parameters [13,26,36]. To determine the toxic effect of exposure and cell number, cell viability and proliferation studies were conducted. Since it was postulated that colloidal Al may be causing an inflammatory response and aged aluminum salts are known to aggregate [32], the effect of Al was studied up to 6 days of exposure. Aluminum and LPS treatment both caused an increase in several glial inflammatory parameters although their responses were different.

2. Materials and methods

2.1. Materials

Human glioblastoma (T98G) cells were purchased from ATCC (Rockville, MD). All tissue culture supplies were obtained from Gibco (Grand Island, NY). Cell viability and cell proliferation kits were purchased from Molecular Probes. The NF-κB gel shift assay compounds were purchased from Promega (Madison, WI). The enzyme immunoassay used for detection of the cytokines was from Neogen (Lexington, KY). All other chemicals used were from Sigma (St. Louis, MO).

2.2. Methods

2.2.1. Cell preparation

Human cell lines were grown in minimum essential medium (MEM) with a modification consisting of nones-sential amino acids, sodium pyruvate, lipoic acid, vitamin B_{12}, biotin and ascorbic acid. The media also contained Earle's salts and L-glutamine. It was supplemented with 10% fetal bovine serum (heat-inactivated). Cells (0.5 million) were seeded in 25-cm^2 flasks and incubated at 37 °C in a humidified atmosphere of 5% CO_2. Once the cells were confluent, they were treated with the salts. The aluminum and LPS solutions were prepared in the cell media and immediately sterile-filtered prior to the first dosing. Although some aggregated aluminum complexes, formed when Al sulfate was brought to neutral pH, was removed, we have previously demonstrated that the filtered solution still contains Al species capable of selectively binding to the Al-specific Morin dye [8]. The solutions, along with the media, were then incubated with the treated cells to insure parallel conditions for the aging of the Al salt for up to 6 days. These were then utilized for further exposure after the cells were harvested for each time point. After the initial 7-h dosing, supernatant and 4:5 of the cells

were collected for the subsequent assays. At this time, the same treated cells were re-seeded at a ratio of 1:5, exposed to the salts prepared previously, and allowed to grow for the other time-points.

2.2.2. Cell proliferation assay

Cells were frozen overnight at −70 °C and the number was determined using the CyQuant cell proliferation assay kit (Molecular Probes, OR). Briefly, cells were thawed at room temperature and 200 μl of the dye, diluted in cell lysis buffer was added. The dye rapidly binds to nucleic acids and exhibits strong fluorescence when bound. The cells were incubated at room temperature for 5 min and the fluorescence was measured at excitation 480 nm and emission 520 nm using a FL600 microplate fluorescence reader. A standard of cells counted with the hemacytometer was used to determine the actual cell number.

2.2.3. Cell viability assay

A viability/cytotoxicity kit (Molecular Probes) was used to measure cell survival after treatment. This assay is based on the simultaneous measurement of the fluorescence of two dyes. Live cells were 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. Upon addition of the dye, samples were incubated at room temperature for 45 min. The fluorescence due to calcein was measured at excitation 485 nm and emission 530 nm, while that of EthD-1 was measured at excitation 530 nm and emission 645 nm using a FL600 microplate fluorescence reader. The percentage of live and dead cells was calculated based on the intensity of fluorescence using a standard of live and dead cells. The dead cells were prepared by treatment with 0.25% digitonin for 10 min.

2.2.4. Electrophoretic mobility shift assay

The gel shift assay was utilized to determine the extent of NF-κB activation in the human glioblastoma (T98G) cells using a protocol developed by Promega (Madison, WI). After treatment, the media was removed and the cells were washed with Tris–HCl/0.7% NaCl (pH 7.4) buffer. Cells were lysed by several freeze–thaw cycles and the resulting cell extract was stored at −70 °C. The amount of protein in 1 μl of the cell extract was determined by the BioRad protein assay [5] and 20 μg of each sample, incubated with ^{32}P-labeled oligonucleotides containing the NF-κB consensus sequence, was loaded onto the gel. A negative control containing no cell extract, as well as competitor reactions were run simultaneously with the samples. The specific competitor contained unlabelled NF-κB consensus nucleotide while the nonspecific competitor

contained unlabelled SP-1 consensus oligonucleotide. The competitor reactions also contained 20 µg of the 6-day LPS-treated sample. X-ray films were manually developed and the intensity of each band was measured and quantitated using the image analyzer, Eagle Eye, from Strategene (San Diego, CA).

2.2.5. Enzyme immunoassay

Levels of TNF-α and IL-6 were determined using sandwich enzyme immunoassay kits from Neogen (Lexington, KY) for the detection of free forms of the proteins in cell culture supernatants. Briefly, 100 µl of the cell supernatant was incubated with rabbit anti-human IL-6 or TNF-α polyclonal antibody for 3 h. The plate was washed and incubated for 45 min with goat anti-rabbit-conjugated alkaline phosphatase. Then, 200 µl of the color reagent solution was added to the plate and the color generated was determined with a spectrophotometric plate reader set at 490 nm. For the IL-6 assay, the samples were diluted 40× because the level of this cytokine in the cell culture supernatant was too concentrated for the detection range of the assay.

2.3. Statistical analysis

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

3. Results

3.1. Cell viability and proliferation

After a 48-h exposure to LPS (1 µg/ml), cell proliferation was increased although there was a significant decline in the viability of the human glioblastoma T98G cells (Figs. 1 and 2). In contrast, Al (500 µM) did not
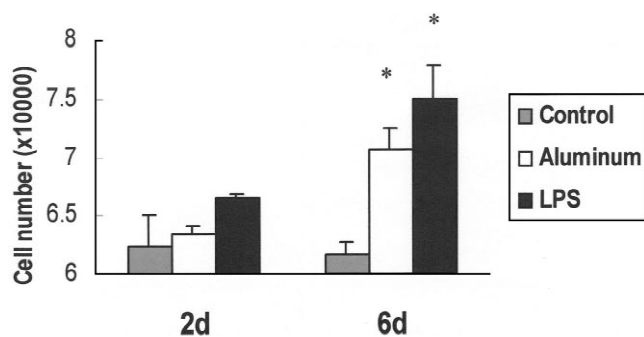

Fig. 1. Cell proliferation in human T98G cells treated with 500 µM aluminum sulfate or 1 µg/ml LPS for 2 and 6 days (2d and 6d). The rate of cell proliferation was determined using the CyQuant cell proliferation assay kit from Molecular Probes. * Value is significantly different (P< 0.05) from the control. Bars represent mean of five to six individual determinations±S.E.
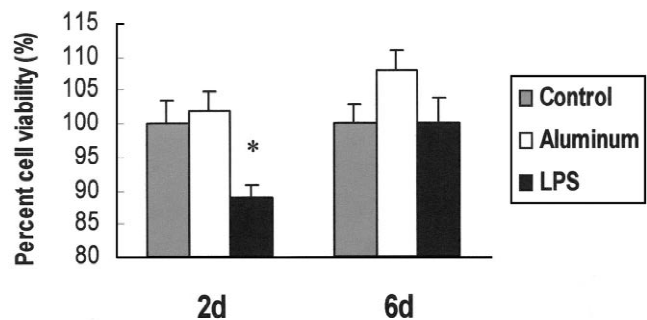

Fig. 2. Viability of human T98G cells treated with 500 µM aluminum sulfate or 1 µg/ml LPS as determined with the viability/cytotoxicity kit. Cells were treated for 2 or 6 days (2d and 6d) * Value is significantly different (P<0.05) from the control. Bars represent mean of five or six individual determinations±S.E.

effect either parameter at the 48-h time-point. After a 6-day exposure, both Al and LPS caused an increase in the rate of proliferation of the glioblastoma cells (Fig. 1). At this later time-point, there was no significant change in cell viability (Fig. 2).

3.2. NF-κB activation

Exposure of the glioblastoma cells for 7 h to LPS caused significant activation of the transcription factor NF-κB. Al exposure also caused a slight increase in the intensity of the shifted band at this time point. However, after a 6-day exposure, Al treatment resulted in a significant activation of the transcription factor while the LPS effect was reduced compared to the control (Fig. 3).

3.3. Cytokine secretion

The level of secreted TNF-α increased after a 2-day treatment with LPS and a 6-day treatment further increased the amount of the protein present in the media. Al treatment also caused an increase in the amount of the cytokine but only after 6 days of treatment with the salt (Fig. 4). LPS also caused a substantial increase in the levels of IL-6 secretion at both time points assayed. In contrast, Al caused a significant reduction in the amount of secreted IL-6 after 6 days of treatment (Fig. 5).

4. Discussion

Inflammation appears to play an important role in the pathogenesis of Alzheimer's disease. Activated astrocytes which produce glial fibrillary acidic protein (GFAP) were associated with both senile plaques and cerebral microvessels [11] and cytokine-producing microglia were abundant surrounding aggregated amyloid deposits in the cerebral cortex [28,33]. Treatment of rabbits chronically with
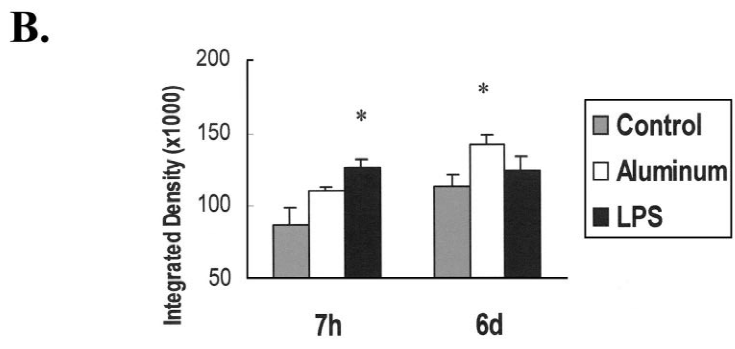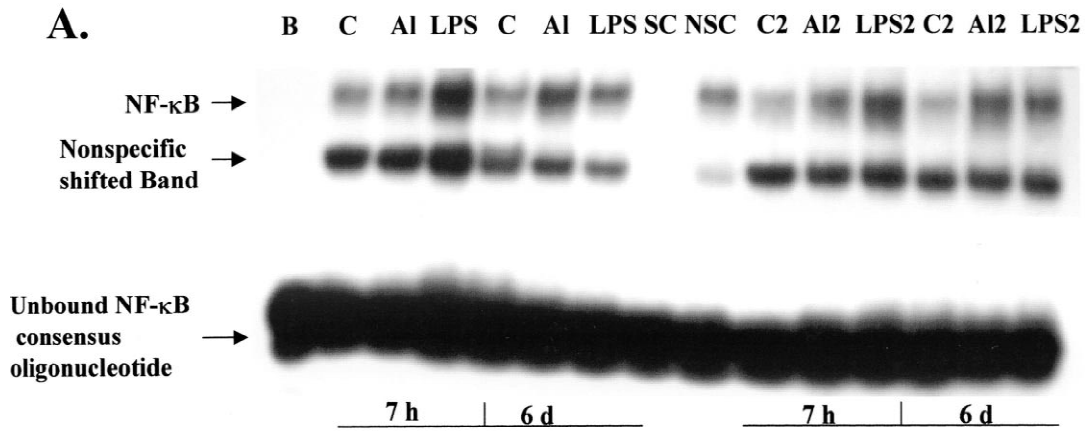
A.

B.

Fig. 3. The effect of treatment with 500 μM aluminum sulfate or 1 μg/ml LPS on NFκB activation in T98G cells. Cell extracts were incubated with ^{32}P-labeled oligonucleotides containing the NF-κB consensus sequence and the shifted bands are shown. (A) T98G cells were treated for 7 h or 6 days. Data for two independent samples are shown. B, blank; C, control; Al, aluminum sulfate; LPS, lipopolysaccharide; SC, specific competitor (unlabelled NF-κB consensus nucleotide and cells treated with LPS for 6 days); NSC, nonspecific competitor (unlabelled SP1 consensus nucleotide and cells treated with LPS for 6 days). Although two shifted bands were observed, the second band appears to be nonspecific since addition of unlabelled SP1 consensus nucleotide (NSC) can also diminish the intensity of the band. (B) The integrated density of the first shifted band. Bars represent mean of four individual determinations±S.E.
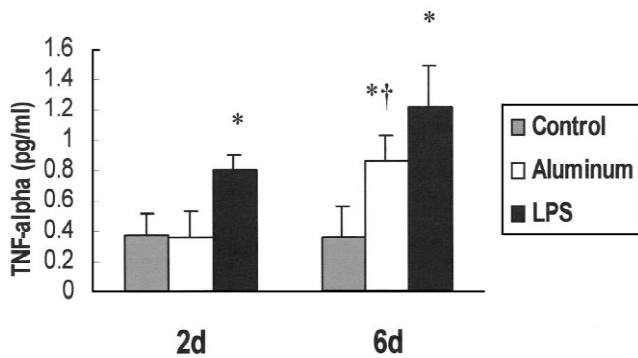

Fig. 4. TNF-α secretion in human glioblastoma T98G cells treated for 2 or 6 days with 500 μM aluminum sulfate or 1 μg/ml LPS. Values are based on log-normal distribution. * Value is significantly different ($P<$ 0.05) than the control. [†] Value at 6 day of Al exposure is significantly greater than the value at 2 days of Al exposure. ($P<0.05$). Bars represent mean of five or six individual determinations±S.E.
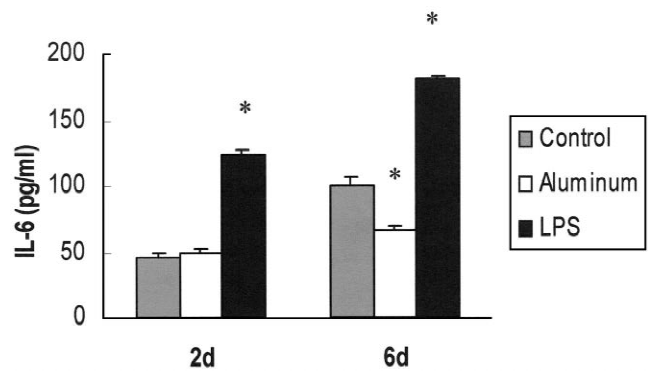

Fig. 5. IL-6 secretion in T98G cells treated with 500 μM aluminum sulfate or 1 μg/ml LPS for 2 or 6 days * Value is significantly different ($P<0.05$) than the control. Bars represent mean of five or six individual determinations±S.E.

aluminum lactate also increased GFAP concentrations in the frontal cortex, reflecting enhanced activation of as-trocytes [39]. In the present study, the rate of proliferation of human glioblastoma cells was increased by a 6-day

exposure to either aluminum sulfate or LPS. This effect may have been due to increased activation of the cells.

Cytokines such as IL-1, IL-6 and TNF-α are generally synthesized by activated microglia and macrophages in response to pathogens and trauma [14]. Levels of cytokines appear to be modulated in Alzheimer's disease. Detectable level of IL-6 was present in the temporal cortex of AD patients but not in control brains [37]. Furthermore, the serum levels of TNF-α was shown to be substantially increased in AD patients compared to age-matched controls [15]. In an animal model of chronic inflammation, induced by intracranial infusion of lipopolysaccharide, there was astrogliosis as well as an increase in the levels of IL-1, and TNF-α mRNA levels. This was followed by hippocampal cell loss and impairment of spatial memory, all of which mirror changes seen in the AD brain [36]. In the present study, a 6-day exposure of human glioblastoma cells to Al caused a significant increase in the levels of TNF-α. This finding is in agreement with a study demonstrating that levels of cerebral mRNA for this cytokine substantially increased in mice chronically exposed to low concentrations of Al in drinking water [35].

Although Al caused a time-dependent increase in the level of TNF-α, the amount of IL-6 was unchanged after a 2-day treatment and decreased after a 6-day treatment. It has also been reported that Al does not effect the levels of IL-1β mRNA in mice cerebral tissue [35]. The two cytokines appear to have different functions. While TNF-α caused expression of the acute-phase protein, α_1-antichymotrypsin mRNA in human astrocytoma cells, IL-6 did not have the same effect [23]. This may underlie the difference in the secretion profile of the two interleukins. The selectivity and extent of the innate immune response, corresponding to the type of pathogen encountered, is controlled by the particular cytokine that is induced after infection [27]. In the present study, the time-course response of cells to Al or LPS was different and it would be interesting to see if this may be due to activation of alternate pathways.

Cytokines such as IL-1β and TNF-α activate NF-κB [21]. This transcription factor promotes the expression of genes involved in inflammation, such as other cytokines, iNOS, and complement factors [2]. Thus, there is a cycle of cytokine secretion and NF-κB activation which if prolonged, may cause neuronal cell death and further proliferation of reactive glial cells. The activation of this transcription factor is involved in a variety of differing neuropathological conditions including experimental autoimmune encephalomyelitis [19] and prion disease caused by the scrapie agent [20]. In temporal lobe neocortex derived from aged or AD patients, NF-κB activation was significantly correlated to an elevation in a key inflammatory enzyme COX-2 [22]. The reason why so many diverse pathogenic stimuli caused induction of this transcription factor is the integral role NF-κB plays in the innate immune response [2,17,27].

It was demonstrated here that Al and LPS cause a time-dependent increase in NF-κB activation. While LPS caused an early acute response that declined after 6 days, Al caused a weak initial induction that increased after 6 days. There are three main categories of Al species in solution. Monomeric complexes are formed immediately upon addition of Al and hydroxylation of the metal in solution. Polymeric species are formed through activation of coordinated OH groups that become deprotonated and form bridges between metal centers. Further aging of the Al solution leads to the formation of metastable polynuclear Al complexes which grow in size and ultimately form microcrystalline gibbsite [32]. The effect of Al on NF-κB activation may be due to the precipitation of Al in solution and the formation of these colloidal species. We have previously shown that the amount of positively charged Al-species capable of interacting with the Al-specific dye, Morin, decreased as the solution aged [8]. This may be due to the formation of these aggregated Al species. In LPS-treated cells, although NF-κB activation was lower after the 6-day time point, it was still above control levels reflecting a sustained increase in pro-inflammatory cytokine secretion at the later time points. The significant LPS-induced induction of NF-κB after the 7-h treatment may underlie the decrease in viability after the 2-day treatment.

There are many similarities between the effect of aluminum salts and amyloid peptide fragments on cells of glial origin. β-amyloid (Aβ) can also activate microglial cells [25,29]. The peptide increased NF-κB activation in primary rat astroglial cells [3,13], and Aβ (25–35) fragment dose-dependently caused release of TNF-α in microglia [25]. In glial-derived cells, β-amyloid caused a response that was similar to Al and in contrast to LPS, Aβ (25–35) did not cause increased levels of IL-6 [26].

While most studies showing parallels between Al and Aβ have been largely performed in isolated systems, it remains to be determined whether such similarities may also apply to more complex in vivo systems. Furthermore, it is not known whether co-exposure to Al and Aβ may modulate the inflammation induced by the peptide. These finding could then more solidly establish aluminum as a relevant contributory factor in the progression of AD. Furthermore, this study does not unequivocally prove aluminum is an immunogen and further thorough investigation in both in vitro and in vivo settings are necessary to reach such conclusion. However, this study does provide ample proof that aluminum does indeed activate cells of glial origin and by doing so, activates several parameters involved in the inflammatory cascade.

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