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# Aluminium blunts the proliferative response and increases apoptosis of cultured human cells: putative relationship to alzheimer's disease

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**Abstract:**
Aluminium (Al) has been investigated as a neurotoxic substance. Al ranks among the potential environmental risk factors for Alzheimer's disease (AD). Epidemiological studies tested the relationship between Al in drinking water and AD, showing a significant correlation between elevated levels of monomeric Al in water and AD, although data to date remain inconclusive with respect to total Al. The aim of this study was to test whether or not Al exacerbates cellular toxicity mediated by the amyloid β (Aβ) peptide. We evaluated the role of Al in modulating programmed cell death (apoptosis) in human cell cultures. We used the osteosarcoma cell line monolayer (SaOs-2) to demonstrate that treatment of SaOs-2 cultures with the Aβ peptide mid-fragment (25 to 35) at nano M, followed by co-incubation with physiological concentrations of aluminium chloride, which release monomeric Al in solution, led to marked expression of caspase 3, but not caspase 9, key markers of the apoptotic process. The same experimental conditions were shown to blunt significantly the proliferative response of normal human peripheral blood mononuclear cells (PBMC) to phytohemagglutinin (PHA) stimulation. Our observations support the hypothesis that Al significantly impairs certain cellular immune responses, and confirm that Al-mediated cell toxicity may play an important role in AD.

**Key Words:** human peripheral blood mononuclear cells; phytohemagglutinin (PHA); apoptosis; caspase; osteosarcoma cultures

**Background:**
The cause of Alzheimer's disease (AD) is closely correlated with the aggregation of the 1 to 42 Amyloid β (Aβ) peptide in the neocortex. The precipitation *in vitro* and *in vivo*, and consequential cell toxicity of Aβ is favored by metal ions, such as aluminium (Al) and iron (Fe). **[1]** An extensive literature has examined the putative role of Al as a risk factor for AD and for other neurological pathologies, including Parkinson's disease and amyotrophic lateral sclerosis, as well as systemic diseases, such as diabetes and cancer. **[2, 3]** The evidence to date remains mixed, primarily because of a range of methodological issues. No data to date demonstrated that the concentration of Al in the brains of AD patients is significantly higher than that of control subjects, however, detectable levels of Al have been reported in senile plaques and neurofibrillary tangles of subjects with AD. **[3]** Most epidemiological studies report total Al (Altot) as an indicator of exposure, perhaps because Al can be present in solution in several forms, not all of which are toxic. The monomeric fraction of Al consists of a non-water soluble component (e.g., monomeric organic complexes), and a water-soluble component. The latter is made up of free $Al^{3+}$ ions, of hydroxy complexes, and of inorganic complexes, including salts of chlorides, fluorides, sulphates, carbonates, phosphates, or silicates. The water soluble form of Al is characteristically toxic to eukaryotic cells, particularly as the $Al^{3+}$ ion and its hydroxy complexes. The pH of the physiological, cellular and cytoplasmic environment greatly influences the relative concentrations of these forms of Al. **[4]**

Epidemiological evidence, albeit rare, suggests a significant correlation between onset of AD and monomeric Al. **[4, 5]** Therefore, it may be important to estimate Al speciation in seeking to establish the association between Al exposure and AD.

The fact of being exposed to high concentrations of Al might accentuate certain neurological, and other diseases. High concentrations of serum total Al (greater than 10 mg per L) were noted, concomitant with high blood iron levels, in foundry workers who demonstrated as well more cognitive deficiencies, compared to control subjects. **[6]** However, oral bioavailability of Al is low (approximately 0.3 percent), and does not reflect stomach contents and water hardness. **[7]** Al may exacerbate cellular toxicity associated with Ab, which is thought to contribute to the etiology and evolution of AD.

**Description:**

We tested two distinct models to characterize the cytochemical mechanisms of Al toxicity. We used the established cell line of osteoblast-like osteosarcoma, SaOs-2 as a model system to test whether or not Al mediated programmed cell death (i.e., apoptosis), and to elucidate which apoptotic pathway might be involved. We also used peripheral blood mononuclear cells (PBMC) obtained from normal healthy elderly and young donors to test whether or not Al impair the proliferative response.

The subjects (n = 26) for this investigation were taken from our common pool of adult male and female donors (18 to 65 years of age) from the principal socio-ethnic groups in the U.S. Subjects signed informed consent. Subjects were screened for history of infectious, neoplastic and autoimmune diseases prior to donation**.**

Blood samples were processed within 12 hours of collection (room temperature, gentle shaking) according to established methods. **[8, 9, 10]**

The established human osteosarcoma cell line, SaOs-2, was obtained from the American Tissue Culture Collection (ATCC, Rockville, MD). SaOs-2 were maintained in exponential growth in AIM-V medium (Gibco) supplemented with 10 percent fetal bovine serum (Gibco). Viability was verified by trypan blue exclusion at each passage, following trypsin-mediated detachment from the plastic substratum (0.2 percent trypsin in 0.9 percent NaCl, 3 min, 37 degree C). On the day of the experiment, cultures were adjusted at a cell concentration of 5 million cells per ml in growth medium for experimental plating.

Aβ peptides from American Peptide Co. (Sunnyvale, CA) were reconstituted and stored at -70°C were used fresh for each experiment**.** Pilot experiments established the optimal conditions for test of cytotoxicity *in vitro* to be co-incubation for 24 to 48 h at 1 nano M to 0.1 nano M). The chloride salt of Al ($AlCl_3$, Sigma, St. Louis, MO) was used in these experiments, over a range of concentrations about nano M, as indicated in individual experiments. In the *in vitro* experiments we report here, the cells were cultured with physiological concentrations of Aβ peptides (i.e., 1 to 42, 1 to 28, 25 to 35, and 40 to 1 as control), and Al in the chloride salt form and at a concentration similar to that reported to resemble bioavailability of Al from drinking waters. **[7]**

To assess the modulation of apoptotic cell death by Al, and unless otherwise indicated in the text, experiments were plated at million cells per ml in: 1) untreated, 2) treated with 0.1 micro gram per ml camptothecin (CAM; Sigma) as control 3) treated with 0.1 micro gram per ml CAM and $AlCl_3$ (nano M), 4) treated with Aβ (40 to 1) anti-sense control peptide and $AlCl_3$ (nano M), 5) treated with the native Aβ (1 to 42) peptide; 6) treated with the native Aβ (1-42) peptide and $AlCl_3$ (nano M), 7) treated with the amino-terminal Aβ (1 to 28) peptide; 8) treated with the amino-terminal Aβ (1 to 28) peptide and $AlCl_3$ (nano M), 9) treated with the mid-Aβ (25 to 35) peptide, 10) treated with the mid-Aβ (25 to 35) peptide and $AlCl_3$ ($10^{-9}$ M).

For apoptotic cell death, we measured the cytoplasmic levels of nucleosomal histone protein (Roche, Indianapolis, IN). We identified the apoptotic pathway by immunocytobiochemistry by standard protocol. **[11]** We monitored caspase 3, the terminal caspase of the apoptotic pathway, which activates the caspase-dependent endonuclease for DNA fragmentation. We also assessed caspase 9 to test whether or not the mitochondrion-mediated pathway of apoptosis was involved.

We assessed traversal of late G1 and G1/S transition by monitoring the expression of cyclin D3 and of the phosphorylated and unphosphorylated forms of the retinoblastoma protein (pRb and Rb), as described elsewhere (Chiappelli and Liu, 1999). Immunoreactive bands were detected by color development, and, when dry, the membranes were scanned and computer analyzed with the Photoshop software.

To assess the modulatory effects of Aβ peptides and Al on T cell-specific functions, we monitored the ability of activated T cells to generate cytotoxic T lymphocytes (CTL) **[11]**, cell proliferation after incubation with phytohemagglutinin (PHA) and lipopolysaccharide (LPS). The generation of CTL involved a 7-day incubation of PBMC effectors in a mixed leukocyte reaction with mitomycin-C-treated (50 micro gram per ml mitomycin-C, 37 degree C, 30 min) RAJI target cells in the presence or the absence of the Aβ peptides and $AlCl_3$, according to our established criteria. **[11]**

All experiments were plated at million cells per ml in 5 conditions: 1) untreated control, 2) control treated with 0.1 micro gram per ml CAM; 3) control treated with (40 to 1, antisense) peptide, 4) treated with the Aβ protein (1 to 42), 4) treated with the Aβ peptide (1 to 28), 5) treated with the Aβ peptide (25 to 35).

PHA and LPS experiments were performed as follows: 1 in million cells per well in complete media were exposed to 5 micro gram per ml of PHA (Sigma Chem. Co., St. Louis, MO) and incubated for 48hours. Thereafter, 0.1 mL of medium containing LPS (100 g per mL) (E. coli; Difco, Detroit, MI, USA) was added to each well. The plate was then incubated for 48 hours in a humidified incubator at 37 degree C in 5 percent $CO_2$, and induction was arrested on ice.

Data, shown as raw data or percentage of control, or transformed as indicated in the text, were analyzed at $\alpha = 0.05$ (Stata5, Stata Corporation, College Station, TX) by ANOVA when the assumptions for parametric statistics (normality, independence and homogeneity of variance) were satisfied, followed by Dunnet post-hoc comparisons to compare the experimental conditions to the control group. If the assumptions for parametric statistics were violated, then the non-parametric Kruskall-Wallis, Friedman and Wilcoxon tests were used. The level of significance was set at 0.05, and corrected by Bonferroni as required. Optima were analyzed as described in Berenbaum. **[12]**

Treatment of SaOs-2 cultures with the Aβ peptide (25 to 35) followed by $AlCl_3$ leads to marked expression of caspase 3, but not caspase 9. These observations suggest that the apoptotic pathway induced by this treatment does not involve the mitochondrial pathway. Those results were confirmed by Western Blot Analysis (Figure 1a).

Figure 1b shows the modulation of the generation of cytotoxic T lymphocyte (CTL) activity by PBMC obtained from elderly healthy subjects. The results of these experiments demonstrate that, whereas the anti-sense Aβ peptide (40 to 1) had little effect upon the generation of CTL activity in either group, Aβ peptides (1 to 42) (1 to 28) and (28 to 35) all blunted by at least 20 percent this specific memory immune function. The data also unequivocally show a greater suppression of the generation of CTL activity when $AlCl_3$ (nano M) was added to culture.

The results obtained with PBMC show that Al significantly reduced the effect of PHA stimulation (Figure 1c), while it enhanced the effect of lipopolysaccharide (LPS) (Figure 1d). Although Aβ alone was able to induce those effects, Al seemed to contribute to boost that effect even at a very low concentration (nano M) at least when the 25 to 35 Aβ fragment was involved. However, Al (nano M) was able to counteract LPS effect on PBMC when it was added to the 1 to 28 and 25 to 35 fragments, as well as the 1 to 42 Aβ peptide, whereas there was no significant effect with the anti-sense Aβ peptide (40 to 1). Al leads to an inhibition of osteoblast function through activation of caspase 3, besides the mechanical effect of accumulating in bone. Al may actively modulate several cell functions, including circulating blood cells.

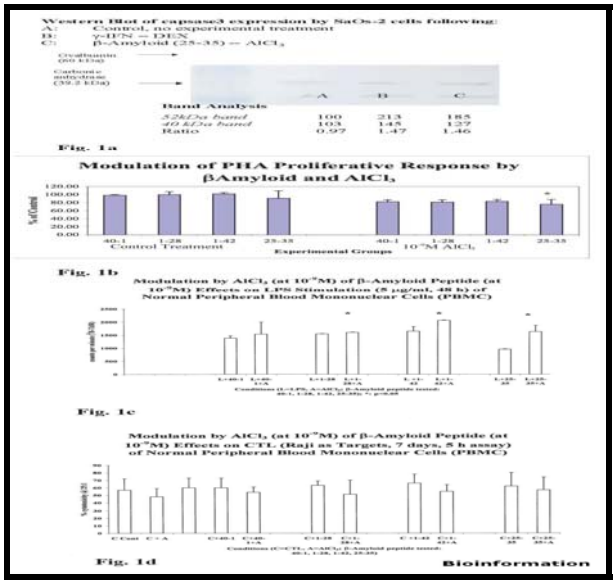


**Figure 1: (a)** Western blot of caspase 3 expression by Saos-2 cells following: A) control, no experimental treatment; B) γinterferon-dexamethasone (nano M; 24 hour); C) Aβ (25 to 35) (nano M; 48 hour) - $AlCl_3$ (nano M; 24 hour); **(b)** Modulation of PHA (5 micro gram per ml, 48 hour) proliferative response by Aβ and $AlCl_3$ (nano M) (* $p < 0.05$); **(c)** Modulation by $AlCl_3$ (nano M) of Aβ peptide. Effects on LPS stimulation (5 micro gram per ml, 48 hour) of normal PBMC (* $p < 0.05$); **(d)** Modulation by $AlCl_3$ (nano M) of Aβ peptide. Effects on CTL (Raji as target, 7 days, 5 hour assay) of normal PBM

T cell changes after small quantities of Al suggest that their putative role in AD etiology and/or progression depend on a cascade of events that may include common environmental factors. A full understanding of this effect is critical not only to identify possible disease mechanisms, but also to make an effective vaccine-based therapeutic approach to this devastating disease. **[13]**

The Aβ protein and its metabolic peptides, by adhering to the endothelial lining of the lumen of blood vessels, trigger and sustain effects on immune cells, which may seriously hamper immune surveillance in subjects with AD. This effect may be further enhanced by monomeric Al both in T cells and in bone precursor cells. **[14]** Alternatively, we can evoke a process by which Al may penetrate the plasma membrane of immune cells and immune bone precursor cells, and alter either directly or via some yet to be identified metabolic step, the chromatin reading frame, or assembly, repair and rearrangement processes. One would invoke a genomic effect of Al on these cells, interactomic and metabolomic sequelae, which would manifest in the decreased T cell function and the increased propensity to apoptosis. The preliminary data we report here provide strong evidence that Al might significantly harm T cell subpopulations, and hinder cellular immune surveillance mechanisms.

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