ALUMINUM NEUROTOXICITY INVOLVES OXIDATIVE STRESS, INFLAMMATION AND APOPTOSIS: IMPLICATIONS FOR NEURODEGENERATION

by

VICTOR JOHNNIE JOHNSON

(Under the Direction of Raghubir P. Sharma)

ABSTRACT

Aluminum is a ubiquitously present metal with no known physiological role. Nevertheless, abnormally high brain aluminum levels are evident in the brains of patients with neurodegenerative diseases including Alzheimer's disease. Epidemiological studies have linked high levels of aluminum in the drinking water with an increased risk of both disease and non-disease dementia and cognitive loss. The objectives of the present studies were to define the role of immune effector functions in aluminum-induced disruption of neuron-glial interactions.

Aluminum has been shown to induce the expression of tumor necrosis factor α (TNFα) in the cerebrum of mice and also in human glioblastoma cells in culture. We show here that aluminum as $AlCl_3$ markedly reduced the production of TNFα in murine microglial cells. This effect was mediated by the production of reactive oxygen species (ROS) and lipid peroxidation since antioxidant treatment prevented the effects. This study indicates that oxidative stress is an integral component of aluminum neurotoxicity.

Neuron-like cells were also affected by aluminum. Treatment of PC-12 cells with $AlCl_3$ resulted in a loss of membrane fluidity concomitant with increased proton extrusion and aberrant membrane physiology including hyperpolarization. These effects were related to an increase in ROS production. Aluminum maltolate (Al-malt), a lipophilic complex of aluminum was more toxic. Apoptosis and necrosis were induced by Al-malt in Neuro-2a cells. Cell death induced by Al-malt was dependent on *de novo* protein synthesis but not on major kinase pathways. In addition, alterations of calcium homeostasis may also be involved. Interestingly, leakage of cytoplasmic protein was evident even in cell dying by apoptosis and indicates that neuronal death may activate secondary inflammation leading to further neurodegeneration.

Al-malt cytotoxicity in rotation-mediated aggregate cultures was correlated with an increase in pro-inflammatory cytokine expression and a marked decrease in neurotrophin expression. An alteration of the cytokine/neurotrophin balance may be central to the etiopathogenesis of aluminum neurotoxicity. Overall the results of these studies further strengthen the role of oxidative stress in aluminum neurotoxicity. We

have also identified a new mechanism by which aluminum can damage the brain, deregulation of the cytokine/neurotrophin balance. Alterations in this balance may also exist in human neurodegenerative diseases.

INDEX WORDS: Aluminum; Neurodegeneration; TNFα; Inflammation; Neurotrophin; Neurotoxicity; Oxidative stress

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DEDICATION

I dedicate this dissertation to my family, my wife Dana and my children Dylan, Brielle and Levi. Their unconditional love, support and understanding were integral to the completion of my studies.

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CHAPTER 1

INTRODUCTION AND SIGNIFICANCE

Progressive loss of coginitive brain functions and dementia are two conditions that plague the aging human population. A diversity of environmental factors and xenobiotics are likely to play an important role in 'normal' loss of brain functions observed in many aging humans. Evidence from epidemiological studies has suggested that chronic exposure to levels of aluminum in the drinking water as low as 0.1 ppm may pose a risk for the development of aging-related neurological impairments (Jacqmin *et al.*, 1994; Forbes *et al.*, 1995; Gauthier *et al.*, 2000). Importantly, Walton *et al*. (1995) demonstrated that aluminum in the drinking water could be taken up into the brain. Subsequent studies have indicated that aluminum can cause neurobehavioral alterations in animal models (Alleva *et al.*, 1998) and humans (Kilburn, 1998). In addition, aluminum increased the expression of nerve growth factor in the central nervous system (Fiore *et al.*, 2000), a key factor in neuronal survival and the plasticity in higher brain functions (Alleva *et al.*, 1998).

Despite its ubiquitous presence in the environment and every organism, no known physiological role for this metal has been established. A mechanistic basis for the toxicity of aluminum remains to be established. The induction of oxidative stress following exposure to aluminum has been demonstrated in both *in vitro* (Campbell *et al.*, 1999) and *in vivo* (Deloncle *et al.*, 1999) studies. We have previously demonstrated that aluminum can alter immune functions within the CNS, specifically increased tumor necrosis factor α (TNFα) mRNA expression in the cerebral cortex (Tsunoda and Sharma, 1999). Many inflammatory genes including TNFα are responsive to changes in oxidative status. Reactive oxygen species, namely hydrogen peroxide is a known potent activator of nuclear factor κB, a transcription factor controlling the expression of many immune

related genes. The possibility for interaction between aluminum-induced changes in oxidative stress and the production of cytokines within CNS tissue requires investigation.

Studies have demonstrated the impact of aluminum on both the immune system and the nervous system but information is lacking on the impact of this metal on the interactions between the two systems. Given the continued use of aluminum in water treatment and the increasing groundwater contamination due to increased solubility from acid rain, a better understanding of the mechanisms of aluminum neurotoxicity will dramatically improve human health risk assessment.

The objective of the research comprising this dissertation was to test the hypothesis that **the toxicity of aluminum is defined by and interplay between oxidative stress induced by aluminum and the production of soluble mediators, including proinflammatory cytokines and neurotrophins in the CNS**. To fulfill this objective the following specific aims were tested;

1) determine the role of aluminum-induced oxidative stress in TNFα production in murine microglial cells, the innate immune system in the brain.

2) investigate the impact of chemical form of aluminum on membrane function and cell death in neuronal cells

3) examine the role of proinflammatory cytokines and neurotrophins in aluminum-induced toxicity using a 3-dimensional primary culture model.

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CHAPTER 2

LITERATURE REVEIW

Aluminum, the metal.

Aluminum is a metal in Group IIIa of the periodic table. It is the third most abundant element in the earth's crust next to oxygen and silicon. Aluminum generally exists in one of two oxidation states, elemental aluminum (Al^0) and an ionic form (Al^{3+}). Due to extremely high reactivity aluminum rarely exists as a free metal in nature. It is usually in a complex with other elements including oxygen, silicon and fluorine (Dinman, 1983). These forms of aluminum are generally considered biounavaiable.

Use and exposure.

As stated above, aluminum is a ubiquitous element in the earth's crust surpassed only by oxygen and silicone (Davis *et al.*, 1987). Surface aluminum is rapidly oxidized and is largely considered to be in a biologically unavailable form (Beliles, 1991). There are no known biological requirements for aluminum but the societal uses are vast and therefore exposure is high. Several aluminum salts are utilized and contacted by humans and animals. Examples included aluminum chloride and aluminum chlorohydrate in antiperspirants (Laden, 1999) and aluminum sulfate and aluminum ammonium sulfate in drinking water treatment (Cohen *et al.*, 1971). Flarend *et al*. (2001) demonstrated that low levels of aluminum were absorbed following antiperspirant application in human subjects. This study indicates that dermal application is a potential exposure route for the general public. Aluminum phosphide is a common pesticide used as an insecticidal grain fumigant (Singh *et al.*, 1996) where oral ingestion and inhalation are common routes of exposure.

Medicinal exposure includes the use of aluminum hydroxide as the active ingredient of antacids (Brunton, 1996). Antacids are commonly used as an initial treatment for acid-reflux disease and peptic ulcers (Brunton, 1996). Aluminum in its elemental form is insoluble in water but salt formation as well as acidic conditions greatly increases its solubility (Boegman *et al.*, 1984). Therefore, it is possible to get systemic absorption of aluminum from antacid use. Acidic beverages and foods are often stored in aluminum containers presenting another potential source of exposure.

Increased aluminum contamination in the groundwater has been associated with increased acid rain fall. Groundwater contamination with aluminum has been increasing in recent years (Boegman *et al.*, 1984), levels being reported as high as 2.7 mg aluminum/l in finished drinking water (Miller *et al.*, 1984). Solubilization of the aluminum makes it bioavailable and thus a potential threat (Davis *et al.*, 1987) and studies have shown uptake of aluminum in rat brain following exposure to aluminum treated drinking water (Walton *et al.*, 1995).

Daily intake of aluminum from the diet in humans has been reported from several sources. Pennington *et al.* (1995) reported that the daily intake for teenage and adult females was 9 mg/day whereas the daily intake for teenage and adult males was 12-14 mg/day. Dutch adults were reported to consume less, 3.1 mg/day, aluminum (Ellen *et al.*, 1990). The current exposure estimate according to the Food and Drug Administration's Total Diet Study dietary exposure model is 8 and 7 mg/day for adult males and females, respectively. The main source of aluminum in the diet is from soil particles left on fresh foods, thus the majority may not be bioavailable. The absorption of aluminum from the diet is reported to be between 0.01% and 0.04% (Greger *et al.*, 1992) although absorption

can reach as high as 1% when consumed with dietary constituents like citrate (DeVoto *et al.*, 1994).

Tissue aluminum concentrations in humans have also been reported. The concentration of aluminum in normal brain has been estimated to range from 1.9 to 4.8 μg/g dry weight (Crapper *et al.*, 1976; Andrasi *et al.*, 1996). Aluminum levels in serum, whole blood and urine have been reported at 0.219, 0.368 and 0.092 μg/ml, respectively (Naylor *et al.*, 1990). The concentration of aluminum in human hair was determined to be 6.42 μg/g dry weight (Naylor *et al*., 1990). These studies indicate that exposure to aluminum in our environment contributes to a body burden of this metal.

Aluminum toxicokinetics.

The toxicokinetics of aluminum are very complex due to factors including speciation of aluminum, route of exposure, vehicle of exposure (i.e. drinking water), pH and co-injested ligands. For a comprehensive details of aluminum toxicokinetics, readers are referred to the review by Yokel and McNamara (2001a). The primary route of exposure to aluminum is oral. Oral absorption of aluminum is dependent on both solubility and bioavaiablility, which are determined by chemical form. Insoluble salts of aluminum including silicates and hydroxides show a very low level of absorption of 0.01% (Priest *et al.*, 1996). The favored aluminum species at low pH is the free Al^{3+} ionic form (Kiss *et al.*, 1998). Thus the acidic pH of the gut would promote the solubilization of the majority of ingested aluminum regardless of the chemical form. There is a possibility for absorption of this species via paracellular routes in the gut. Once in the Al^{3+} state, aluminum will compete for ligands, which ultimately determine

the bioavaiability of this metal. If the Al^{3+} binds to it original anion, the absorption tends to be well below 1% (Yokel *et al.*, 1988). In contrast, if the Al^{3+} binds to an organic anion such as citrate, absorption approaches 1% in humans (Day *et al.*, 1991; DeVoto *et al.*, 1994) and has been reported as high as 2.18% when administered to rabbits as aluminum citrate (Yokel *et al.*, 1988). When excess sodium citrate was ingested with aluminum citrate, the oral absorption was estimated at 5.1% in rats (Schonholzer *et al.*, 1997). The lack of complexation in the stomach accounts for the low absorption in the absence of organic ligands. This is due to the abrupt pH change to near neutrality in the lower intestine. Any Al^{3+} remaining will rapidly precipitate as non-absorbable aluminum hydroxide complexes and be excreted in the feces.

Dermal and inhalation are also significant routes of exposure to aluminum from the application of antiperspirants (contact and aerosol) and in the workplace with aluminum fumes and dust. All antiperspirants can potentially be absorbed transdermally although the contribution to total daily burden is expected to be low. This is due to the water-soluble nature of the aluminum compounds used in antiperspirants, which should not diffuse across the lipid rich epidermis. Flarend *et al*. (1997) showed that only 0.012% of an applied dose of ^{26}Al-labled aluminum chlorohydrate, a common constituent of antiperspirants was absorbed following application to the underarm of human volunteers. Aerosol forms of aluminum-based antiperspirants present a unique opportunity for this source of aluminum to enter the body via dermal, inhilation, oral and nasal-olfactory routes. Aluminum fumes in the workplace can also be absorbed through inhalation and nasal-olfactory routes. Indirect evidence suggests that aluminum fumes may result in absorption in the lung. Urinary aluminum dropped from 82 to 29 mg/L in welders

following a 16-37 day exposure-free interval (Sjogren *et al.*, 1988). Direct uptake of aluminum via the nasal-olfactory route has also been demonstrated in laboratory animals (Perl *et al.*, 1987). This exposure route is significant because once the aluminum enters the olfactory region of the brain it can be transported by axonal transport to other regions of the brain, thus circumventing the blood-brain barrier. This route requires further investigation.

Once absorbed into the body, aluminum is carried in the blood bound to transferin (~80%) and citrate (~20%) and transported to tissues. Aluminum is equally distributed between plasma and cells within the blood. It has been estimated that bone and lung contain ~ 50 and 25% of the total body burden of aluminum (Yokel *et al.*, 2001a). Distribution to the brain is also possible despite the presence of the blood-brain barrier, total concentration being estimated at 1% of total body burden (Yokel *et al.*, 2001a). Studies have demonstrated that aluminum can enter the brain via transferring receptor-mediated endocytosis of aluminum-transferin complexes (Roskams *et al.*, 1990) and also monocarboxylate transporter mediated uptake of aluminum-citrate (Yokel *et al.*, 1999; Yokel *et al.*, 2002). This low level of aluminum distribution to the brain indicates that the nervous tissue is a susceptible target tissue and only low levels are needed to induce aberrant pathophysiological changes.

The elimination of aluminum from the body is very slow. The half life for total body aluminum has been estimated at 7 years in humans and likely reflects redistribution from bone stores (Yokel *et al.*, 2001a). Of more relevance to the neurotoxicity of this metal, the half life of aluminum elimination in the brain is estimated at >100 days (Greger *et al.*, 1997). A more recent study confirmed the retention of aluminum in the

brain showing a half life of 150 days (Yokel *et al.*, 2001b). The long half-life coupled with the unavoidable and continuous exposure to aluminum suggests that this metal may bioaccumulate with age. Studies have demonstrated that aluminum absorption is higher in aged Alzheimer's disease patients than in age-matched controls (Moore *et al.*, 2000) and that the brain aluminum content increases with age (Markesbery *et al.*, 1981).

Toxicity of Aluminum.

The overt clinical toxicity of aluminum is highly dependent on its chemical form. This is due to the great differences in bioavailability among the various salt forms of aluminum (Beliles, 1991). Rabbits are known to be sensitive to the effects of aluminum although the repeated administration of aluminum oxide via intravenous injection failed to cause toxicity (Wada, 1985). A similar lack of toxicity was demonstrated following acute administration of aluminum phosphate in rabbits and aluminum hydroxide in humans (Wada, 1985). Other salts of aluminum are toxic. The LD_{50} for aluminum chloride was reported at 3700 mg/kg and 3805 mg/kg in rats and mice, respectively. Aluminum sulfate was shown to be more toxic, the LD_{50} being 770 mg/kg and 270 mg/kg following oral and intraperitoneal injection in mice, respectively. The LD_{50} for aluminum sulfate was even lower in rabbits at 100 mg/kg via subcutaneous injection (Venugopal *et al.*, 1978).

The contribution of organic ligands to the toxicity of aluminum has been controversial. Citrate increases the absorption of aluminum both from the gastrointestinal tract and into the brain. At the same time, citrate may promote the exflux of aluminum from the brain via the monocarboxylate transporter and may lower total brain levels

(Yokel *et al*., 1999). Maltolate is another organic ligand that can increase the distribution of aluminum into the brain. Repeated intraperitoneal injection of aluminum-maltolate complex was shown to significantly increase brain aluminum burden (Ogasawara *et al.*, 2002). The lack of increased brain aluminum in the citrate group may be due to the promotion of aluminum efflux by citrate and a net decrease in overall brain levels. Aluminum-transferin is the predominant species in the blood and administration of this form has been shown to increase brain levels (Yokel *et al*., 1999). Sufficient evidence indicates that absolute levels of aluminum ingested in the diet may not be a proper indicator of exposure and ultimately toxicity. It may be more appropriate to consider the level of aluminum in the context of available organic ligands also present in the diet.

Neurotoxicity of Aluminum.

Biologically available aluminum has been shown to be a neurotoxic substance. Intracerebral injection of aluminum was shown to induce neurofibrillary degeneration in rabbits (Klatzo *et al.*, 1965) and cats (De Boni *et al.*, 1976). Similar lesions were reported in rabbits exposed systemically to aluminum (De Boni *et al*., 1976; Katsetos *et al.*, 1990). These lesions were composed primarily of intermediate neurofilament proteins thus differing from the paired helical filaments of *tau* seen in Alzheimer's disease tangles. Later studies demonstrated that some aluminum-induced tangles contained aberrantly phosphorylated *tau* proteins (Singer *et al.*, 1997)). The exact relationship of these tangles to the lesions seen in Alzheimer's disease brains remains controversial (Muller *et al.*, 1994). Aluminum has been shown to associate with chromatin (De Boni *et al.*, 1980) and to alter the properties of neurofilaments *in vitro*

(Muller *et al.*, 1994). It has been suggested that aluminum may alter the phosphorylation of neurofilament, thus altering axonal transport and promoting filament aggregation (Bizzi *et al.*, 1986).

Aluminum has been shown to induce neurobehavioral alterations in the absence of pathological changes in the cytoskeleton. Aluminum-lactate treatment was shown to decrease motor activity, grip strength and the startle response in mice following dietary exposure (Golub *et al.*, 1992). Exposure to aluminum lactate during early development (conception to 35 days of age; Golub *et al.*, 2001) and during puberty (Golub *et al.*, 1999) also caused motor and neurobehavioral deficits in mice. All of these changes were observed in the absence of gross pathological changes in the brain. Therefore, aluminum must impact cellular systems in addition to cytoskeletal elements.

Cellular membranes are integral to cell function and alterations in composition and fluidity can be detrimental to the survival of the cell. Numerous studies demonstrate that aluminum reduces membrane fluidity in liposomes and artificial phospholipid membrane vesicles (Deleers *et al.*, 1985; Deleers *et al.*, 1986; Akeson *et al.*, 1989). Aluminum has also been shown to induce lipid peroxidation (Oteiza *et al.*, 1993) or exacerbate iron-mediated lipid peroxidation (Bondy *et al.*, 1998a). Lipid peroxidation is known to induce membrane rigidity (Bolotina *et al.*, 1985) and represents a plausible mechanism for the effect of aluminum on membrane fluidity. Membrane rigidification was suggested as the mechanism by which aluminum altered the electrophysiological response to hypertonic shock in N1E-115 neuroblastoma cells (Sorek *et al.*, 1992) indicating the possibility of disrupted signaling in the brain upon aluminum exposure.

Further research is needed to define the role of membrane alterations in aluminum neurotoxicity.

Neurotransmitters and their metabolites are also sensitive to aluminum exposure. (Tsunoda *et al.*, 1999a) reported that administration of aluminum in the drinking water caused a decrease in dopamine and its metabolites in the hypothalamus of mice. Effects of aluminum on the dopamine system have been reported in other studies. Flora *et al*. (1991) found that oral exposure to aluminum nitrate lead to a significant decrease in dopamine as well as serotonin. A possible explanation for this decrease in dopamine and other neurotransmitters is an increase in uptake and metabolism following exposure to aluminum (Lai *et al.*, 1981). Other studies have indicated that aluminum has no effect on neurotransmitters in the brain (Wenk *et al.*, 1981; Wenk *et al.*, 1982). The exact effects of aluminum on neurotransmission remain unclear due to these inconsistencies.

Aluminum and neurodegenerative diseases.

The similarity between aluminum-induced changes in the central nervous system (CNS) and those apparent in many neurodegenerative diseases has prompted researchers to consider aluminum as a possible etiologic agent in these diseases. Dialysis dementia is a progressive neurological syndrome common among patients undergoing dialysis for chronic renal failure (Arieff *et al.*, 1979). The dementia that is a hallmark of this condition is very similar to that seen in Alzheimer's disease. Analysis of aluminum body burden has indicated a 15-fold increase in patients suffering from dialysis dementia (Dunea *et al.*, 1978). Further corroborating evidence came from Scotland where dialysis dementia only occurred in three geographical regions where aluminum content in the

water supply was elevated (Elliott *et al.*, 1978) and high aluminum concentrations (1000 μg/l) in the dialysate was associated with encephalopathy (Flendrig *et al.*, 1976). In addition, the use of oral aluminum hydroxide as a phosphate binder to prevent hyperphosphataemia was strongly linked to the development of dialysis encephalopathy in patients on renal dialysis and those not on dialysis (Dewberry *et al.*, 1980; Rotundo *et al.*, 1982). Additionally, desferrioxamine, a strong chelator of aluminum, was shown to be an effective treatment for dialysis dementia (Ackrill *et al.*, 1980). These studies and others implicate aluminum as a key etiological agent in dialysis dementia.

The neurotoxicity of aluminum manifests in many forms that are dependent on factors including aluminum speciation, animal species, exposure route and duration. Resulting from this complexity in response, the use of aluminum as an experimental neurotoxicant has recapitulated virtually every feature of the neurodegenerative spiral afflicting Alzheimer's patients (Strong, 2002). Early studies in rabbits showing neurofibrillary aggregations produced by aluminum exposure suggested that aluminum may be the agent responsible for senile plaques seen in Alzheimer's patients. In addition, the concentration of aluminum in the brains of Alzheimer's patients was found to be higher than in normal brains (Crapper *et al.*, 1980). Perl and Brody (1980) reported that the increase in aluminum was concentrated in neurofibrillary tangle-bearing neurons and senile amyloid plaques. Further investigations revealed that aluminum-induced tangles in experimental animals differed both chemically and structurally from the tangles characteristic of dementia (Crapper McLachlan *et al.*, 1992). Despite these findings, epidemiological studies identified high in aluminum in the drinking water and soil to be a risk factors for Alzheimer's disease (Neri *et al.*, 1991) and amyotrophic lateral sclerosis

and Parkinson's disease (Perl *et al.*, 1982), respectively. Recently, Praticò *et al*., (2002) demonstrated that aluminum increased amyloid β levels and accelerated senile plaque formation in a murine model of Alzheimer's disease. This study strengthens the contention that aluminum is an environmental factor that contributes to the progression of Alzheimer's disease.

A recent epidemiological study has reported a link between aluminum levels greater than 100 μg/L in drinking water low in silica and Alzheimer's disease in elderly subjects (Rondeau *et al.*, 2000). Re-analysis of the data in the Rondeau *et al*. (2000) study indicated that there was a positive correlation between cognitive decline in the elderly and aluminum levels in the drinking water in excess of 100 μg/L (Rondeau *et al.*, 2001). This suggests that aluminum in the drinking water may be an environmental factor involved in the cognitive loss associated with aging. Aluminum may also be a factor involved in the cognitive loss that is often observed years prior to demonstrable neurodegeneration in Alzheimer's patients. Markesbery *et al*. (1981) demonstrated progressive aluminum accumulation in the brain during aging. The accumulation of aluminum in the human brain was shown to be region specific, significant accumulation occurring in the hippocampus and neocortex (Xu *et al.*, 1992), areas of the brain important in cognition and memory. Thus, aluminum may be involved in the progressive cognitive loss observed in Alzheimer's disease even prior to observable neurodegeneration.

Cytokines in the brain.

The long-standing contention of the CNS being an immune privileged organ has eroded in the last few decades (Akiyama *et al.*, 2000). Immune cell recruitment is suppressed but not excluded from the CNS by the presence of the blood-brain barrier. Mechanistically, this suppression represents a safe-guard against secondary immune-mediated brain damage (Bechmann *et al.*, 2001). Consequently and to the detriment of the brain, this organ does not benefit from the tissue regenerative properties of cytokines and other immune mediators associate with inflammation to the same extent as other tissues. Nevertheless, cytokines and their network of interactions play vital roles in normal brain physiology and homeostasis.

The primary cells of the immune system within the brain are the glial cells. Under normal physiological condition, glial cells are involved in the production of trophic factors and in the maintenance of neuronal survival within the CNS (Ridet *et al.*, 1997; Anderson *et al.*, 2000). Among the trophic factors released are various cytokines. Cytokines can act in an autocrine and/or paracrine manner. The physiological consequence of these molecules within the CNS is defined by the expression of functional receptors for the specific cytokines. The presence of cytokines and their receptors is due mainly to local synthesis within the brain. Other sources include uptake via circumventricular organs where there is no blood-brain barrier and also by migration of cells and cytokines across the blood-brain barrier under condition of compromise such as peripheral inflammation following extreme bacterial infection (Turrin *et al.*, 2000). Cytokines can share common physiological functions and this can lead to positive and negative interactions within the brain. An example of a positive interaction is the

relationship between tumor necrosis factor α (TNFα) and interleukin-1β (IL-1β). Both cytokines can induce a fever response and together their actions are synergistic (Bluthe *et al.*, 1994). In contrast, IL-1β and IL-1β receptor antagonist interact in antagonist manner. Over expression of IL-1β receptor antagonist has been reported to confer resistance to the lethal effects of lipopolysaccharide (LPS) in transgenic mice (Doughty *et al.*, 1997).

Cytokines in the CNS play a role in a diverse array of physiological responses including sleep, feeding, fever and peripheral immune regulation (Turrin *et al.*, 2000). The dual nature of many cytokines, neuroprotective and neuropathological, becomes evident when examining their role in these processes. TNFα is a classic example of a pro-inflammatory cytokine often associated with neurotoxicity. Prolonged synthesis of TNFα has been shown to result in cognitive dysfunction and neurobehavioral alterations (Fiore *et al.*, 1996; Fiore *et al.*, 2000). In contrast, TNFα plays a vital role in the control of sleep-wake cycles and the lack of TNFα has been shown to reduce sleeping time (Krueger *et al.*, 1999). Cytokines are also important in feeding control and the development of anorexia. LPS-induced overproduction of proinflammatory cytokines including TNFα and IL-1β is associated with a decreased feeding response and the onset of anorexia, the severity of which is dependent on the level of these cytokines in the brain (Plata-Salaman *et al.*, 1998). Muramyl dipeptide is another bacterial cell wall component capable of inducing anti-inflammtory cytokines in addition to pro-inflammatory cytokines. Plata-Salaman *et al*. (1998) showed that anorexia induced by muramyl dipeptide was reduced compared to that of LPS. This effect was due to a greater increase in IL-1β receptor antagonist expression in the muramyl dipeptide treated animals compared to that induced by LPS. Studies like these demonstrate that a balance between

pro- and anti-inflammatory cytokine may be more important to maintenance of homeostasis than any one cytokine. Disruption of this balance can lead to neuropathological manifestations and has been implicated in neurodegenerative diseases.

Nerve growth factor (NGF; Fiore *et al*., 2000) is a cytokine that is constituatively expressed in the brain and functions in differentiation and survival of many populations of neurons. Spatial learning and memory are also influenced by the expression of NGF implicating this cytokine in neural plasticity (Thoenen, 2000). Aged anti-NGF transgenic mice display a severe cholinergic deficit in the basal forebrain concomitant with marked decreases in learning and memory function (Capsoni *et al.*, 2000). Given the opposing role of pro-inflammatory cytokines like TNFα in cognitive function, research into the interaction between pro-inflammatory cytokines and neurotrophic factors has recently gained focus. TNFα inhibits the development of long-term potentiation in hippocampal slices (Tancredi *et al.*, 1992), a process regarded as important for memory function. Transgenic mice over-expressing TNFα show a reduced expression of NGF in the hippocampus that correlates with learning deficits (Fiore *et al*., 2000). Biochemical studies indicated that signaling induced by non-toxic levels of TNFα was able to diminish the capacity of insulin-like growth factor I to promote survival in primary cerebellar granule neurons (Venters *et al.*, 2000). Aluminum treatment has been shown to increase the levels of TNFα (Tsunoda *et al.*, 1999b) and NGF (Santucci *et al.*, 1994; Alleva *et al.*, 1998) in the brain of mice. Collectively, these studies demonstrate an antagonistic relationship between TNFα and NGF, the balance of which is critical for proper brain function. The balance between TNFα and NGF signaling may play an integral role in aluminum neurotoxicity and involvement in neurodegenerative disease.

Cytokine balance and inflammation in Alzheimer's disease.

A large body of evidence indicates that the cytokine balance in the Alzheimer's disease brain is shifted towards a state of pro-inflammation (Akiyama *et al*., 2000; Barja, 2002). Researchers are now asking: Does the inflammation occur as a result of the pathological changes in the brain or are inflammatory events contributory to disease development? Elevated levels of TNFα and IL-1β have been detected in brain tissue from Alzheimer's disease patients (Akiyama *et al*., 2000). Similarly, increased production of TNFα, IL-1β and interleukin-6 (IL-6) from non-stimulated cerebral microvessels is evident in Alzheimer's disease brains compared to microvessels from normal brain (Grammas *et al.*, 2001). Interestingly, these inflammatory cytokines are also rapidly increased following head injury (Shohami *et al.*, 1994) and in patients with epilepsy (Ichiyama *et al.*, 1998), two known risk factors for the development of Alzheimer's disease. The appearance of elevated levels of pro-inflammatory cytokines in conditions that predispose patients to the development of Alzheimer's disease suggests that the cytokines may be involved in development of the disease.

Activated microglial cells have been shown to associate with the smallest and earliest plaques in amyloid precursor protein (APP) overexpressing transgenic mice supporting an early involvement of inflammation in Alzheimer's disease progression (Stalder *et al.*, 1999). Cells aggregating around diffuse plaque from human brain were shown to be immunoreactive for IL-6 even in the absence of dystrophic neuritis (Bauer *et al.*, 1991). Similar association of IL-1β immunoreactive cells with early plaques has been established (Griffin *et al.*, 1995). These data strongly implicate inflammation in the development of Alzheimer's disease pathology and not simply a consequence of it. In

fact, once a senile plaque has completely matured, cell association and cytokine overexpression is no longer detectable (Hull *et al.*, 1996).

In addition to cytokines, free radicals are important mediators of inflammatory events in the brain. The brain is exquisitely sensitive to oxidative stress mainly due to a high rate of energy consumption and the vast abundance of lipids susceptible to free radical damage (Pratico, 2002). Reactive oxygen species (ROS) are commonly generated by breakdown of the electron transport in the mitochondria of respiring cells. Another source of copious amounts of ROS are activated inflammatory cells including microglia (Colton *et al.*, 2000). The oxidative burst capacity of microglial cells functions to eradicate infectious agents from the CNS. Under conditions of inflammation, the production of ROS may become aberrant resulting in host tissue damage. Evidence for the involvement of ROS and oxidative stress in neurodegeneration and Alzheimer's disease include the detection of lipid peroxidation (Good *et al.*, 1996) and increased brain isoprostane levels (Pratico *et al.*, 1998; Pratico *et al.*, 2000). In addition, increased activation of NADPH oxidase and superoxide production are evident in Alzheimer's disease brain (Shimohama *et al.*, 2000). Reactive nitrogen species are also produced under conditions of inflammation and have been implicated in the pathogenesis of Alzheimer's disease (Akiyama *et al*., 2000)). Increased expression of endothelial and inducible nitric oxide synthase (NOS), enzymes responsible for NO production, has been detected Alzheimer's disease brains (Luth *et al.*, 2001). Luth *et al*. (2001) also demonstrated increase expression of NOS in APP23 transgenic mice over-expressing the Swedish double mutation in the APP gene.

As for cytokines in Alzheimer's disease the questions remains: Does the aberrant generation of free radicals induce the formation of pathological lesions characteristic of Alzheimer's disease or is oxidative stress a secondary event? Isoprostane levels in urine, plasma and CSF fluid are regarded as excellent indicators of *in vivo* oxidative stress (Pratico *et al*., 2000). Recently, it was demonstrated that isoprostane levels were higher in the CSF of Alzheimer's disease patients than non-demented controls (Montine *et al.*, 1999). Further investigations indicated that the level of isoprostanes in CSF were positively correlated with Alzheimer's dementia severity as measured by the mini-mental state examination and the dementia severity rating scale (Pratico *et al*., 2000). Since pathological time course is not feasible in human studies, these studies still have not definitively demonstrated that oxidative stress is a possible instigator of Alzheimer's disease. Studies using APP transgenic mice Tg2576 confirmed that isoprostane levels correlated with disease progression. In fact, Pratico *et al*. (2001) showed that increased isoprostane levels were detectable starting at 8 months of age, a full 4 months prior to the appearance of a few diffuse amyloid plaques. Therefore, oxidative stress likely plays an important role the early stages of Alzheimer's disease development. It is clear from the above discussion that inflammation and many of the mediators produced during inflammation play a critical role in neurodegeneration and disease progression in Alzheimer's disease patients.

Aluminum and cytokines.

Studies on the effects of aluminum on the immune system are rare. Golub *et al*. (1993) investigated the effects of dietary aluminum on mitogen-induced cytokine

production. This study indicated that concanavalin A-induced production of interleukin-2, interferon-γ and TNFα were reduced in mice that were exposed to aluminum in the feed. The opposite effect was reported for TNFα expression in the brain of aluminum treated mice. Tsunoda and Sharma (1999b) found that the expression of TNFα mRNA increased in the cerebrum of mice treated with aluminum via the drinking water. Recently cDNA microarray analysis in human neural progenitor cells demonstrated that aluminum up-regulated the expression of several pro-inflammatory genes including APP, IL-1, Fas binding protein and nuclear factor κB (NFκB) (Lukiw *et al.*, 2001). Supporting these findings, *in vitro* exposure to aluminum salts for 6 days resulted in increase TNFα production in human T98G glioblastoma cells (Campbell *et al.*, 2002). Collectively, these studies demonstrate that inflammation and pro-inflammatory cytokines are important mediators of aluminum neurotoxicity and further mechanistic research is warranted.

Aluminum and reactive oxygen species.

Aluminum is not a transition metal and thus cannot initiate oxidative injury. Interestingly, aluminum has the ability to greatly enhance the oxidative properties of iron, which is a transition metal (Xie *et al.*, 1996a; Xie *et al.*, 1996b). Several studies have reported the ability of aluminum to facilitate iron-mediated lipid peroxidation (Evans *et al.*, 1992; Xie *et al.*, 1996a; Xie *et al.*, 1996b; Verstraeten *et al.*, 1997; Savory *et al.*, 1999). Lipid peroxidation is a consequence of ROS and reactive nitrogen species production and is an indirect measure of a metal's ability to promote oxidation. Aluminum has also been shown to significantly enhance the iron-mediated production of

ROS *in vitro* (Xie *et al.*, 1996a; Bondy *et al.*, 1998b). Brain levels of iron are increased, especially in the basal ganglia, in patients with Alzheimer's disease (Bartzokis *et al.*, 2000). Therefore, it is conceivable that an interaction between aluminum and iron could take place in the brain leading to increased oxidative stress. Recent evidence suggests that aluminum is capable of stabilizing iron in the Fe^{2+} state, thus supplying fuel for the Fenten reaction leading to increased ROS production (Yang *et al.*, 1999).

Oxidative stress is an important factor influencing the progression of neurodegenerative diseases like Alzheimer's (Pratico *et al.*, 2002) and aging associated declines in cognitive brain functions (Barja, 2002). As mentioned previously, Praticò *et al*. (2002) demonstrated that aluminum treatment increased amyloid plaque formation in the Tg2576 APP transgenic mouse model. Aluminum treatment significantly increased brain isoprostane levels, a sensitive marker of *in vivo* lipid peroxidation. The increase in isoprotane levels correlated with the accumulation of amyloid β and plaque formation. Interestingly, administration of vitamin E prevented the aluminum-induced increase in isoprostane levels and ameliorated the acceleration of disease in aluminum-treated animals. These results strongly suggest that aluminum-induced oxidation promoted the Alzheimer's-like amyloidosis and therefore, it is possible for the same outcome in human Alzheimer's patients exposed to aberrant levels of aluminum.

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CHAPTER 3

DECREASED TUMOR NECROSIS FACTOR α PRODUCTION IN MURINE MICROGLIAL CELLS TREATED WITH ALUMINUM IS MEDIATED BY REACTIVE OXYGEN SPECIES: IMPLICATIONS FOR NEURODEGENERATION[1]

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Abstract

Oxidative stress and inflammation are components of many neurodegenerative conditions and the role of environmental factors is not fully understood. Reactive oxygen species and lipid peroxidation play an integral role in aluminum-induced neurotoxicity. We investigated the effects of aluminum (Al) on reactive oxygen species (ROS) production and inflammation in BV-2 murine microglial cells. Al exposure resulted in a significant increase in ROS production and lipid peroxidation. Notwithstanding the increase in ROS production, Al caused a dose-dependent decrease in the production of tumor necrosis factor α (TNFα). Examination of the activation status of nuclear factor-κB (NF-κB) revealed that Al exposure inhibited the nuclear localization of this transcription factor. Co-treatment with vitamin E, a factor known to prevent lipid peroxidation abrogated the inhibitory effect of Al on TNFα production. Results indicate that Al-induced lipid peroxidation is likely responsible for the observed decrease in TNFα production in microglial cells.

Keywords: Microglia, Reactive Oxygen Species, TNFα, Cytokine, NFκB, Oxidative Stress, Aluminum

1. Introduction

Oxidative stress and inflammation are characteristic of many neurodegenerative and dementing diseases of the central nervous system (CNS) including dialysis dementia and Alzheimer's disease (Pratico, 2002). Microglial cells are the primary mediators of innate immunity in the CNS and are known to produce ROS and inflammatory cytokines. In addition, microglial activation is a common response to environmental agents that cause CNS inflammation (Terrazzino et al., 2002). Studies suggest that glial cells accumulate metals that are known CNS toxins including aluminum (Levesque et al., 2000; Campbell et al., 1999). Aluminum is a known neurotoxicant and several studies have suggested a link to Alzheimer's disease and dialysis dementia (Crapper McLachlan and De Boni, 1980; Neri and Hewitt, 1991).

Aluminum is a ubiquitous element being the third most prominent in the earth's crust, next to oxygen and silicon. Increasing acid rain is leading to groundwater contamination with Al salts thus increasing exposure and availability to humans and animals (Davis and Turlington, 1987). Levels as high as 380 μg/l have been reported in the groundwater (Davis and Turlington, 1987). Compounding this problem is the use of Al sulfate and Al ammonium sulfate in drinking water treatment and purification (Boegman and Bates, 1983; Cohen and Hannah, 1971).

Aluminum exposure in cats and rabbits has been shown to cause a condition known as experimental Al encephalopathy (De Boni et al., 1976). Neurological and neuropathological changes including neuronal degeneration have been reported following Al exposure in these species (De Boni et al., 1976; Selkoe et al., 1979). In addition, neurofibrillary tangles reminiscent of those seen in Alzheimer's disease were reported in

rabbits exposed to Al lactate (Wen and Wisniewski, 1985). Low-level exposure to Al has been postulated as a possible contributing factor in several human neurodegenerative diseases including Alzheimer's disease (Crapper McLachlan and De Boni, 1980; Walton et al., 1995), dialysis dementia (Elliot et al., 1978), amyotrophic lateral sclerosis (Perl and Brody, 1980) and Parkinson's disease (Perl and Brody, 1980; Hirsch et al., 1991). In addition, exposure to low levels of Al may also impair cognitive function (Rifat et al., 1990; Jacqmin et al., 1994).

The precise mechanisms responsible for Al-induced neurotoxicity remain to be elucidated. Evidence suggests that ROS and oxidative stress are important factors in neurodegenerative diseases (Evans et al., 1992) as well as immune regulation (Muller et al., 1997; Mattson et al., 1997). Exposure to Al has been shown to increase lipid peroxidation in myelin (Verstraeten et al., 1997) and the production ROS (Bondy and Kirstein, 1996; Xie and Yokel, 1996) and reactive nitrogen species (RNS) (Garrel et al., 1994) in isolated neurons and glia. ROS are potent activators of the transcription factors NFκB and AP-1 (Muller et al., 1997). Alternatively, ROS-induced lipid peroxidation results in the release of lipid hydroperoxides that have been shown to potently inhibit the activation of NFκB. 4-Hydroxy-2,3-nonenal (4-HNE) inhibits both constitutive and inducible activation of NFκB through prevention of IκB degradation (Camandola et al., 2000). NFκB is the dominant transcription factor controlling the expression of proinflammatory cytokines including TNFα (Goldfeld et al., 1993), IL-1β (Grilli et al., 1993) and IL-6 (Baldwin, 1996).

In vivo exposure to Al in mice was shown to depress the production of interleukin-2, interferon γ and tumor necrosis factor α (TNFα) in splenic cells stimulated

with mitogens *in vitro* (Golub et al., 1993). In contrast, Tsunoda and Sharma (1999) reported elevated expression of TNFα mRNA in the cerebrum of mice treated with Al ammonium sulfate in the drinking water. Recently, extended exposure to colloidal aluminum resulted in increased TNFα production in cultured T98G human microglial cells (Campbell et al., 2002). The involvement of ROS production and oxidative stress in Al-induced effects of TNFα production remain to be investigated. The interplay between ROS production and cytokine synthesis may have a significant impact on our current understanding of Al neurotoxicity and the role of this metal in human neurodegenerative diseases.

In the present study, BV-2 murine microglial cells were treated with Al in order to assess the effects of this agent on oxidative stress and TNFα production. The BV-2 microglial cell line represents a valuable tool for assessment of the impact of agents such as Al on the innate immune system of the CNS. Exposure to Al increased ROS production in dose- and time-dependent manner. Lipid peroxidation was also evident in cultures treated with Al. Alternatively, the production of TNFα was diminished following exposure to Al. Decreased NFκB activation may be responsible for the effects of Al on cytokine production.

2. Materials and Methods

2.1. Materials

The BV-2 murine microglial cell line was graciously provided by Dr. Kostantinos Vekrellis of Harvard Medical School. This cell line represents a v-raf/v-myc retrovirally transformed murine microglial cell. They have been confirmed as microglial cells by the

presence of inwardly rectifying K^+ channels (Blasi et al., 1990). The phenotypic and functional properties of the BV-2 cell are very similar to native murine microglial cells (Blasi et al., 1990). Dihydrorhodamine 123 was purchased from Molecular Probes, Inc. (Eugene, OR, USA). The NFκB (rabbit anti-mouse NFκB IgG) antibody used for western blotting was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA) and the NFκB (anti-NFκB p65) antibody used for flow cytometry was obtained from Roche Molecular Biochemicals (Indianapolis, IN, USA). The latter antibody is specific for the nuclear localization sequence of NFκB and therefore binds only to the IκB-free activated form of the transcription factor (Kaltschmidt et al., 1995). Cell culture reagents were obtained from GIBCO Life Technologies, Inc. (Grand Island, NY, USA). All other reagents used were purchased from Sigma Chemical Co. (St. Louis, MO, USA) and were tissue culture grade.

2.2. Cell culture

BV-2 microglial cells were grown in RPMI supplemented with 10% non-heat inactivated fetal calf serum (FCS) and 1% penicillin-streptomycin (Pen-Strep®, GIBCO). Cultures were maintained in plastic tissue culture vessels in a humidified atmosphere at 37°C with 5% CO_2. Cells were seeded at 1 x 10^6/ml for each experiment and allowed to grow to 80% confluence prior to treatment with Al. All treatments were performed in RPMI 1640 with 10% FCS unless stated otherwise.

Aqueous solutions of Al^{+++} undergo extensive hydrolysis over time yielding colloidal complexes that may influence the bioavailability of Al (Campbell and Bondy, 1999). To control for this, Al stocks were made immediately prior to treatment by

dissolving $AlCl_3$ in millipore water at a concentration of 10 mM and this stock was used to make the treatment solutions (1-1000 μM $AlCl_3$).

2.3. Cell viability and mitochondrial enzyme activity

Cell viability was determined using the trypan blue (Gibco) exclusion method (McLimas et al., 1957). BV-2 cells were seeded in 6-well culture plates and allowed to grow to 80% confluence. Cells were treated with Al for 24 h followed by removal from the substrate using 0.25% trypsin. Cells were resuspended in 0.04% trypan blue and counted using a hemocytometer. Viability was determined as the percentage of cell capable of excluding trypan blue from the cytoplasm.

Mitochondrial enzyme activity was determined using the 3(4,5-dimethyl thiazolyl-2)2,5-diphenyl tetrazolium bromide (MTT) assay. BV-2 cells were seeded in 96-well culture plates and treated with Al when 80% confluent. Twenty h later, MTT was added to each well at a final concentration of 0.5 mg/ml for 4 h. Media was removed and the purple formazan crystals were dissolved in 100% DMSO. Absorbance at 570 nm was determined using a Spectra SLT absorbance reader (Tecan, Durham, NC, USA).

2.4. Reactive oxygen species production

The production of ROS was monitored in real-time using dihydrorhodamine 123 (DHR 123), which is a cell permeable non-fluorescent ROS probe. Upon interaction with ROS (hydrogen peroxide) DHR 123 is oxidized resulting in the liberation of rhodamine 123, a highly fluorescent mitochondrial specific dye. The resulting fluorescent signal is proportional to the amount of free radicals being formed. BV-2 cells were grown to 80% confluence in black 96-well microplates and then washed in Lockes buffer (8.6 mM HEPES pH 7.4, 5.6 mM KCl, 11.8 mM NaCl, 1 mM MgCl2, 2.3 mM CaCl2, 5.6 mM

glucose and 100 μM glycine) prior to treatment. Lockes buffer was used because preliminary experiments showed that RPMI 1640 with 10% FCS prevented loading with DHR 123 presumably due to binding of the dye to membrane impermeable serum constituents. Lockes buffer containing 25 μM DHR 123 was added to the cells and the intra- and extracellular dye concentrations were allowed to equilibrate for 30 min at 37°C. Al (5-500 μM) in the presence or absence of exogenous iron ($FeSO_4$, 30 μM) was prepared in Lockes buffer and added to respective wells. Production of a fluorescent signal was immediately monitored using a Spectramax Gemini fluorescent plate reader (Molecular Devices, Irvine, CA, USA). The excitation and emission maxima were scanned prior to analysis and were set at 507 nm and 529 nm, respectively. A 515 nm cutoff filter was used to eliminate stray signal from the excitation source. The fluorescence signal was digitized and analyzed using SoftMax Pro™ (version 3.1.1, Molecular Devices, Irvine, CA, USA).

2.5. Analysis of mRNA expression

Total cellular RNA was isolated from microglial cells following stimulation, as outlined in the figure legends using TRI reagent LS (Molecular Research Center, Cincinnati, OH) according to manufacturer's protocol. Reverse-transcriptase polymerase chain reaction (RT-PCR) was used to analyze the expression of mRNA for TNFα and β-actin (internal control) as described elsewhere (Johnson et al., 2000). The sense and antisense primers used in this study were 5'CTCTTCAAGGGACAAGGCTG3' and 5'CGGACTCCGCAAAGTCTAAG3' for TNFα and 5'ATGGATGACGATATCGCT3' and 5'ATGAGGTAGTCTGTCAGGT3' for β-actin, respectively. The thermal cycles consisted of denaturation at 94^{o}C for 30 seconds, annealing (TNFα, 54^{o}C and β-actin,

48°C) for 30 seconds and extension at 72°C for 1 min followed by a final extension at 72°C for 5 min. The optimum number of cycles for each primer set was 25 cycles for TNFα and 30 cycles for β-actin.

The amplification products were fractionated on 2% agarose gel and documented using a Kodac DC290 digital camera. The resulting images were digitized and quantified using UN-SCAN-IT software (Silk Scientific, Inc., Orem, UT) and the pixel values for TNFα were normalized to that of β-actin.

2.6. Cytokine quantification

Supernatants from microglial cultures were collected following 24 h exposure to Al or LPS and used to analyze for TNFα protein secretion. Supernatants were frozen and maintained at –85°C until analyzed. TNFα was quantified by ELISA using a commercial kit (Genzyme Diagnostics, Cambridge, MA) according to manufacturer's instructions.

2.7. Analysis of NFκB activation

BV-2 cells were grown in 6-well plates to 80% confluence followed by treatment with 500 μM of $AlCl_3$ with or without 50 ng/ml LPS for 1 h. Western blotting was used to determine the effects of Al treatment on translocation of NFκB to the nucleus. The nuclear protein fraction was isolated and subjected to western blot analysis as previously described (Sharma et al., 2000). Briefly, nuclear protein (3 μg) was subjected to vertical gel electrophoresis in 7.5% SDS-polyacrilamide and subsequently transferred to a 0.45 nm pore-size nitrocellulose membrane by electro-blotting. The membranes were blocked and then stained with rabbit anti-mouse NFκB IgG primary antibody followed by horseradish peroxidase conjugated goat anti-rabbit IgG secondary antibody. The

membranes were washed, treated with chemiluminesence substrate (Amersham Pharmacia, Piscataway, NJ, USA), and exposed to X-ray film.

The decrease in nuclear translocation of NFκB was confirmed using flow cytometry as follows. Cells were collected by trypsinization and washed two times with PBS with 0.1% sodium azide and 1% BSA (PAB) followed by permeabilization for 5 min with 1% Triton X-100. The anti-NFκB p65 antibody was added to the cell pellet and incubated on ice for 30 min. Cells were washed two times in PAB followed by staining with a mouse-anti mouse IgG secondary antibody conjugated to biotin for 30 min on ice. Cells were again washed and incubated with streptavidin-Quantum Red™ for 30 min on ice. The fluorescence of individual cells was examined using an EPICS XL/MCL (Coulter Cytometry, Hialeah, FL, USA) flow cytometer. The fluorochrome was excited using the 488 nm line of an argon-ion laser and emission was collected at 670 nm. A total of 20000 events were acquired for each sample.

2.8. Analysis of lipid peroxidation

Lipid peroxidation results in the production and release of soluble malondiadehyde (MDA). Therefore, quantification of MDA equivalents in the cell culture media is a measure of the extent of lipid peroxidation. Interaction of MDA with thiobarbituric acid (TBA) results in a chromogenic reaction with an absorbance peak at 532 nm. Briefly, cells were treated with 500 μM Al in the presence or absence of vitamin E (100 μM) for 24 h in RPMI 1640 plus 0.5% FCS. Three hundred μl of culture media was mixed with 300 μl of 0.1 N HCl and 40 μl of 1% phosphotunstic acid. Three hundred μl of 0.7% TBA was then added and the mixture was heated at 95 °C for 30 min to facilitate the complexation of TBA and MDA. After cooling in an ice bath to room

temperature, 400 µl of *n*-butanol was added and the tubes were shaken vigorously. Following centrifugation at 1000 x g for 10 min, 200 µl of the *n*-butanol phase was added to the well of a microtiter plate and the absorbance at 532 nm was read using a PowerWaveX microplate spectrophotometer (Bio-Tek Instruments, Inc., Winooski, VT). MDA equivalents were calculated from an MDA standard curve and results are expressed as % of control levels.

2.9. Statistical analysis

All statistical analyses were performed using the SAS statistical software (SAS Institute, Cary, NC). Treatment effects were analyzed using one way analysis of variance (ANOVA) followed by Dunnett's t-test. A value of $P < 0.05$ was considered significant unless indicated otherwise.

3. Results

3.1. Cell viability and mitochondrial enzyme activity

Determination of cell viability using trypan blue exclusion indicated that Al did not cause cell death in the BV-2 cell. Viability was above 95% at all doses tested following treatment for 24 h (Fig. 3.1a). The final cell number after 24 h did not change (data not shown). Aluminum treatment did not alter mitochondrial dehydrogenase activity since there was no change in MTT reduction to formazan (Fig. 3.1b).

3.2. Production of reactive oxygen species and oxidative stress

Oxidation of DHR 123 resulting in the liberation of fluorescent rhodamine 123 is proportional to the amount of H_2O_2 produced within the cell. The addition of exogenous iron to BV-2 cells resulted in a significant increase in the generation of free radicals (Fig.

3.2a, 3.2b, 3.2c). A representative graph of the kinetics of ROS production is given in Fig. 3.2a. In addition, figure 3.2b shows that 500 μM Al significantly augmented iron-mediated free radical generation in BV-2 cells. Interestingly, Al alone significantly increased the production of reactive intermediates capable of oxidizing DHR 123 (Fig. 3.2c). The results shown in figure 2c indicate that Al caused a dose-dependent increase in free radical production. Treatment of BV-2 cells with 500 μM Al resulted in a subtle but significant increase in the liberation of soluble MDA (Fig. 3.3), an indicator of lipid peroxidation.

3.3. Expression of TNFα mRNA

There was a dose-dependent decrease in the expression of TNFα mRNA in cultures treated with Al for 24 h (Fig. 3.4). Aluminum also significantly attenuated LPS-induced TNFα mRNA expression in microglia stimulated with LPS as indicated in figure 3.4. The effects of Al on LPS-mediated TNFα mRNA expression increased with increasing Al concentration (Fig. 3.4). Fig. 3.5 shows that the expression of TNFα mRNA in microglial cells was decreased in a time-dependent manner in response to 500 μM Al; inhibition of mRNA expression began as early as 1 h after exposure and reached a maximum by 4 h. The decrease was stable for up to 24 h.

3.4. Secretion of TNFα protein

The secretion of TNFα protein into the medium was also measured in BV-2 cells exposed to Al (0-1 mM). The amount of TNFα produced was decreased in a dose-dependent manner in response to Al treatment as shown in figure 3.6a. LPS was used as a positive control for TNFα production and markedly increased the secretion in a dose-dependent fashion (Fig. 3.6b).

3.5. Status of NFκB activation

The activation of NFκB was examined using two independent methods, western blotting and flow cytometry. Exposure to 500 μM Al for 24 h resulted in a marked reduction in the amount of NFκB that was translocated to the nucleus (Fig. 3.7a). Aluminum also partially inhibited translocation of NFκB induced by LPS. These results indicate that Al may inhibit the function of NFκB by preventing its translocation. The observed decrease in nuclear translocation of NFκB was confirmed using flow cytometry using an antibody specific for the nuclear localization sequence. This antibody only binds in the absence of IκB, thus to the activated form of the transcription factor. The mean fluorescence intensity of cells treated with Al was decreased indicating lack of activation of NFκB (Fig. 3.7b).

3.6. Effects of Al are antagonized by antioxidant treatment

Vitamin E is a powerful antioxidant known to inhibit ROS-induced lipid peroxidation. Treatment of BV-2 cells with vitamin E completely prevented Al-induced lipid peroxidation (Fig. 3.3). In addition, this antioxidant abrogated the Al-induced decrease in TNFα mRNA expression (Fig. 3.8) suggesting that the decrease in TNFα mRNA expression by Al is due to lipid hydroperoxides.

4. Discussion

Aluminum has clearly been established as a neurotoxic metal. However, the role of Al in neurological disorders such as Alzheimer's disease remains both controversial and unresolved. There is ample evidence to suggest that oxidative stress plays a role in many neurodegenerative conditions including Alzheimer's disease (Akiyama et al.,

2000). A recent breakthrough demonstrated clearly that Al in the diet accelerated the disease progression in transgenic mice over expressing the human amyloid precursor protein (Pratico et al., 2002). Al-induced oxidative stress was clearly shown to be the cause of increased amyloid-β and plaque formation since vitamin E eliminated the effects. Microglial cells represent a major source of reactive intermediates within the CNS (Colton et al., 2000). *In vitro* experiments have shown that exposure to Aβ peptide, a factor involved in the pathogenesis of Alzheimer's disease, results in the activation of NADPH oxidase complexes in microglia leading to the liberation of free radicals (Della-Bianca et al., 1999). The present study employed BV-2 murine microglial cells to investigate the impact of Al on free radical generation and cytokine production.

The data presented demonstrates that Al is capable of potentiating ROS production induced by iron, a redox active metal. Intriguingly, treatment of microglial cells with Al alone also promoted the generation of ROS. This finding is paradoxical given that Al is a trivalent metal incapable of initiating free radical production on its own (Campbell and Bondy, 1999). Therefore, exogenous Al must be interacting with endogenous redox active metals including iron. In this system, Al may be augmenting the basal iron-mediated ROS production to an aberrant level. Bondy and Kirstein (1996) demonstrated that Al was capable of potentiating ROS production in neural tissue treated with iron. Iron-induced lipid peroxidation was also potentiated by Al treatment (Xie and Yokel, 1996). The mechanism by which aluminum can potentiate or induce ROS production is not well understood but may involve deregulation or iron metabolism. The ability of Al to compete with iron for binding sites on transferin may elevate local levels of free iron in tissues like the brain (Cochran et al, 1984). More applicable to the present

research is the observation that Al can stabilize iron in the Fe^{2+} state thereby feeding the Fenten reaction (Yang et al., 1999). The resulting increase in hydroxyl radicals would contribute to lipid peroxidation. Notwithstanding the increased ROS production, treatment with Al did not affect the viability of the BV-2 cells in this study. The production of ROS is part of the normal function of microglial cells and therefore these cells may have a high capacity to buffer free radicals and therefore prevent their cytotoxic action.

In addition to oxidative stress, inflammation has been identified as a key mediator of the pathology seen in Alzheimer's disease brains. The principle source of inflammatory mediators in the CNS is the microglial population. These cells produce proinflammatory cytokines including TNFα which can act upon cognate receptors on other cells leading to cell damage and death. Numerous studies have demonstrated elevated levels of TNFα in the CNS (Tarkowski et al., 1999) and periphery (Fillit et al., 1991) of Alzheimer's patients. We have previously demonstrated that administration of Al in the drinking water for four weeks caused a significant increase in TNFα mRNA in the cerebrum of mice (Tsunoda and Sharma, 1999). Campbell et al. (2002) showed increased TNFα production following long-term treatment of human glioblastoma cells with colloidal aluminum sulfate. In the present study, culture of BV-2 microglial cells in the presence of Al resulted in a dose-dependent decrease in the expression of TNFα mRNA and secretion of TNFα protein. Also, Al treatment dose-dependently decreased LPS-induced expression of TNFα mRNA. In the study by Campbell et al. (2002), there was no increase in TNFα in cultures exposed to Al for 2 days. Al salts undergo extensive hydrolysis at physiological pH leading to the formation of colloidal suspensions over

time (Smith, 1996). Therefore, it is likely that the increase in TNFα observed was due to chronic activation by aluminum complexes. Similar activation of microglial cells was observed following treatment with insoluble aluminosilicate particles (Evans et al., 1992). It is unlikely that aluminum would exist in a colloidal state *in vivo* given its high affinity for biological ligands including transferin and citrate. We used fresh solutions of aluminum chloride for a shorter duration of treatment and therefore the effects seen in the present study may be due to soluble aluminum and this may account for the differences between the two studies.

It is well established that free radicals are potent activators of NFκB, a transcription factor controlling the expression of TNFα (Muller et al., 1997). Therefore, one would expect aberrant ROS generation to increase the expression of this cytokine. Nuclear translocation of NFκB to the nucleus is required for biological activity. In this study, Al inhibited the translocation of this transcription factor thereby decreasing its activity. Lipid peroxidation often is the end result of aberrant free radical production and is a common feature in neurodegenerative diseases. Here we show that treatment of microglial cells with Al leads to lipid peroxidation and many studies support this finding (Verstraeten et al., 1997; Xie and Yokel, 1996; Deloncle et al., 1999). Aberrant free radical production may lead to inhibition of NFκB through the lipid peroxidation byproduct, 4-HNE. It has recently been demonstrated that 4-HNE suppresses the basal and inducible activity of NFκB (Camandola et al., 2000). The mechanism of this inhibition was shown to be prevention of IκB phosphorylation and proteolysis (Page et al., 1999). To the best of our knowledge, the effects of Al on 4-HNE have not been investigated but represent a plausible explanation for our results. The possible role of

this aldehyde mediator in Al neurotoxicity is currently under investigation using our model system.

The possibility exists that effects of xenobiotics on isolated cell systems may not be indicative of exposure *in vivo*. Vital interactions with other cell types in the CNS may be required. The lack of interaction with other cells may explain the apparent differences between treating BV-2 cells *in vitro* and our previous finding of increased TNFα expression *in vivo* (Tsunoda and Sharma, 1999). It was previously demonstrated that exposure of pure neuronal cultures to Al caused very little change in viability. In contrast, the presence of glial cells in the culture greatly enhanced the sensitivity of neurons to Al-mediated cell death (Suarez-Fernandez et al., 1999). More recent research has shown that protease products released from dying neurons are capable of activating glial cells to release TNFα (Viviani et al., 2000). Further investigation of the effects of Al on cytokine production in mixed neuron-glial cultures is warranted.

In support of our findings that Al ($AlCl_3$) inhibits the production of TNFα in BV-2 cells, He and Strong (2000) showed that *in vivo* treatment with $AlCl_3$ also inhibited microglial migration and activation. Acute and chronic exposure to $AlCl_3$ resulted in motorneuron degeneration but inhibited microglial recruitment and transformation to cytotoxic effector cells at the site of injury. Our data present a plausible oxidative stress-related mechanism by which $AlCl_3$ can inhibit the effector functions of microglial cells. Studies are currently underway in our laboratory to determine if a similar microglial response will be observed in the CNS of mice treated systemically with $AlCl_3$. There is evidence that microglial cells play an important role in clearing neuronal debris at sites of injury (Flugel et al., 2001) and also in axonal regeneration (West et al., 2001). Therefore,

Al may directly potentiate oxidative stress-induced damage in the CNS by potentiating ROS production and indirectly through the inhibition of microglial function.

In summary, Al inhibits the production of TNFα in isolated microglial cells without inducing cell death. This effect appears to be mediated through inhibition of NFκB activation and occurs even in the presence of enhanced ROS production. Attenuation of this effect by inhibiting ROS production and lipid peroxidation with vitamin E suggests the effects of Al on BV-2 cells are mediated by oxidative stress. Continuing studies are needed to further investigate the relationship between free radicals, TNFα and NFκB and how this information may relate to *in vivo* exposures and neurodegenerative conditions.

Acknowledgements

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Abbreviations

4-HNE – 4-Hydroxy-2,3-nonenal; Al – Aluminum; DHR 123 – Dihydrorhodamine 123; MDA – Malondialdehyde; MTT - 3(4,5-dimethyl thiazolyl-2)2,5-diphenyl tetrazolium bromide; NFκB – Nuclear factor κB; NO – Nitric oxide; RNS – Reactive nitrogen species; ROS – Reactive oxygen species; TBA – thiobarbituric acid; TNFα – Tumor necrosis factor α

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Fig. 3.1. Viability and mitochondrial dehydrogenase activity remain unaffected by Al. BV-2 cells were cultured in the presence of Al (0-500 μM) for 24 h. Viability (a) was determined using the trypan blue exclusion technique at 24 h. Mitochondrial dehydrogenase activity (b) was determined using the MTT assay. Mean ± SEM (n = 3). Data are representative of three independent experiments.

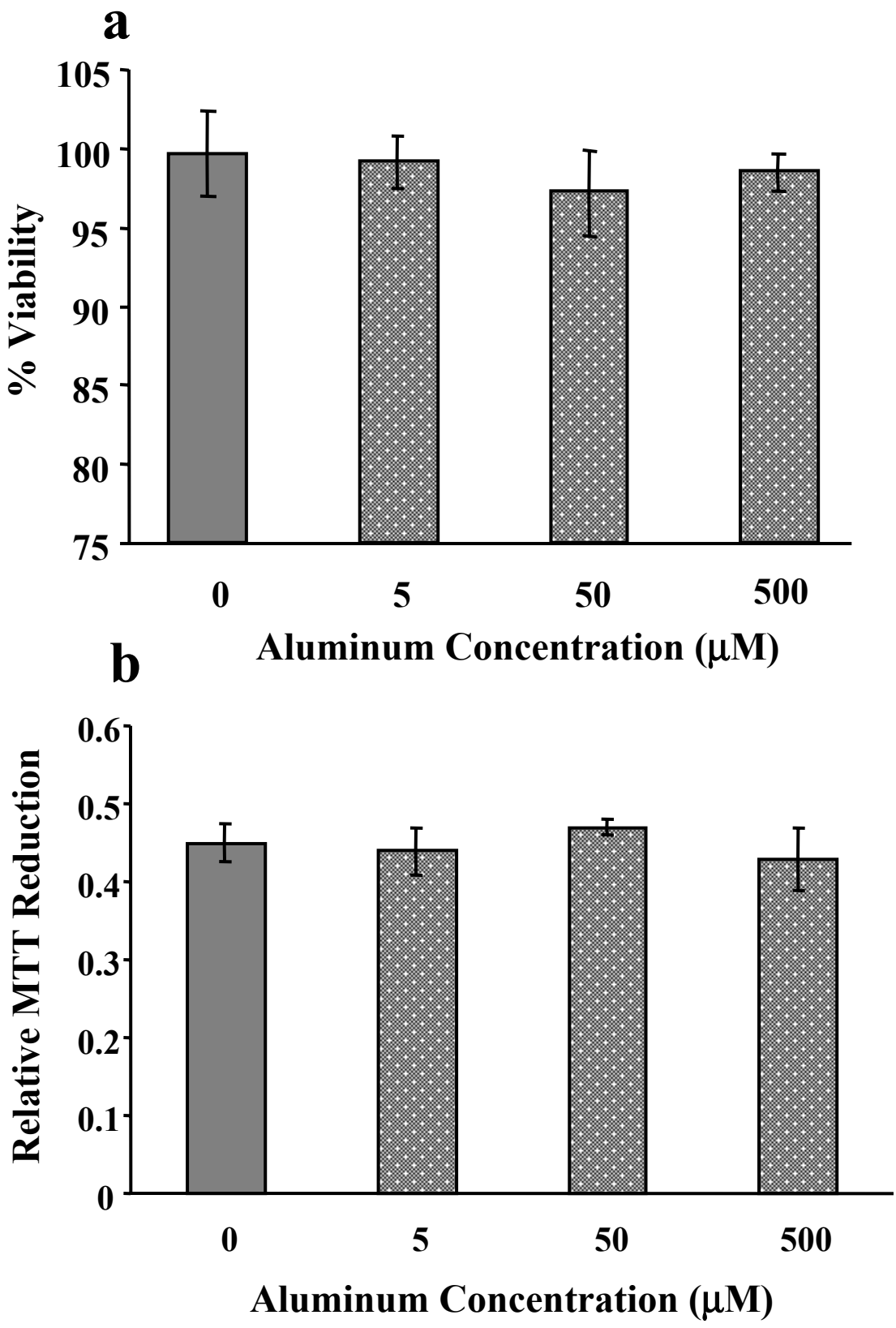

a
105
100
95
90
85
80
75
% Viability
0
5
50
500
Aluminum Concentration (μM)
b
0.6
0.5
0.4
0.3
0.2
0.1
0
Relative MTT Reduction
0
5
50
500
Aluminum Concentration (μM)

Fig. 3.2. Aluminum increased the production of reactive oxygen species in murine microglial cells. BV-2 cells were exposed to Al (0-500 μM) in the presence or absence of iron ($FeSO_4$; 30 μM) and the generations of ROS were monitored using DHR 123. A representative trace of the kinetics of ROS production in BV-2 cells is shown in Fig. 2a. The level of ROS production (b) at 1 h after treatment was increased by Al alone and Al potentiated iron-induced ROS. The increase in ROS production induced by Al was dose-dependent (c). Mean ± SEM (n = 3). Data are representative of three independent experiments. * Significantly different from control group at $P < 0.05$. # Significantly different from iron-treated control group at $P < 0.05$.

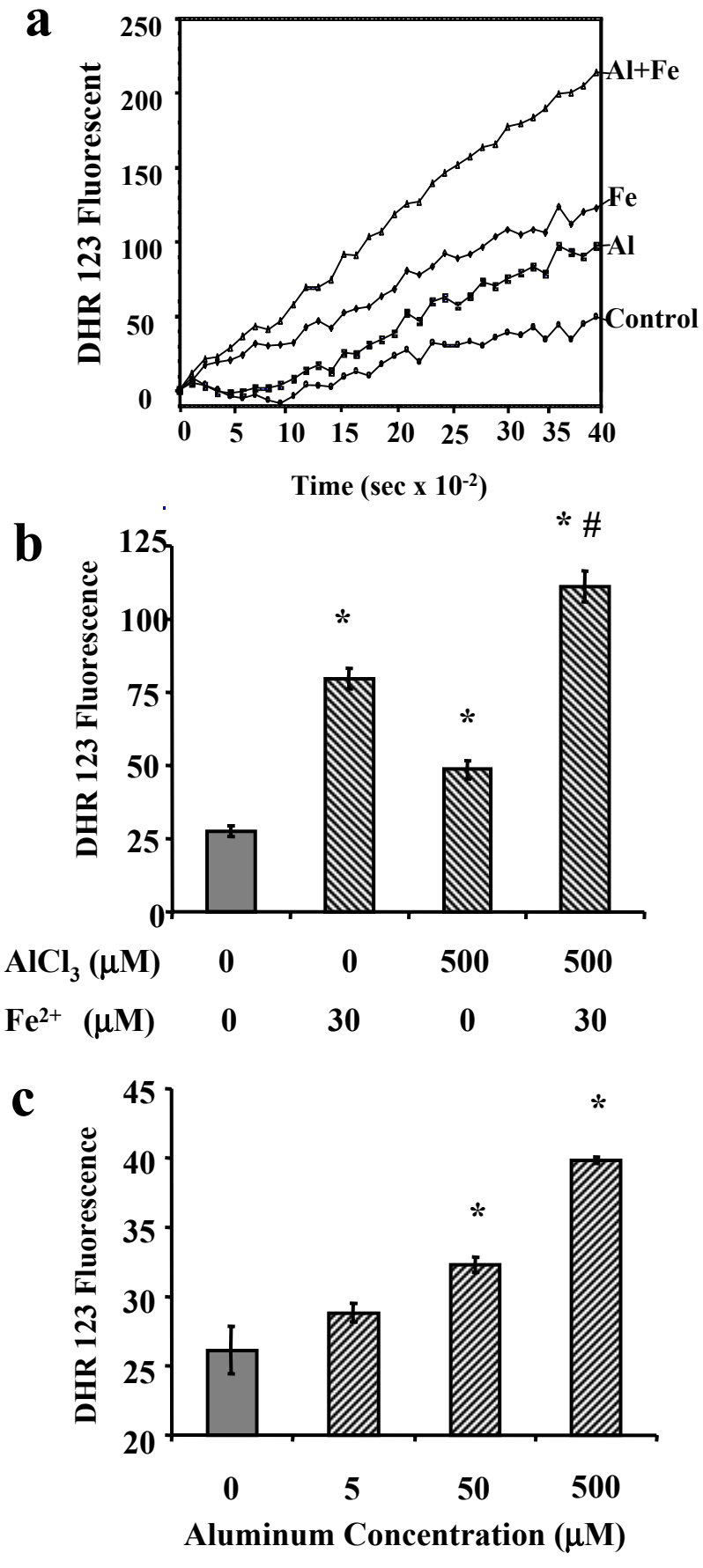

a
DHR 123 Fluorescent
250
200
150
100
50
0
0 5 10 15 20 25 30 35 40
Time (sec x 10-2)
Al+Fe
Fe
Al
Control
b
DHR 123 Fluorescence
125
100
75
50
25
0
* #
*
*
AlCl3 (μM) 0 0 500 500
Fe2+ (μM) 0 30 0 30
c
DHR 123 Fluorescence
45
40
35
30
25
20
*
*
0 5 50 500
Aluminum Concentration (μM)

Fig. 3.3. Aluminum treatment increased lipid peroxidation in BV-2 cells. BV-2 cells were cultured in media containing 500 μM Al for 24 h with and without vitamin E (100 μM) following which, soluble MDA in the media was quantified using the TBARS assay as outlined in Materials and Methods. Mean ± SEM (n = 3). Data are representative of three independent experiments. * Significantly different from control group at $P < 0.05$. ** Significantly different from control group at $P < 0.01$.

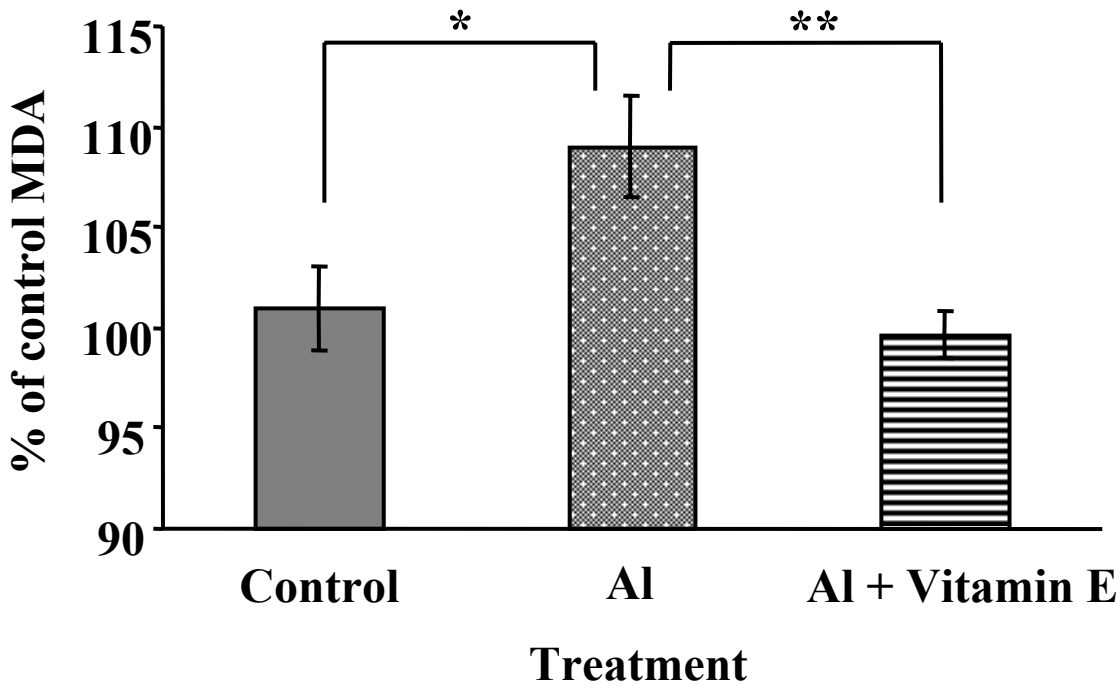

*
**
115
110
105
100
95
90
% of control MDA
Control
Al
Al + Vitamin E
Treatment

Fig. 3.4. Treatment of microglial cells with Al resulted in a dose-dependent inhibition of TNFα mRNA expression. BV-2 cells were cultured in the presence of Al (0-500 μM) for 24 h alone or followed by LPS (50 ng/ml) for an additional 6 h. Gene expression was analyzed by RT-PCR and normalized to the expression of β-actin (internal control). Mean ± SEM (n = 3). Data are representative of three independent experiments. *Significantly different from control group at $P < 0.05$. # Significantly different from LPS-treated control group at $P < 0.05$.

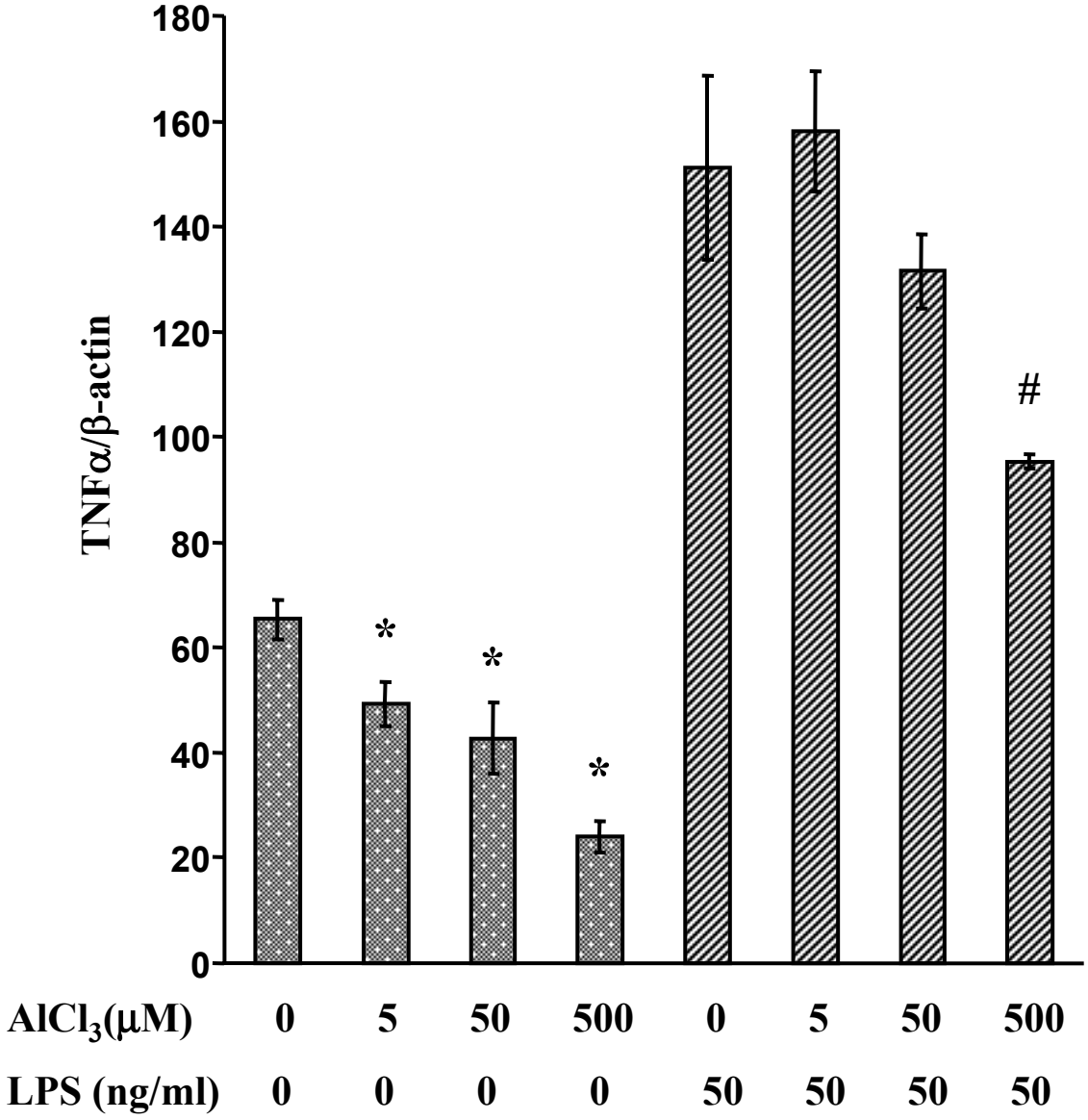

TNFα/β-actin
180
160
140
120
100
80
60
40
20
0
*
*
*
#
AlCl3(μM) 0 5 50 500 0 5 50 500
LPS (ng/ml) 0 0 0 0 50 50 50 50

Fig. 3.5. Aluminum-induced inhibition of TNFα mRNA expression is time dependent. BV-2 cells were exposed to 500 μM of Al for 1, 2, 4, 6, 12 and 24 h following which, gene expression was analyzed by RT-PCR. Expression was normalized to the expression of β-actin (internal control). Mean ± SEM (n = 3). Data are representative of three independent experiments. * Significantly different from control group at $P < 0.05$.

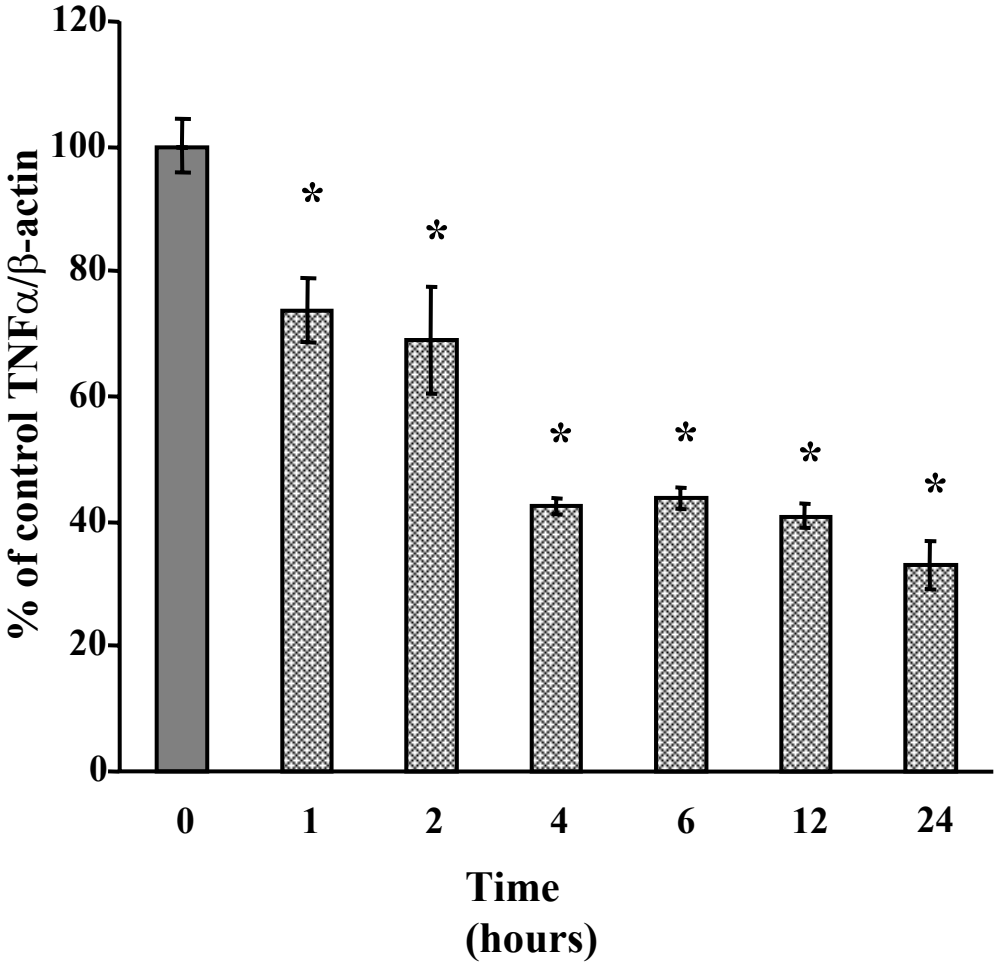

120
100
80
60
40
20
0
% of control TNFα/β-actin
*
*
*
*
*
*
0
1
2
4
6
12
24
Time
(hours)

Fig. 3.6. Al inhibits secretion of TNFα protein by microglial cells. BV-2 cells were exposed to 0-1000 μM of Al (a) or 0-10 μg/ml of LPS (b) for 24 h following which, TNFα protein in the medium was determined using a commercial ELISA matched antibody pair. Mean ± SEM (n = 3). Data are representative of three independent experiments. * Significantly different from control group at $P < 0.05$.

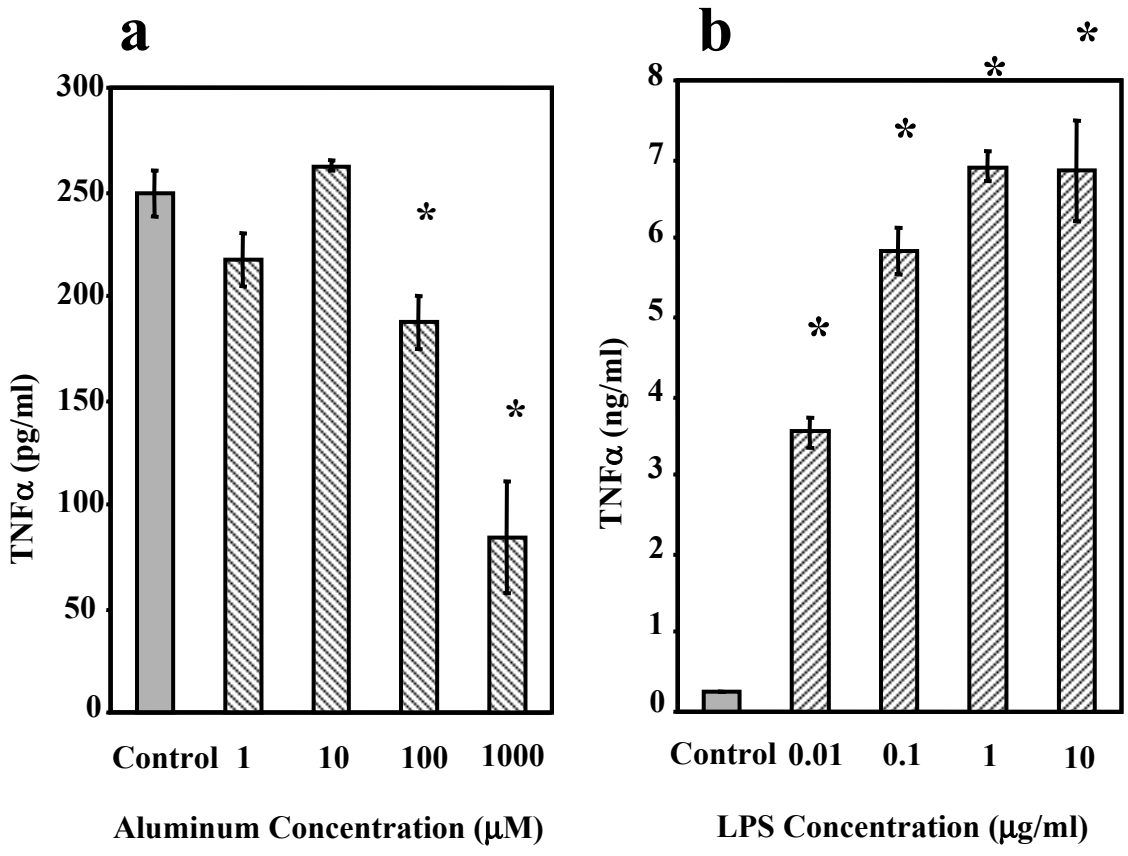

a
300
250
200
150
100
50
0
TNFα (pg/ml)
*
*
Control
1
10
100
1000
Aluminum Concentration (μM)
b
8
7
6
5
4
3
2
1
0
TNFα (ng/ml)
*
*
*
Control
0.01
0.1
1
10
LPS Concentration (μg/ml)

Fig. 3.7. Nuclear translocation of NFκB is inhibited by Al exposure in microglial cells. BV-2 cells were cultured with 500 μM Al for 24 h followed by 50 ng/ml LPS for 1 h. Nuclear translocation of NFκB as determined by western blotting (a) of the nuclear protein fraction was decreased by treatment with Al for 24 h. LPS-induced translocation of NFκB was also reduced by Al treatment. The level of activated (IκB-free) form of NFκB was determined using flow cytometry (b) as outlined in Materials and Methods. This method confirmed the reduction in nuclear translocation of NFκB in response to Al. Data are representative of two separate experiments.

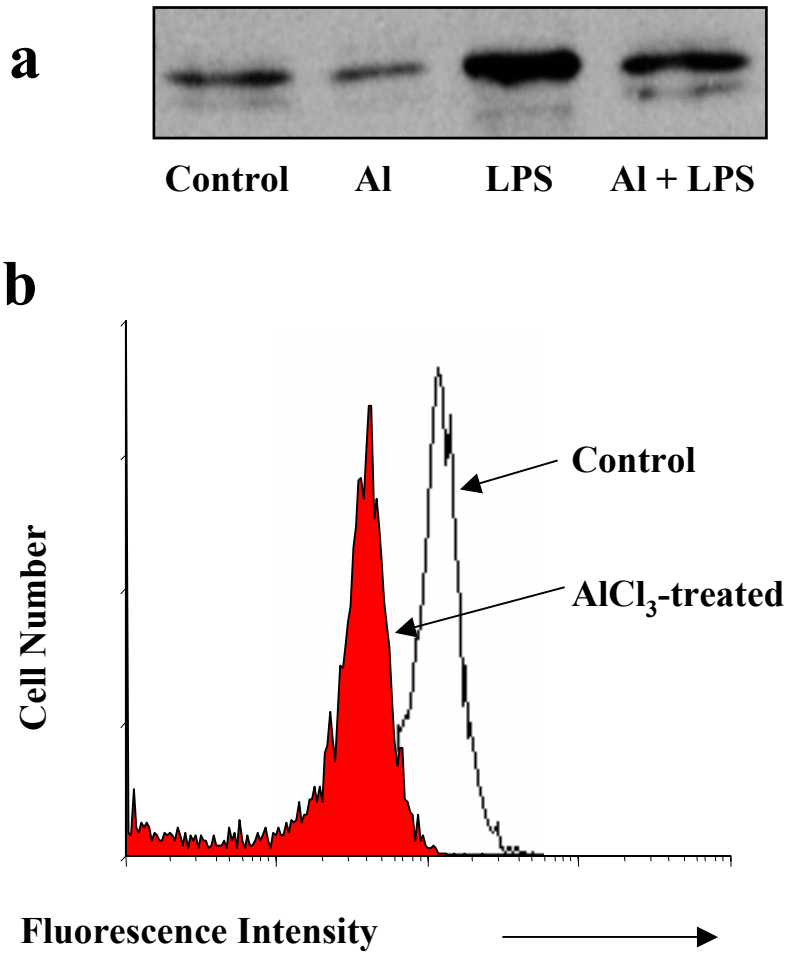
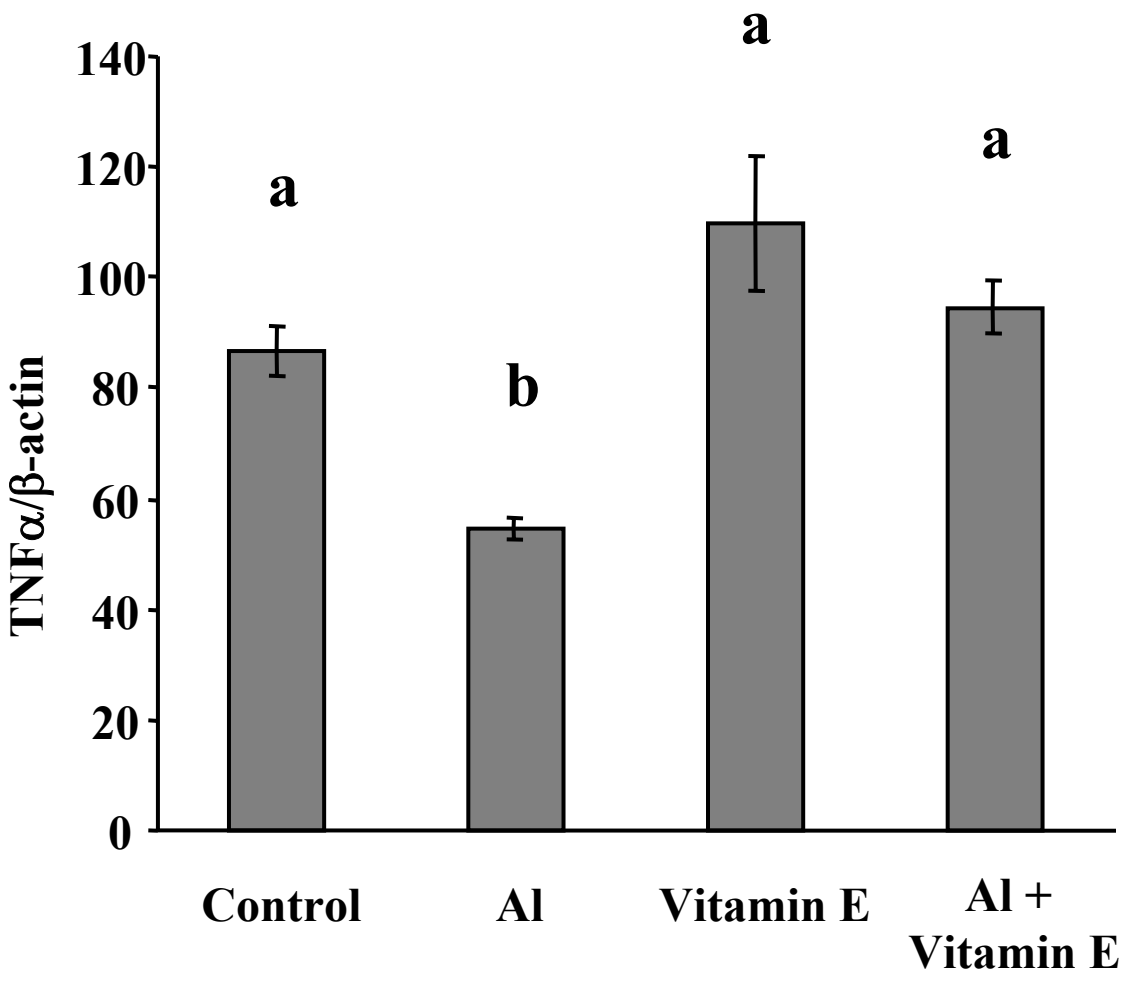

a
Control
Al
LPS
Al + LPS
b
Cell Number
Control
AlCl3-treated
Fluorescence Intensity

Fig. 3.8. Antioxidant treatment abrogates the effect of Al on TNFα mRNA expression in BV-2 cells. BV-2 cells were treated with 500 μM Al in the presence of vitamin E (100 μM) for 24 h. The expression of TNFα mRNA was quantified RT-PCR and normalized to the expression of β-actin in the same sample. Mean ± SEM (n = 3). Data are representative of three independent experiments. Bars with a different superscript letters are significantly different at $P < 0.05$.


TNFα/β-actin
140
120
100
80
60
40
20
0
a
b
a
a
Control
Al
Vitamin E
Al +
Vitamin E

CHAPTER 4

DECREASED MEMBRANE FLUIDITY AND HYPERPOLARIZATION IN ALUMINUM-TREATED PC-12 CELLS CORRELATES WITH INCREASED REACTIVE OXYGEN SPECIES PRODUCTION[1]

[1]Johnson, V.J., Tsunoda, M., Murray, T.F., and Sharma, R.P. To be submitted to Brain Research.

Abstract

Effects of aluminum (Al) on membrane properties of excitable cells are not fully understood. The effects of *in vitro* Al exposure in resting rat pheochromocytoma (PC-12) cells, a model that exhibits neuron-like properties, were investigated. Treatment of PC-12 cells with Al (>0.01 mM) resulted in a concentration-dependent decrease in membrane fluidity. Similar concentrations of Al increased the rate of extracellular acidification, measured by a cytosensor microphysiometer, indicating stimulation of proton extrusion from cells. Al caused a rapid and concentration-dependent hyperpolarizion of the cell membrane as determined by decreased fluorescence of a potential-sensitive dye, *bis*-[12]trimethine oxonol [$Dibac_4(3)$]. These membrane alterations corresponded with an increased production of reactive oxygen species, indicated by increasing dihydrorhodamine 123 oxidation. We previously demonstrated a selective decrease in hypothalamic DA and metabolites in mice treated with different concentrations of Al in drinking water for 4 weeks. Therefore, it was desirable to relate the observed alterations in membrane properties caused by Al to metabolic function of PC-12 cells, a model previously employed for neurotransmitter synthesis and release. Incubation of PC-12 cells with up to 3 mM Al for 6 h caused no alterations in the production or release of neurotransmitters or their metabolites. Results indicated that acute exposure to Al modifies membrane properties of neuron-like cells but had no effect on neurotransmitter biogenic amine pathways under the current treatment conditions.

Theme: Disorders of the Nervous System

Topic: Neurotoxicity

Keywords: Aluminum; membrane fluidity; reactive oxygen species, membrane potential, proton extrusion; neurotransmitter

1. Introduction

Aluminum (Al), a metal in Group IIIa of the Periodic Table, is a well-known neurotoxic agent. The administration of Al induces neurological and pathological changes in the central nervous system of sensitive species such as cats and rabbits [6,41,51]. In addition, low-level exposure to Al has been postulated as a possible contributing factor in several neurodegenerative diseases including dialysis dementia and Alzheimer's disease [10,29,34]. The mechanism of Al neurotoxicity remains unclear. There have been several reports that examined the effect of Al on isolated neuronal cell cultures suggesting that one possible mechanism of action of this metal may involve membrane-related effects, including alterations in oxidative stress and lipid peroxidation [4,14,52] and alterations in membrane fluidity [48-50]. Oxidative stress [37] and loss of membrane fluidity [11,30] have been implicated in the pathophysiology and Alzheimer's disease and may represent mechanisms for the involvement of aluminum in disease progression.

Previous studies have shown that reactive oxygen species (ROS) are involved in the toxicity of Al. Al has been shown to significantly enhance the production of iron-mediated ROS production in primary rat hippocampal neurons [52], primary cerebellar granule cells [33] and cerebral tissue [5]. Al alone has also been shown to increase ROS production in glial cells treated *in vitro* [8]. Overproduction of ROS can lead to alterations in membrane properties and damage. Several studies have reported the ability of Al to facilitate iron-mediated lipid peroxidation [2,45,55] and melanin-induced lipid peroxidation [31]. Lipid peroxidation has been shown to have a profound affect on membrane stability and fluidity [9], changes in which can affect membrane protein

channel functions and ion regulation [23]. Additionally, it has been proposed that aluminum-induced changes in membrane fluidity increase the susceptibility of lipids to peroxidative damage [49]. Therefore, a synergistic and reciprocal role between membrane fluidity changes and lipid peroxidation may be involved in the pathogenesis following Al exposure.

Aluminum has been shown to bind to lipids in phospholipid membranes [18] indicating the potential for aluminum to disrupt fluidity and function of cellular membranes. Subsequent studies showed that aluminum caused rigidification of liposome membranes and that loss of membrane fluidity correlated with lipid peroxidation [49,50]. Intact plasma membranes in human IMR-32 neuroblastoma cells were also susceptible to aluminum-induced membrane rigidification [48]. Variations in membrane fluidity have been associated with changes in membrane bound ion regulation across the membrane due to alterations in ATPase function [59]. Aluminum treatment reduced the function of plasma membrane H^+-ATPase [18] and brain synaptosomal Ca^{2+}-ATPase [19]. Although not investigated, these effects of aluminum may be related to changes in membrane fluidity.

In a previous study [46], we showed that administration of Al through drinking water caused the decreases in both DA and its metabolite levels in the hypothalamus of mice. The mechanism of the effects of Al on DA biosynthesis and metabolism was not apparent from the *in vivo* study. The observed effects of Al may be related to other cellular or metabolic effects of Al including membrane alterations. Zaleska *et al*., [56] demonstrated that the synthesis of dopamine in rat striatal synaptosomes was markedly decreased in the presence of ADP-chelated Fe^{3+}/ascorbate. The decrease in dopamine

synthesis correlated with levels of lipid peroxidation and malondialdehyde levels but the direct application of malondialdehyde did not affect synthesis. Treatment also increased membrane microviscosity and the authors concluded that the decreased dopamine synthesis was due to alterations of the structural properties of nerve ending membranes [56]. Since aluminum has been shown to increase lipid peroxidation and also decrease membrane fluidity, it is of interest to determine if these changes affect catecholamine synthesis, dopamine in particular.

The purpose of the present study was to investigate the relationship between Al-induced alterations in membrane properties and metabolic changes in PC-12 cells. PC-12 cells have been used as a model system to examine the effects of other chemicals on DA and norepinephrine (NE) synthesis and release [21]. These neuron-like cells synthesize, store, release and metabolize DA and NE in a similar manner to that observed in the mammalian central nervous system [40]. The effects of aluminum treatment on membrane fluidity, membrane potential, ROS production and proton extrusion were examined in PC-12 cells. We also examined the levels of neurotransmitters NE, DA, serotonin (5-hydroxytryptamine, 5-HT), and their metabolites, dihydroxyphenylacetic acid (DOPAC), and 5-hydroxyindoleacetic acid (5-HIAA) in these cells following acute exposure to Al.

2. Materials and Methods

2.1. Cell culture of PC-12 cells

PC-12 cells (American Type Culture Collection, CRL-1721) were maintained in plastic cell culture flasks. Before plating cells the flask was coated with collagen IV

(Life Technologies, Grand Island, NY) at 0.5 μg/cm^2. Cultures were maintained in Dulbecco's Modified Eagle's Media (DMEM) with 1000 units/ml of penicillin G, 100 μg/ml of streptomycin (Gibco BRL, Grand Island, NY), 10% horse serum and 5% fetal bovine serum (FBS; Atlanta Biologicals, Atlanta, GA) at 37ºC in 5% CO_2. This study investigated the effects of $AlCl_3$ and Al ammonium sulfate as sources of Al^{3+} on membrane integrity and function. Cytotoxicity of Al at various concentrations was determined in 96-well collagen-coated culture plates by the 3(4,5-dimethyl thiazolyl-2)2,5-diphenyl tetrazolium bromide (MTT) assay [32].

2.2. Measurement of plasma membrane fluidity

A molecular rotor, a molecule that exhibits viscosity-dependent fluorescence, was used to examine changes in membrane fluidity in cultured PC-12 cells. An improved derivative of the classical molecular rotor 9-(dicyanovinyl) julolidine was constructed through the addition of a farnesyl chain and will be referred to as FCVJ. The addition of the farnesyl chain resulted in a greater affinity for the plasma membrane and dramatically reduced cytoplasmic staining [17]. This compound was generously synthesized and provided to us by Dr. Emmanuel Theodorakis of the University of California, San Diego. The protocol used was essentially as described previously [17] with modification where noted. Briefly, PC-12 cells were loaded with FCVJ by incubating for 20 min in staining solution. The FCVJ staining solutions was made by adding 50 μl of a 20 mM dimethyl sulfoxide stock to 2 ml of FBS. The mixture was vortexed vigorously and 10 ml of DMEM was added. Following staining, cells were washed once with Locke's buffer [3] and seeded in 96 well poly-L-lysine coated black plates with clear bottoms at 1.5 x 10^6 cells/well. The plates were centrifuged at 300 x *g* for 4 min with no break to allow rapid

adherence of the cells to the plate. Cultures were treated with Al (0-3 mM) in Locke's buffer using the automated pipetter of a fluorescence imaging plate reader (FLIPR, Molecular Devices, Irvine, CA) and images of all 96 wells were acquired simultaneously using a 488 nm argon laser to excite the FCVJ and a charged coupled device camera to document the fluorescence as previously described [25] with the following modifications. Cells were incubated at 37°C throughout the experiment. Images were captured every 6 sec for 1 min to establish baseline and then 100 μl Al (2X) was added from a reagent plate at 25 μl/sec. Images were then captured once/second for 1 min followed by one image every 30 sec for 2 h. The concentration to produce 50% of maximal change (EC_{50}) was calculated by using Prism version 2.0 (GraphPad, San Diego, CA). The fluorescence of individual cells treated in suspension with Al was examined using an EPICS XL/MCL (Coulter Cytometry, Hialeah, FL, USA) flow cytometer. The FCVJ was excited using the 488 nm line of an argon-ion laser and emission was collected at 525 nm. A total of 20,000 events were acquired for each sample. Membrane fluidity is inversely proportional to fluorescence output from FCVJ and the data are expressed as mean fluorescence intensity. The experiment was repeated three times with quantitatively similar results. The results of a representative experiment (n = 4 wells for each concentration) have been depicted in Fig. 4.1 for the FLIPR experiment.

2.3. Measurement of extracellular acidification rates (ECAR)

Measurement of the effects of Al compounds on ECAR of PC-12 cells followed the method described by Twiner and coworkers [47]. PC-12 cells were cultured on 3 μm pore-size porous polycarbonate membrane capsules (Corning Costar, Cambridge, MA) in DMEM with 10% fetal bovine serum and 5% horse serum until confluent in 5% CO_2 at

37ºC. In the evening prior to ECAR determinations, the media was changed to Dulbecco's modified Eagle medium (DMEM) (Gibco) supplemented with 11.1 ml of sterilized 4 M NaCl per liter and cells incubated overnight. The pH was adjusted to 7.3 ± 0.2 by using sodium hydroxide at 37ºC. Membrane units with cells were installed in the microflow chambers of a cytosensor microphysiometer (Molecular Devices) and positioned above silicon-based potentiometric sensors and subsequently the cells were perfused with DMEM (100 μl/min). ECAR was monitored at 2 min intervals. Every 90 sec, the flow of DMEM was halted for 30 sec, during which time extracellular acid accumulated. A 30 min cycle consisted of 14 min for exposure to Al and 16 min for recovery to base line. The microphysiometer measures the rate of linear change in proton concentration in the surrounding medium when the flow of the medium is stopped [16]. The baseline ECAR was adjusted to 100% prior to beginning each 30 min cycle. Cells were exposed to Al ammonium sulfate in DMEM at pH 7.3 at 0, 1, 10, 100, 300 μM, 1 mM, or 3 mM. Two chambers were exposed to the control solution, and each unit was exposed to different concentrations of Al for 5 cycles. The mean acidification rate for each unit for 5 cycles was calculated. The experiment was repeated using $AlCl_3$. Equimolar concentrations of boron (as boric acid) were also employed to determine if the effects of Al were specific to Al or can be induced by a similar metal. The EC_{50} was calculated using the percent change at 6 min as mentioned above.

2.4. Measurement of membrane potential

The membrane-potential-sensitive dye $Dibac_4(3)$ was used to measure Al-induced changes in membrane potential. The distribution of this probe within the membrane and the resulting fluorescence is dependent on the transmembrane potential of the cell [44].

Hyperpolarization induces a decrease in fluorescence whereas depolarization induces an increase in fluorescence. A linear relationship between membrane potential and the $DiBac_4(3)$ fluorescence in various cell types has been established [12]. PC-12 cells, cultured as above, were washed with Locke's buffer [3] and aliquoted at 100,000 cells/well in 96-well black microplates. The Locke's buffer was then supplemented with 5 μM $DiBac_4(3)$ (Molecular Probes, Eugene, OR) and the intracellular and extracellular dye concentrations were allowed to equilibrate at 37°C/5% CO_2 for 30 min. The cells were then incubated in the presence of various concentrations of Al for 1 h and $DiBac_4(3)$ fluorescence was recorded using a Spectramax Gemini fluorescent plate reader (Molecular Devices) . The excitation and emission wavelengths were optimized prior to analysis and set at 488 nm and 525 nm, respectively, with a 515 nm cutoff wavelength. The fluorescent readings were stored in a computer using SoftMax Pro Version 3.1.1 (Molecular Devices). The concentration to produce 50% of maximal change (EC_{50}) was calculated by using Prism version 2.0 (GraphPad, San Diego, CA). The experiment was repeated three times with a similar trend, results of a representative experiment (n = 4 wells for each concentration) have been depicted in the results.

2.5. ROS generation in PC-12 cells

The production of ROS was monitored using dihydrorhodamine 123 (DHR 123, Molecular Probes), which is a cell permeable non-fluorescent molecule. Upon interaction with free radicals, DHR 123 is oxidized resulting in the liberation of rhodamine 123, a highly fluorescent mitochondrial specific marker. The fluorescence of DHR 123 is proportional to the production of ROS such as H_2O_2 [38] or peroxynitrile [22]. PC-12 cells were washed with Locke's buffer and aliquoted at 100,000 cells/well in

96-well black microplates. The Locke's buffer was then supplemented with 25 μM DHR 123 and the intracellular and extracellular dye concentrations were allowed to equilibrate at 37°C/5% CO_2 for 30 min. The cells were incubated in the presence of various concentrations of Al and the kinetics of DHR 123 fluorescence was recorded using Spectramax Gemini fluorescent plate reader. The optimum excitation and emission wavelengths were determined by spectral scanning and set at 507 nm and 529 nm, respectively, with a 515 nm cutoff wavelength. The fluorescent readings were digitized using SoftMax Pro Version 3.1.1. The EC_{50} was calculated as mentioned above. The results were similar in two additional repetitions and data from a representative experiment (n = 4 wells) have been illustrated here.

2.6. Determination of neurotransmitters and their metabolites

PC-12 cells were grown in collagen-coated 24-well culture plates at 5×10^5/well. Triplicate wells were exposed to 1 μM to 3 mM of Al ammonium sulfate in the medium. Six wells were used as controls. After six h exposure, the supernatant of each well was removed and 0.5 ml of the supernatant was mixed with 0.5 ml of ice-cold 0.2 M perchloric acid (J. T. Baker, Phillipsburg, NJ) with 0.1% cysteine and used to determine biogenic amine levels. The cells were washed with 0.5 ml of ice-cold phosphate-buffered saline twice, and extracted with 0.5 ml of 0.1 M perchloric acid with 0.1% cysteine directly in each well. The cell lysate was filtered by centrifugation using a 0.2 μm pore-size filter (Poretics, Livermore, CA) and biogenic amines and their metabolites were determined. Catecholamines NE and DA, DA metabolites DOPAC and homovanillic acid (HVA), indoleamine 5-HT, and its metabolite 5-HIAA, were analyzed using high-performance liquid chromatography with electrochemical detection according to method

described previously [27]. Description of the analytical system and conditions were same as reported earlier [46].

2.7. Statistics

Data were analyzed by one-way analysis of variance (ANOVA) using Statview version 5.0. A value of $P \leq 0.05$ was employed to indicate significant differences between different treatments.

3. Results

3.1. PC-12 cell viability was not changed by Al treatment.

Viability of PC-12 cells was determined with varying Al concentrations of up to 3 mM. Treatment of cells for 6 h with different Al concentrations did not produce any cytotoxicity as determined by MTT assays (data not presented). Our experiments on membrane potential and ROS determinations involved treatment of these cells for 1 h, suggesting that the observed changes were not due to alteration of cell viability.

3.2. Membrane fluidity was reduced in PC-12 cells treated with Al.

Plasma membrane fluidity of PC-12 cells treated with Al for 1 h was determined using FCVJ fluorescence. Molecular rotors are fluorescent molecules that can release or lose excited state energy in one of two ways, 1) liberation of light energy or 2) intramolecular rotation, the ratio of which depends on the free volume of the surrounding environment [26]. Therefore, the fluorescence emission is directly proportional to the viscosity of the molecular rotors environment, in this case membrane fluidity. The addition of the farnesyl chains greatly reduces cytoplasmic staining and increases the signal-to-noise ratio of FCVJ [17]. Treatment of PC-12 cells with Al for 2 h resulted in a

concentration- and time-dependent increase in the cellular FCVJ fluorescence (Fig. 4.1a). This indicates that Al decreased membrane fluidity since the rotational release of energy from FCVJ was converted to photon release and increased fluorescence. Concentration-response analysis indicates an EC_{50} value of ~0.1 mM Al (Fig. 4.1b). Examination of the effects of Al on membrane fluidity using flow cytometry revealed that there was a uniform response in the cell population (Fig. 4.2). This is indicated by the low variation in MFI in individual cells. Figure 4.2a-d shows the fluorescence of cell populations treated with 0-1 mM Al, respectively. Since membrane fluidity is reduced by lipid peroxidation [15], the hydroxyl radical generating system, H_2O_2 (500 μM) + Fe^{2+} (100 μM) [42] was used as a positive control for membrane rigidification. PC-12 cells treated with H_2O_2/Fe^{2+} showed a reduction in membrane fluidity after 1 h of exposure (Fig. 4.2e). The dotted line in Fig. 4.2 indicates the mean fluorescence intensity of the control cells. The difference in the fold increase in FCVJ fluorescence between the FLIPR assay and the flow cytometry may be due to treatment of adherent cells versus suspended cells, respectively.

3.3. Al caused proton extrusion and hyperpolarization of PC-12 cells.

Alterations in membrane fluidity are often associated with changes in ion fluxes across the membrane [23,60]. In order to verify if the changes in membrane fluidity were associated with the exchange of cations across the membrane, the extrusion of protons in extracellular medium was investigated using a microphysiometer. This technique measures the alteration of proton concentration in the extracellular medium as live cells are constantly extruding protons. The rate of proton extrusion increased in the presence of Al, reaching a maximum in about 6 min, and stayed elevated until after 14 min when

the cells were washed and the cycle repeated. Al increased the ECAR in a concentration-dependent manner (Fig. 4.3a). Values presented in the figure reflect the rate of acidification as percent of basal (control) rate normalized by the system software [16]. The data in Fig. 4.3a are averages of 5 cycles in a single experiment indicating repeatability of observations. Experiments involving borate at equimolar concentrations produced no changes in the ECAR (data not shown). Concentration-response data provided an EC_{50} of 1.5 mM Al in ECAR experiments; similar values were obtained whether Al was used as $AlCl_3$ or Al ammonium sulfate in two independent experiments.

Changes in ion flux across the plasma membrane can cause an alteration in the potential across the membrane. To investigate the effects of Al on membrane potential, we treated PC-12 cells loaded with the potential sensitive fluorescent dye $Dibac_4(3)$. Addition of Al to PC-12 cells caused a concentration-dependent decrease of $Dibac_4(3)$ fluorescence, indicating hyperpolarization of the membrane (Fig. 4.4). The effect was significant at 10 μM Al, increasing with Al concentrations of up to 3 mM. At the highest concentration of Al employed, $Dibac_4(3)$ fluorescence was reduced to one-third of the control value. These data indicate that the potential across the membrane is becoming more negative inside the cells, thus hyperpolarized.

3.4. The production of ROS is increased in PC-12 cells treated with Al.

It is well established that oxidative stress and lipid peroxidation can reduce membrane fluidity [23,49,50]. Above changes in cell membrane properties were accompanied by an increase in ROS production in these cells (Fig. 4.5a and b). The oxidation of DHR 123 increased with time in the control and treated samples indicating time-dependent basal and Al-induced ROS production (Fig. 4.5a). A comparison of the

concentration-responses of Al with both membrane hyperpolarization and ROS production is depicted in Fig. 4.6. It is interesting to note that the EC_{50} for Al obtained for the two parameters was very similar, ~0.5 mM, which was slightly lower than the EC_{50} for the ECAR (1.5 mM). The values for EC_{50} were within the same order of magnitude, the difference may be due to the fact that the ECAR determination involved DMEM which contained a number of Al complexing compounds, and the EC_{50} in membrane potential and ROS experiments were conducted in Locke's buffer.

3.5. Al treatment did not alter biogenic amine metabolism in PC-12 cells.

The effects of Al on cell membrane properties were related to functional changes in PC-12 cells. These cells effectively synthesize and metabolize DA and 5-HT. To evaluate the effect of Al on biogenic amines and their metabolites, PC-12 cells were treated with Al as Al ammonium sulfate for 6 h. It may be assumed that the influence of cytotoxicity on the parameters evaluated here was minimal during the short treatment period since no change in viability was evident (data not shown), although cytotoxic effects ultimately could cause cellular dysfunction and metabolic changes at later times. Incubation of PC-12 cells with different concentrations of Al for 6 h did not alter the concentration of DA or 5-HT in cell lysates (Table 4.1). These were the only major biogenic amines observed in PC-12 cells. The incubation medium contained measurable amounts of secreted NE, 5-HT and metabolites DOPAC and 5-HIAA, none of which were influenced by the presence of Al in the medium for 6 h (Table 4.2). Quantitatively similar results were obtained when cells were treated with $AlCl_3$.

4. Discussion

Aluminum is a metal with neurotoxic properties, and due to its vast distribution, exposure is unavoidable. The first encounter with Al on the cellular level is the plasma membrane. Evidence for the entry of Al into mammalian cells is controversial and therefore, it is necessary to understand the effects of Al on membrane properties in order to better characterize the mechanisms of Al-induced neurotoxicity. Results from these current experiments indicated that Al is capable of modifying membrane properties of PC-12 cells, a neuron-like culture model [21]. *In vitro* exposure to Al resulted in a loss of membrane fluidity as evidenced by an increase in FCVJ fluorescence. Membrane rigidification was accompanied by hyperpolarization of the cell membrane as demonstrated by a decrease in $Dibac_4(3)$ fluorescence. In addition, Al exposure also increased the ECAR possibly indicating an increase in ion permeability. PC-12 cells exposed to Al also showed an increased production of ROS indicated by increased DHR 123 oxidation.

Loss of membrane fluidity in a cell will lead to dramatic alterations in ion regulation and normal cellular physiology. Alterations in membrane fluidity were shown to markedly affect the proton permeability of lysosomal membranes *in vitro* [60]. In addition, treatment of lysosomal membranes with Al has been shown to reduce the activity of lysosomal H^+-ATPase thereby greatly reducing lysosomal acidification [57]. Prevention of proton uptake by lysosomes will have an impact on cytoplasmic pH leading to an accumulation of protons and acidification of the cytoplasm. Strict regulation of intracellular pH is critical for proper electrical excitability of neurons. Inhibition of proton extrusion using inhibitors of acid extrusion exchangers resulted in an initial

increase in action potential frequency followed by a prolonged inhibition of nerve function [7]. Intracellular acidosis also has an affect on neuronal viability. Ying *et al*. [54] showed that acidosis potentiated oxidative stress-induced cortical neuron death possibly through acidosis-induced inhibition of antioxidant defenses. To prevent these detrimental effects, a cell will regulate intracellular proton concentrations to eliminate acidosis. One mechanism for lowering intracellular pH is through the extrusion of protons. Here we observed an increase in ECAR indicative of proton extrusion in cells treated with Al.

The effect of Al in ECAR was not simply due to the physical presence of the metal ions because boron that shares the properties with Al in the Periodic Table (both are Group IIIa elements) did not produce similar changes in this system. The technique employing alterations in ECAR has been utilized as a useful tool to measure the membrane and metabolic effects of unrelated chemicals and different cell systems [28,35] and has been used to monitor metabolic changes in PC-12 cells previously [36]. For example, Pitchford *et al*. [36] showed an increase in ECAR in PC-12 cells treated with nerve growth factor [13] and demonstrated that the Na^+/H^+ exchanger was involved. The mechanism by which the ECAR is increased by Al is not clear but may be related to Al-induced changes in intracellular pH as discussed above. The proton economy in a cell includes proton production, excretion and regulatory mechanisms and is vital to the control in intracellular pH [39].

An alternative mechanism for the increase in ECAR is through the inhibition of carbonic anhydrase activity. Previous studies have shown a profound inhibition of rat brain carbonic anhydrase when treated with Al [58]. Inhibition of carbonic anhydrase in

CA3 hppocampal neurons using sulthiame or acetazolamide resulted in intracellular acidificaiton [24] and has been proposed as a mechanism for the anti-epileptic properties of these drugs. Therefore, it is possible that inhibition of carbonic anhydrase by Al [58] and subsequent intracellular acidification induces a compensatory mechanism to control pH manifesting in the extrusion of protons and increased ECAR. Further studies will be required to precisely define the interaction between Al-induced inhibition of carbonic anhydrase and increased ECAR.

Disruption of ion fluxes across the plasma membrane can also have an impact on membrane potential. In the present study, treatment of PC-12 cells with Al resulted in a decrease in $Dibac_4(3)$ fluorescence indicative of hyperpolarization [44]. Hyperpolarization is an increase in negative charges, or a loss of positive charges on the intracellular side of the membrane. Increased proton extrusion may be responsible for the observed effects of Al on membrane potential. The absolute value of the potential change cannot be determined from the present data and would require patch clamp techniques. Swallow *et al*. [43] showed that acid loading resulted in hyperpolarization of macrophage cell membranes. This effect was not sensitive to charybdotoxin precluding the involvement of K^+ efflux through Ca^{2+}-activated K^+ channels. Instead the authors suggested that the electrogenic H^+-ATPase pumping of protons across the plasma membrane was responsible for the hyperpolarization. The presence of bafilomycin sensitive H+-ATPase in PC-12 cells has been shown [1] and therefore, it is possible that this channel is in part responsible for the observed effects of Al on ECAR and membrane potential.

In the current study, Al induced the production of ROS in PC-12 cells. The observed increase in ROS production may involve interactions between Al and endogenous cellular iron. Xie and Yokel [41] reported that Al facilitated iron-mediated lipid peroxidation of several bovine brain fractions and of pure phospholipids. Iron is a known inducer of ROS [42]. It has also been shown that Al can interfere with the cellular utilization of iron in the erythroleukemia K562 cells [43]. In addition, Al has been shown to inhibit the aconitase activity [44]. This enzyme requires iron for its function and the action of Al may involve the loss of iron from aconitase leading to an increase in intracellular redox-active iron and subsequent ROS production. Yang *et al*. [53] showed that Al was capable of stabilizing iron in the Fe^{2+} state thereby feeding the Fenten generation of hydroxyl radicals. Lipid peroxidation has been shown to have a profound affect on membrane stability and fluidity [9] which may in turn increase the susceptibility of membrane lipids to peroxidative damage [49]. This synergistic and reciprocal relationship between membrane fluidity changes and lipid peroxidation may be involved in the effects observed in the present study and also in the pathophysiology of Al exposure *in vivo*.

We previously reported that Al administered through drinking water caused a decrease in DA and its metabolites, DOPAC and HVA, in the hypothalamus of mice given Al ammonium sulfate as 5 parts per million of Al. Our intention was to examine the relationship between the observed membrane changes and the effects of Al on biogenic amine metabolism in PC-12 cells. The validity of PC-12 cells as a model for toxicant-induced alterations in biogenic amine metabolism has been reported. Seegal and coworkers [40] assessed the effect of a mixture of polychlorinated biphenyls on DA and

NE metabolism in PC-12 cells following the exposure for 6 h. There was a concentration- and time-dependent decrease of these biogenic amines in cells with a corresponding increase in the medium suggesting extrusion of neurotransmitter from the cells. Despite the extensive membrane alterations induced by Al, there were no alterations in the metabolism of any neurotransmitters or metabolites observed in the cells or in the surrounding medium. It appears that the *in vivo* effects of Al on the dopaminergic system [46] are due to prolonged neurological changes and may not be evident following the short-term treatment protocol used in the present study.

Although Al modified the membrane properties in PC-12 cells in a concentration-dependent manner, the concentration of Al in the extracellular fluid may not reflect the available Al. The chemistry of Al is complex and at physiological pH, the fraction of Al available in ionized and thus biologically active form may be minimal. At pH 4 or above most the Al may appear in a colloidal form [20] and very little may be available for biological activity. The use of complexing agents such as citrate has been used to keep the Al in solution and make it available for cellular uptake. While the citrate complex of Al may be taken up by cells and citrate is metabolized releasing free Al, the membrane effects of citrate complex will likely be decreased because the complexed metal may not be available at the membrane. The effects of Al on dopamine in mice [46] may be independent of membrane changes or the lack of effects on PC-12 biogenic amine metabolism may reflect the complex solution chemistry of Al. Standardization of Al preparations used in *in vivo* and *in vitro* studies is required before direct and reliable extrapolations can be made.

In conclusion, the data reported here suggest that Al may have a direct effect on PC-12 cells, particularly on the cell membranes. This effect may either involve or lead to generation of ROS and subsequent changes in cellular function. PC-12 cells can be differentiated into functional neurons by incubation with a nerve growth factor and it would be of interest how differentiated cells will respond to Al in these systems. Whether long-term treatment or treatment of NGF differentiated PC-12 cells will show altered biogenic amine metabolism in response to Al treatment is of future interest.

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Table 4.1

The concentrations of dopamine (DA) and serotonin (5-HT) in the extract of PC-12 cells exposed to Al ammonium sulfate.[a]

Treatment	DA	5-HT
Control	174.05±45.87	35.16±2.02
Al 1 μM	130.68±32.34	35.09±3.05
Al 10 μM	142.34±43.23	40.33±4.16
Al 100 μM	154.43±30.08	41.21±1.04
Al 300 μM	225.82±14.19	36.29±3.40
Al 1 mM	140.22±20.80	37.49±5.77
Al 3 mM	224.80±65.58	34.91±2.23

[a] mean value±standard error (ng/10^6 cells), n=6 for control, and n=3 for Al exposed groups. The *P* values for ANOVA were 0.65 and 0.70, for DA and 5-HT, respectively.

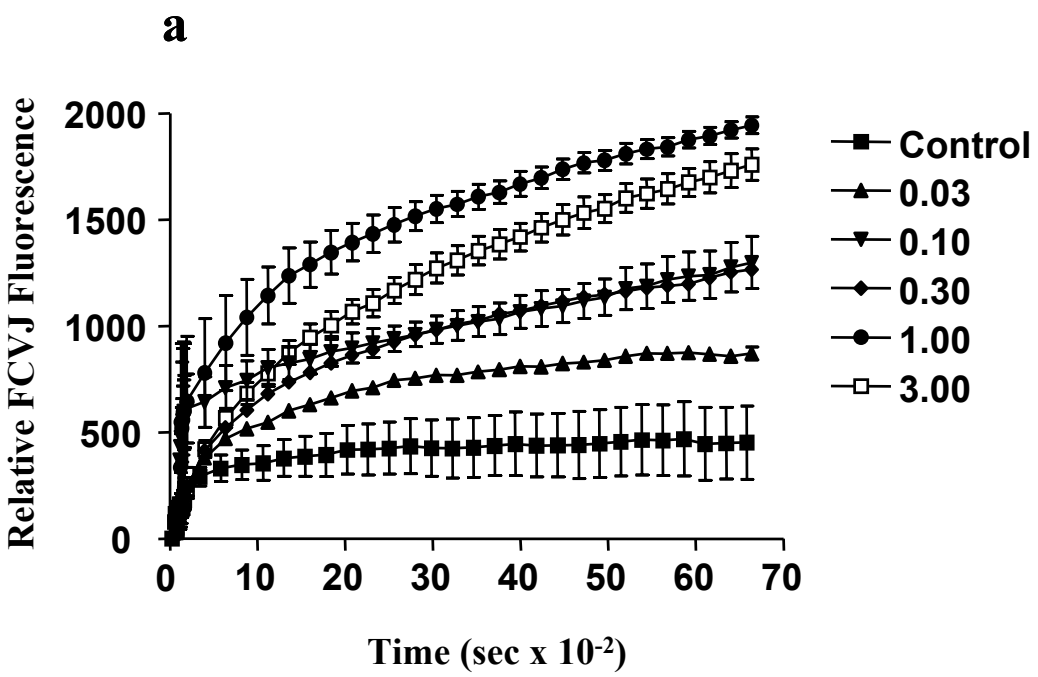
Table 4.2

The concentration of neurotransmitters and metabolites in the supernatant of PC-12 cells exposed to Al ammonium sulfate.[a]

Treatment	NE	5-HT	DOPAC	5-HIAA
Control	179.83±9.64	141.49± 6.00	100.01±5.40	42.14±2.92
Al 1 μ M	204.87±2.78	148.79± 9.31	102.52±11.08	47.31±1.74
Al 10 μM	199.83±8.17	142.05± 2.09	106.01±10.63	39.64±2.47
Al 100 μM	208.74±3.01	139.23± 2.99	105.93±5.42	42.93±2.06
Al 300 μM	189.48±10.53	139.82± 5.29	108.10±0.51	41.90±3.22
Al 1 mM	195.45±0.33	140.33±12.50	97.05±7.21	38.42±3.66
Al 3 mM	211.76±1.06	133.02±10.44	105.95±2.63	40.69±7.24

[a] mean value ± standard error (ng/ml), n=6 for control, and n=3 for Al treated cultures. The corresponding *P* values for ANOVA for each column (left to right) were 0.08, 0.92, 0.92, and 0.78, respectively.

Fig. 4.1. Membrane fluidity is concentration- and time-dependently decreased in PC-12 cells treated with Al. PC-12 cells were loaded with FCVJ following which cells were treated with Al as $AlCl_3$ (0.03-3 mM) and imaged using a FLIPR for 2 h. Kinetics of the fluorescence change over time is shown in Fig. 4.1a. Concentration response analysis shows an EC_{50} of ~0.1 mM Al (Fig. 4.1b). Mean ± SEM (n = 4 wells/group/time point) Data are representative of three independent experiments with similar results.


a
Relative FCVJ Fluorescence
2000
1500
1000
500
0
0
10
20
30
40
50
60
70
Time (sec x 10-2)
Control
0.03
0.10
0.30
1.00
3.00

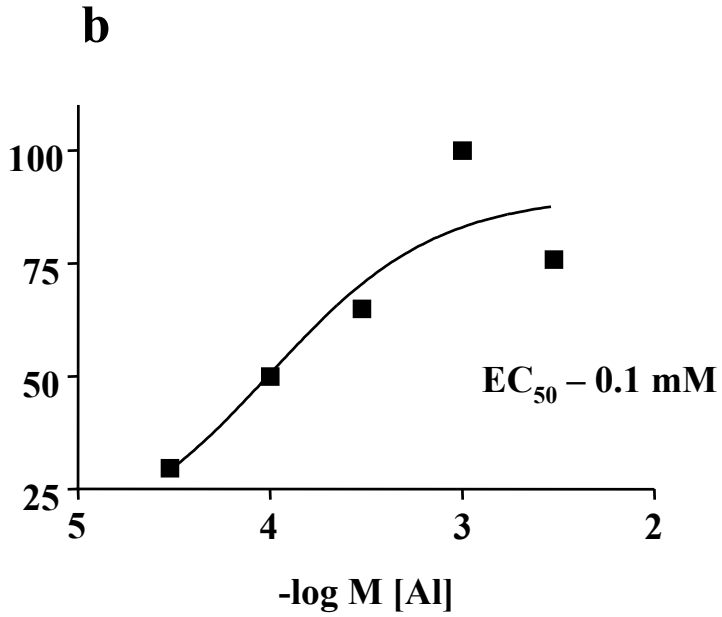

b
100
75
50
25
5
4
3
2
-log M [Al]
EC50 – 0.1 mM

Fig. 4.2. Al treatment reduces membrane fluidity in PC-12 cells. PC-12 cells were loaded with FCVJ following which cells were treated with Al as $AlCl_3$ (0.01-1 mM) for 1 h. FCVJ fluorescence was then examined in individual cells using a flow cytometer. The histograms displayed represent FCVJ fluorescence of PC-12 cell treated with (a) Locke's buffer (control), (b) 0.01 mM Al, (c) 0.1 mM Al, (d) 1 mM Al and (e) H_2O_2 (500 μM)/Fe^{2+} (100 μM) [42] hydroxyl radical generating system (positive control for free radical induced loss of membrane fluidity). The mean fluorescence intensity (MFI) of each population is given in the figure. Data are representative of three independent experiments with similar results. The dotted line represents the MFI on the control cells and is to be used as a reference to visualize the increase in FCVJ fluorescence caused by Al treatment.

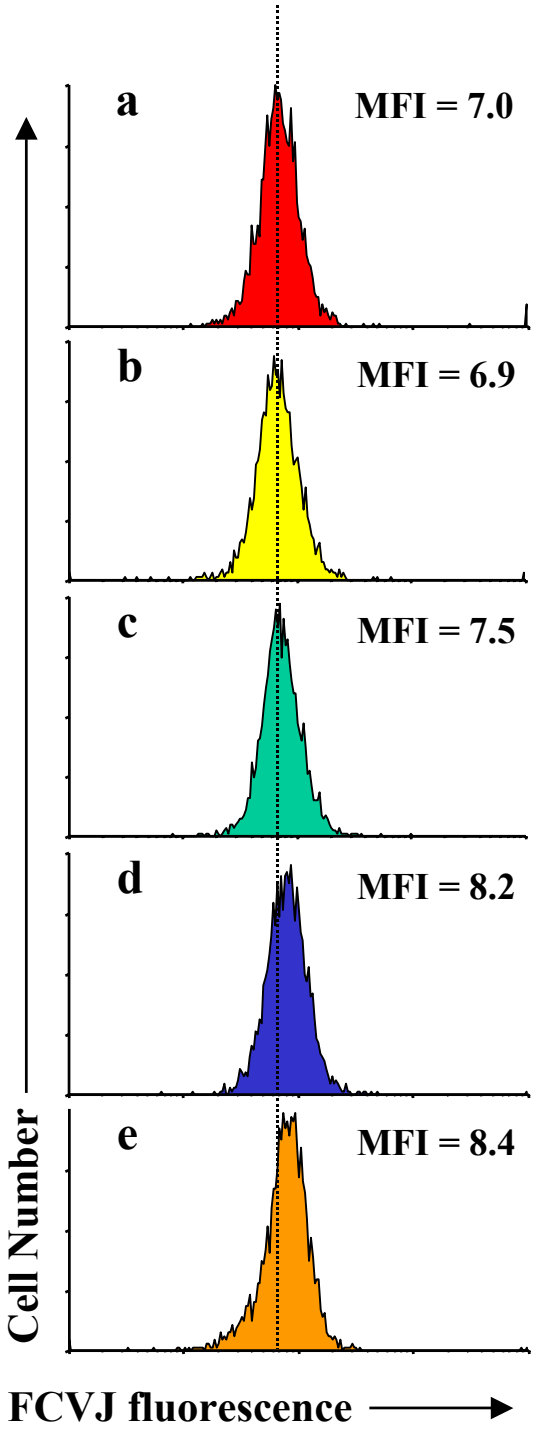

a
MFI = 7.0
b
MFI = 6.9
c
MFI = 7.5
d
MFI = 8.2
e
MFI = 8.4
Cell Number
FCVJ fluorescence

Fig. 4.3. Extracellular acidification rate (ECAR) of PC-12 cells after addition of different concentrations of Al. Data are expressed as percent of basal linear rate of proton extrusion prior to addition of Al obtained directly from the instrument read-out. Results have been shown (a) using Al as $AlCl_3$; mean ± SEM of 5 consecutive cycles, from one representative experiment. The lower panel (b) depicts the concentration-response for ECAR. All points were significantly different ($P \leq 0.05$) from the respective control value at that time point.

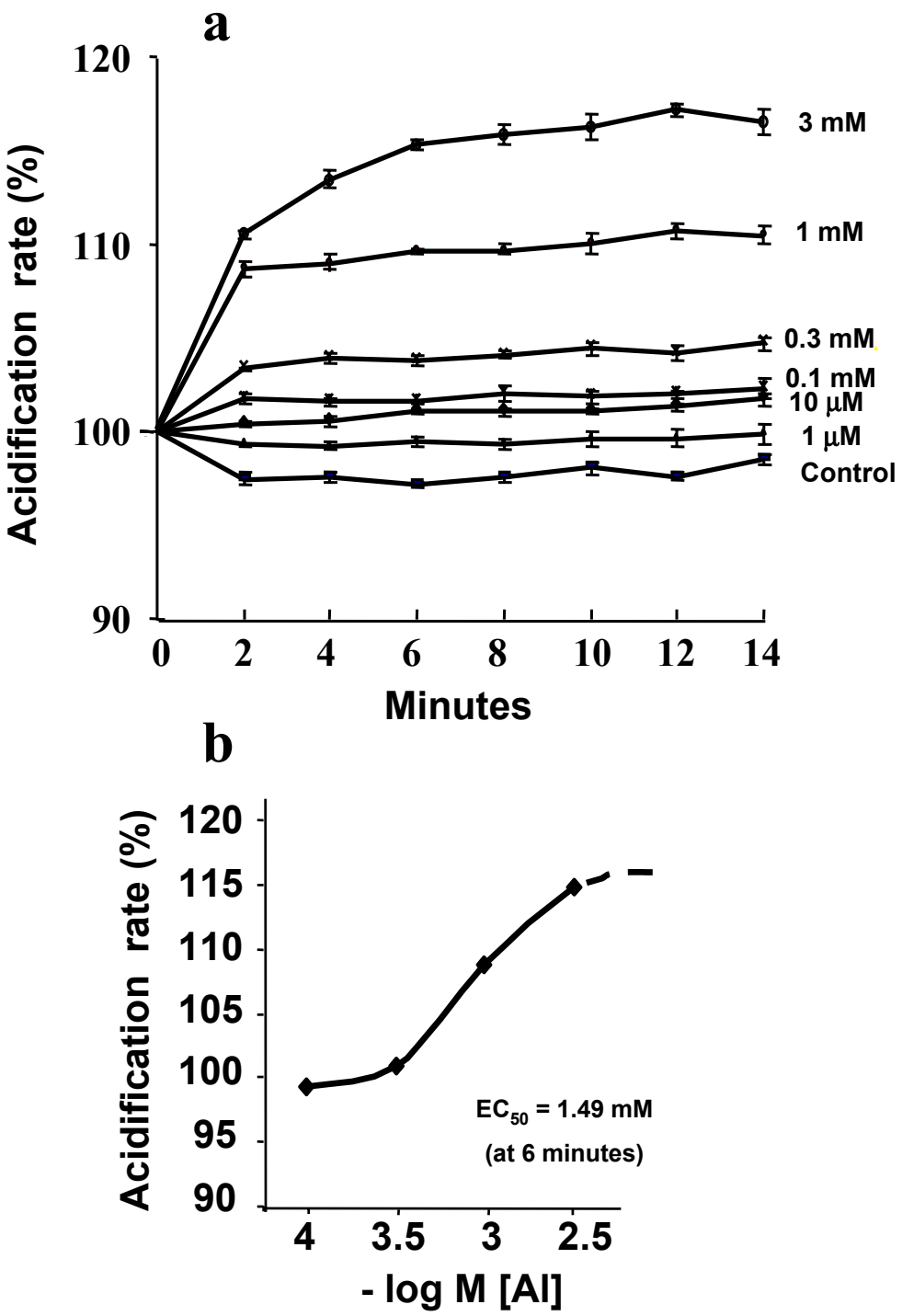

a
120
110
100
90
Acidification rate (%)
0
2
4
6
8
10
12
14
Minutes
3 mM
1 mM
0.3 mM
0.1 mM
10 μM
1 μM
Control
b
120
115
110
105
100
95
90
Acidification rate (%)
4
3.5
3
2.5
- log M [Al]
EC50 = 1.49 mM
(at 6 minutes)

Fig. 4.4. Relative $Dibac_4(3)$ fluorescence (arbitrary units) in PC-12 cells 90 min after addition of different amounts of Al as $AlCl_3$. Results are from one trial, representative of 2 independent experiments. Error bars indicate mean ± SEM of 4 wells from a representative experiment (C = control). * Indicates a significant difference ($P \leq 0.05$) from the control.

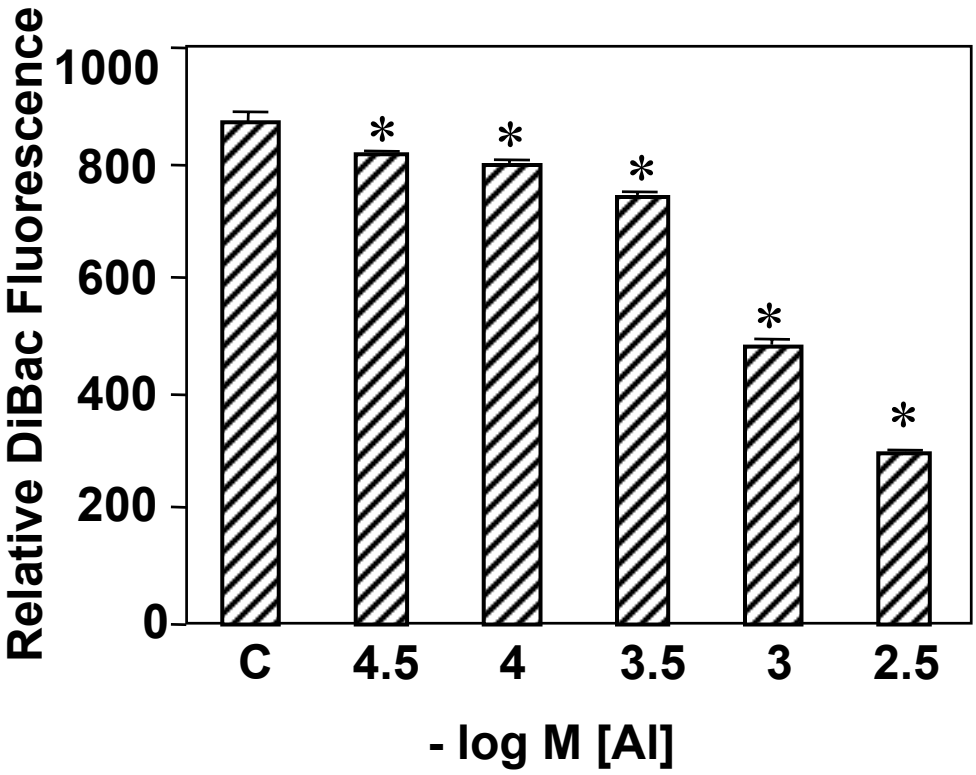

Relative DiBac Fluorescence
1000
800
600
400
200
0
*
*
*
*
*
C
4.5
4
3.5
3
2.5
- log M [Al]

Fig. 4.5. Increased dihydrorhodamine 123 fluorescence (arbitrary units) after addition of Al as $AlCl_3$ to PC-12 cells. The real-time change is illustrated in the top panel (a) for selected Al concentrations. The lower graph (b) represents the fluorescence change at 90 min. Error bars indicate mean ± SEM of 4 wells from a representative experiment of three independent trails with similar trends. * Indicates a significant difference ($P \leq 0.05$) from the control.

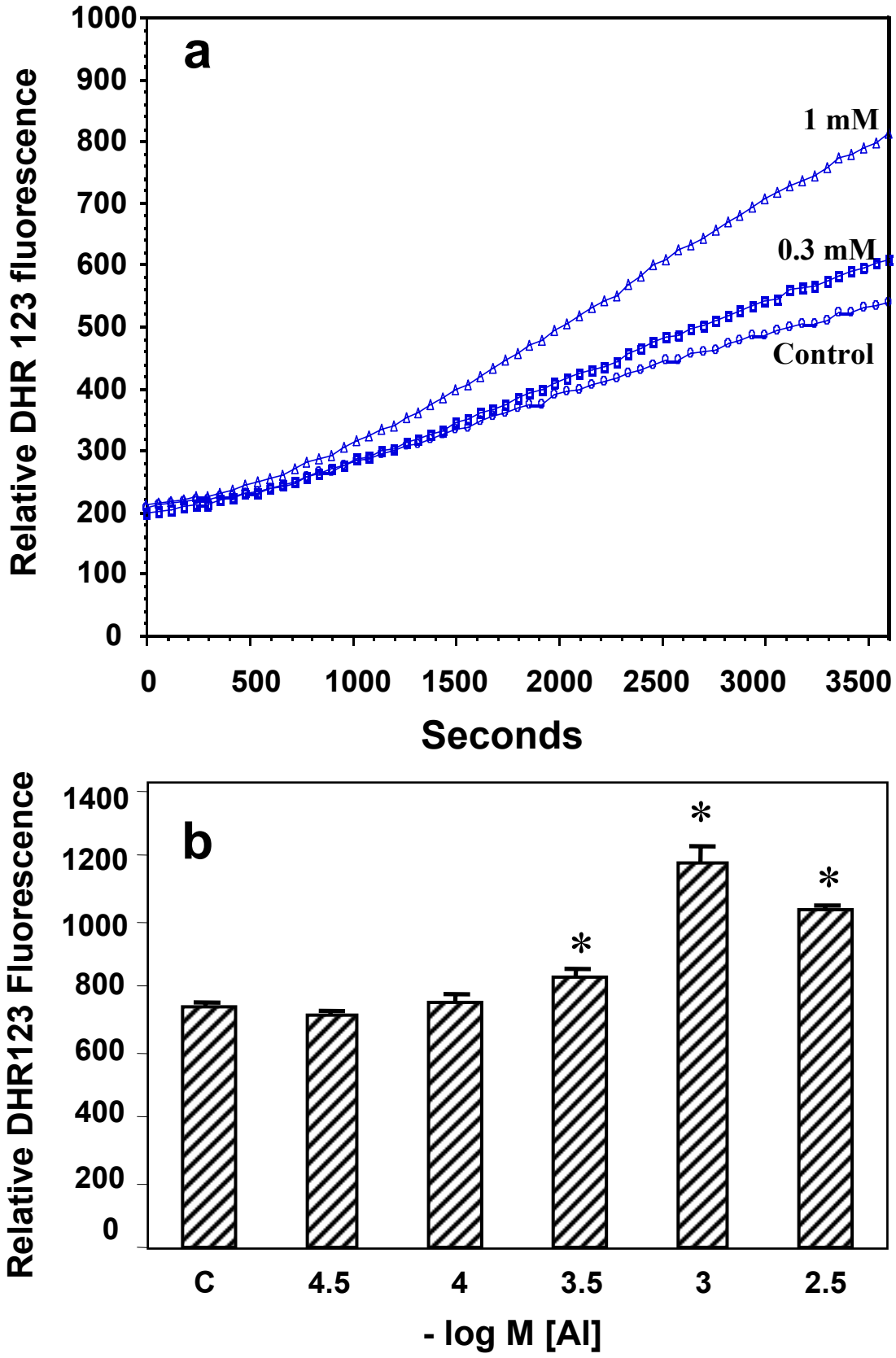
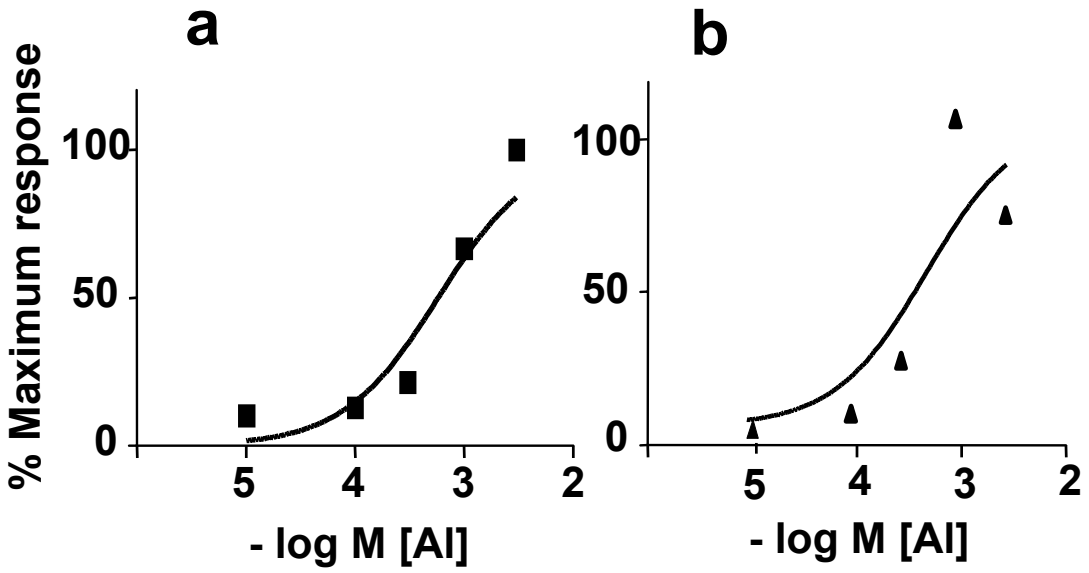
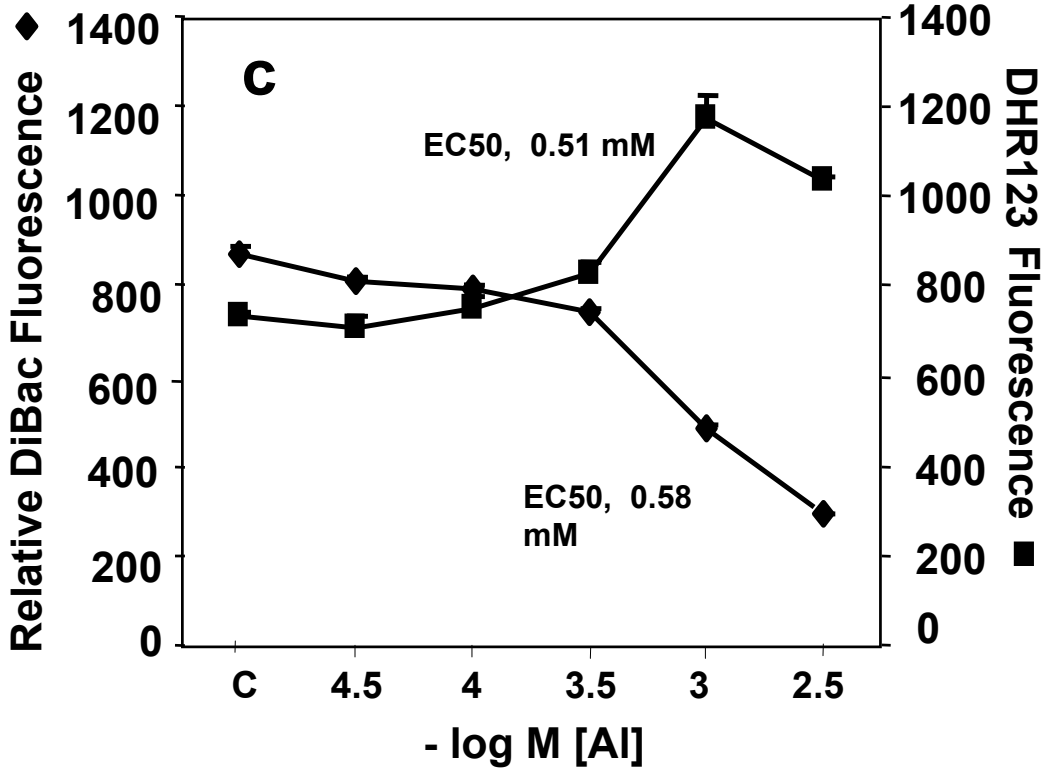

a
Relative DHR 123 fluorescence
1000
900
800
700
600
500
400
300
200
100
0
1 mM
0.3 mM
Control
0
500
1000
1500
2000
2500
3000
3500
Seconds
b
Relative DHR123 Fluorescence
1400
1200
1000
800
600
400
200
0
*
*
*
C
4.5
4
3.5
3
2.5
- log M [Al]

Fig. 4.6. Concentration-response curves of Al-induced changes in (a) membrane potential and (b) the generation of ROS, depicted as percent of maximal responses. The actual fluorescence changes (arbitrary units) are shown in (c) indicating the relationship between the two parameters. The EC_{50} values shown in "c" are calculated from the curves in "a" and "b", respectively, using the Prism program.


a
b
% Maximum response
100
50
0
5
4
3
2
- log M [Al]


c
EC50, 0.51 mM
EC50, 0.58 mM
Relative DiBac Fluorescence
DHR123 Fluorescence
1400
1200
1000
800
600
400
200
0
C
4.5
4
3.5
3
2.5
- log M [Al]

CHAPTER 5

ALUMINUM-MALTOLATE INDUCED APOPTOSIS IN NEURO-2a CELLS IS DEPENDENT ON PROTEIN SYNTHESIS BUT NOT MAJOR KINASE SIGNALING PATHWAYS[1]

[1]Johnson, V.J. and Sharma, R.P. To be submitted to Cell Death and Differentiation.

Abstract

Aluminum maltolate (Al-malt) has been shown to cause neurodegeneration following *in vivo* exposure and apoptosis plays a prominent role. The objective of this study was to define the form of cell death induced by Al-malt. Neuro-2a cells were treated with Al-malt for 24 h with or without pretreatment with a variety of pharmacological agents. Al-malt concentration-dependently increased cell death. The mode of cell death was a mix of apoptosis and necrosis. Treatment with Al-malt resulted in caspase 3 activation and the externalization of phosphatidyl serine, both indicative of apoptosis. In addition, nuclear condensation and fragmentation were evident. Interestingly, pretreatment with cyclohexamide markedly reduced Al-malt-induced apoptosis indicating that the death process is dependent on *de novo* protein synthesis. Further studies showed that the toxicity was independent of oxidative stress and major kinase signaling pathways. The results provide insight into the mechanisms of Al-malt neurotoxicity and support the involvement of this metal in neurodegeneration.

Keywords: aluminum; aluminum maltolate; maltolate; apoptosis; necrosis; caspase 3; neurodegeneration; neurotoxicity; Neuro-2a

Abbreviations: AD, Alzheimer's disease; Al, aluminum; Al-malt, aluminum maltolate; CHX, cyclohexamide; H33258, Hoechst 33558; LDH, lactate dehydrogenase; NO, nitric oxide; PMA, phorbol 12-myristate 13-acetate; PS, phosphatidyl serine; SEM, scanning electron microscopy

Introduction

The etiology of Alzheimer's disease (AD) is exceedingly complex with contributing factors including oxidative stress[1,2], inflammation[3] and extensive protein and membrane modification.[4] All of these pathophysiological processes are also associated with non-disease aging. It is not clear why some patients develop AD and others remain healthy into advanced age. Two major contributing factors in the development of AD are the existence of a genetic predisposition[5] and exacerbation of normal aging related changes by environmental factors.[4] Aluminum (Al) represents one such environmental factor that has been linked to the development of AD. In fact, it has been stated that the use of Al as an experimental neurotoxicant has recapitulated virtually every feature of the neurodegenerative spiral afflicting Alzheimer's patients.[6] Additional evidence suggests that Al accumulates in the human brain with aging.[7] Given that Al is the third most abundant element in the earths crust and exposure is unavoidable, further research into the neurotoxicity of this metal are both warranted and essential.

The hallmark diagnostic features of AD include neurofibrillary tangles and neuritic amyloid plaques both of which are considered to be late events in disease progression. Research is now focusing on identification of early signs of disease, which may provide sufficient time for therapeutic intervention. Pratico *et al*.[8] recently showed that the levels of isoprostanes, indicators of *in vivo* oxidative stress, were higher in AD urine and plasma than in non-demented controls and that isoprostane levels correlated with disease severity. The same group definitively showed that an increase in ispoprostane levels preceded the development of plaques in an animal model of AD.[9] In addition, Su *et al*.[10] found that neuronal DNA damage indicative of apoptosis preceded

the formation of tangle pathology in human AD patients. Oxidative stress and apoptosis are related events and both have been associated with Al-induced neurotoxicity.[11] The presence of Al in AD brains[12] together with the ability of Al to produce oxidative stress and apoptosis, both identified as early events in AD, provide strong evidence for an early involvement of this environmental factor in disease progression.

Aluminum maltolate (Al-malt) is a lipophilic complex of aluminum that forms a meta-stable solution of pH 7. This Al complex is advantageous for use in *in vitro* mechanistic studies because it dose not form insoluble precipitates of Al-hydroxide at physiological pH, as do most other salt forms of Al.[13] Maltolate is also a common component of the human diet. It is a byproduct formed during sucrose pyrolysis or thermal degradation of starch[14] and can be found in coffee, soybean, baked cereals and camalized and browned foods.[15] Given the extremely high affinity of maltolate for Al, there is a potential for this species to form in the gastrointestinal tract. Therefore investigating the enhanced toxicity of Al-malt is relevant to human health. Numerous studies have demonstrated that Al-malt is a potent neurotoxin. There is also evidence to suggest that maltolate may facilitate the entry of Al into the brain[16] thereby increasing the potential for neurotoxicity. Intracisternal injection of Al-malt produces cytoskeletal alterations[17,18] that are reminiscent of the tangles seen in AD. Recent findings show that the neurofibrillary degenerations induced by Al also contain hyperphosphorylated *tau* proteins as in AD.[19] Increasing evidence suggests that oxidative stress and apoptosis are key events in the neuropathological changes following exposure to Al-malt.[11]

Al-malt-induced apoptosis in the rabbit hippocampus involves perturbation of the Bcl-2/Bax ratio.[11] More recently, it was shown that the endoplasmic reticulum is

involved in Al-malt-induced apoptosis.[20] Translocation of Bax to the endoplasmic reticulum and a loss of Bcl-2 have been demonstrated in rabbits treated with Al-malt. These effects may be involved in the observed release of cytochrome c from the mitochondria in Al-malt treated rabbits.[21] Once released, cytochrome c can bind to Apaf-1 in the cytoplasm forming a complex that can activate caspase 9 with subsequent activation of death-inducing caspase 3.[22] Ghribi *et al*.[20] demonstrated that caspase 3 was activated following intracisternal injection of Al-malt and the site of activation was the endoplasmic reticulum.[21] These biochemical changes were shown to culminate in DNA fragmentation as evidenced by TUNEL staining in the hippocampus of Al-malt treated rabbit.[23,11] Collectively, these studies provided evidence for the involvement of apoptotic cell death in Al-induced neurodegeneration and strengthen the possible involvement of Al as an early factor in the development of AD.

Neuronal cell lines are also sensitive to the toxic effects of Al-malt and may provide models to study the mechanisms involved in apoptosis. Al-malt-induced apoptosis has been demonstrated in differentiated PC-12 cells[24,25] and human NT2 neuroblastoma cells.[26] Neuro-2a (N2a) murine neuroblastoma cells are also sensitive to Al treatment which up-regulated the expression and accumulation of neurofibrillay tangle protein.[27] Treatment also increased the uptake of iron in N2a cells[27] possibly contributing to Al-induced oxidative stress and neurotoxicity. The objective of the present study was to define the nature of Al-malt-induced cell death, apoptosis versus necrosis. In addition we pharmacologically manipulated Al-malt-induced cell death using inhibitors and activator of kinase pathways, extracellular calcium disposition and oxidative stress with the intention of identifying sites for intervention. The results clearly demonstrate the co-

involvement of apoptosis and necrosis in Al-malt neurotoxicity. The apoptotic cell death induced by Al-malt was dependent on new protein synthesis as evidenced by inhibition of apoptosis in the presence of cyclohexamide (CHX). Both the apoptosis and the necrosis were independent of oxidative stress and major kinase pathways. The influx of calcium from the extracellular milieu may play a role in apoptosis induction by Al-malt. These results suggest that increasing anti-apoptotic gene expression may be effective in preventing Al-malt neurotoxicity.

Results

Al-malt induces cell death in Neuro-2a cells

The complex speciation chemistry of Al presents a difficulty when comparing the cytotoxicity of various forms used for *in vitro* experiments. Our first goal was to determine the susceptibility of N2a cells to an inorganic and an organic form of Al. Treatment of N2a cells for 24 h with up to 1000 μM of $AlCl_3$ did not induce cell death as indicated by the MTT cytotoxicity assay (Figure 5.1). In contrast, a dose-dependent increase in cell death was observed in N2a cells treated with Al-malt. Mitochondrial function was reduced to 80 and 40% of control levels in cells treated with 250 and 500 μM Al-malt, respectively for 24 h (Figure 5.1). Previously it was reported that maltolate can cause cell death in N2a cells.[28] Therefore, we tested the ability of maltolate to induce cell death in our system. Since three molecules of maltolate are bound to each ion of Al, we used 3X molar concentrations of maltolate as an important control group. Converse to the results of Hironishi *et al*.[28] we did not observe any change in viable cell number by the MTT assay (Figure 5.1). It can be concluded that 24 h exposure is insufficient for the

manifestation of maltolate toxicity in N2a cells. N2a cells exposed to 1500 μM maltolate did show a small decrease in viability although the effect was not significant. Perhaps toxicity would be evident at 72 h as seen previously in this cell line.[28] The findings of the mitochondrial MTT assay were confirmed by examining the release of lactate dehydrogenase (LDH) from the cells. Figure 5.1 indicates that Al-malt induced a dose-dependent increase in the release of LDH indicating a loss of integrity of the cell membrane. The increase was significant at concentrations above 62.5 μM. Maltolate alone did not increase LDH release from N2a cells (data not shown).

The toxicity of Al-malt was also evident in the marked drop in DNA synthesis by 24 h of treatment (Figure 5.2). Incorporation of ^{3}H-thymidine into N2a was completely abolished by 500 μM Al-malt. Maltolate alone did not decrease DNA synthesis at the doses tested. Interestingly, all concentrations of maltolate except for 1500 μM induced DNA synthesis in N2a cells indicative of a proliferative effect (Figure 5.2). These findings confirm that the enhanced toxicity of Al-malt is specific for the complex and not simply due to the presence of maltolate.

Apoptosis and necrosis are involved in Al-malt neurotoxicity

Dual staining with cell permeant acridine orange and cell impermeant ethidium bromide is an effective flow cytometric method for discriminating apoptotic from necrotic cell death.[29] Live cells (Figure 5.3, population L) stain with acridine orange and fluoresce green with low orange-red signal from ethidium bromide. Apoptotic cells (Figure 5.3, population Ap) exhibit a reduced green fluorescence but maintain a low ethidium bromide signal. The reduction in acridine orange signal is likely due to nuclear fragmentation and cell shrinkage. Necrotic cells (Figure 5.3, population N) loose the

ability to exclude ethidium bromide and as a result fluoresce bright orange-red (Figure 5.3). Treatment of N2a cells with Al-malt resulted in a dose-dependent increase the apoptotic and the necrotic populations (Figure 5.3c-f). The group treated with maltolate alone (Figure 5.3b) showed a slight increase in the apoptotic population over that of the control (Figure 5.3a). This may reflect the beginning of an increase in apoptosis as reported earlier[28] or may represent a slowing of the normal apoptotic turnover of cells observed in all cell cultures. These cell changes in response to Al-malt treatment, nuclear condensation and cell shrinkage, are early events in the apoptotic cascade.

The externalization of phosphatidyl serine (PS) is another early event in apoptosis and represents a flag to induce phagocytosis by mononuclear cells.[30] This event was determined using the Annexin V binding assay and flow cytometry. Al-malt concentration-dependently increased the externalization of PS as shown in Figure 5.4c-f. A slight increase in Annexin V binding was also observed in the maltolate treated group (Figure 5.4b) confirming the findings of the acridine orange/ethidium bromide assay. Annexin V can also bind to PS on the inner leaflet when the intergrity of the plasma membrane is compromised and is indicative of necrosis or secondary necrosis following apoptosis. To control for this, propidium iodide (PI), a cell impermeant nuclear stain was used. Live cells are displayed in the lower left quadrant and are negative for both stains. The upper right quadrant of the panels in figure 5.4 represent cells that are staining for Annexin V and PI and have thus undergone necrotic cell death. The cells in the lower right quadrant are the early apoptotic cells showing a characteristic redistribution of PS. Both the upper and lower right quadrants were increased by Al-malt. Thus, the Annexin

V binding assay confirms the coexistence of apoptotic and necrotic cell death in N2a cells treated with Al-malt.

Microscopic analysis of cellular and nuclear morphology also suggests that apoptosis and necrosis are both induced in N2a cells following treatment with Al-malt. Cells treated with 500 μM Al-malt show cellular shrinkage (Figure 5.5c) as well as nuclear condensation and fragmentation (Figure 5.5d inset, arrow), both hallmark features of apoptosis. In addition, treated cells exhibit dramatic membrane blebbing (Figure 5.5j, arrow). Signs of necrosis are also evident in the same cultures that display apoptosis. Cell swelling (Figure 5.5c) and loss of membrane integrity without nuclear condensation (Figure 5.5d inset, arrow head) are evident in cultures treated with 500 μM Al-malt. The loss of membrane integrity is also apparent in the scanning electron micrograph of cells treated with Al-malt (Figure 5.5j, arrow head). All of these features of cell death were not evident in control cultures (Figure 5.5a,b,i). Pretreatment with CHX (CHX controls are shown in figure 5.5e,f) greatly reduced the cell shrinkage (Figure 5.5g) and nuclear alterations (Figure 5.5h) induced by Al-malt. In addition, neuronal processes were still evident in cultures pretreated with CHX (Figure 5.5g, arrows) whereas treatment with Al-malt alone completely eliminated process formation (Figure 5.5c).

Caspase 3 is activated in N2a cells treated with Al-malt

The activation of caspase 3 is an integral step in the majority of apoptotic events. This enzyme belongs to the cysteine protease family and is responsible for cleaving substrates such as DNA fragmentation factor that can go one to damage DNA[31] and is thus considered to be an effector caspase. Caspase 3 activity was markedly increased in a

concentration-dependent manner in N2a cells treated with Al-malt (Figure 5.6). Again, a slight increase in caspase 3 activtiy was noted in the maltolate treated cells (open bar in Figure 5.6) indicative of a small increase in apoptosis. The specificity of the assay was demonstrated by using a specific inhibitor of caspase 3, Ac-DEVD-CHO, which abolished the activity in Al-malt treated lysates (solid bar in Figure 5.7). Interestingly, the caspase 3 activity of washed cell was much lower than that present in cell lysed in the treatment media (data not shown). This could be due to removal of apoptotic cells during washing or more likely due to the release of caspase 3 from dying cells into the media as reported previously.[32]

Al-malt-induced apoptosis requires *de novo* protein synthesis

Apoptosis is an active form of cell death and can require *de novo* protein synthesis to initiate and/or complete the process.[33] Cyclohexamide is a potent inhibitor of gene translation and has been used extensively in apoptosis research. Pretreatment of N2a cells with 0.5 μg/ml of CHX for 30 minutes prior to the addition of Al-malt produced a marked inhibition of cell death as evidenced by the release of LDH into the media (Figure 5.7, solid bars). Flow cytometric examination using acridine orange/ethidium bromide dual staining showed that CHX almost completely inhibited apoptosis but not necrosis (Figure 5.8). These finding were confirmed by cell cycle analysis and the detection of hypodiploid nuclei. Al-malt dose-dependently increased the hypodiploid population and this was abolished in cells pretreated with CHX (Figure 5.8). Interestingly, Al-malt appeared to induce cell cycle arrest in the G_2/M phase, a phenomenon often associated with toxicant-induced apoptosis in cultured cells.[34]

Therefore perturbations of cell cycle progression may be involved in Al-malt-induced neurotoxicity.

Confirming a role for *de novo* protein synthesis in the initiation of apoptotic cell death induced by Al-malt is the finding that pretreatment of N2a cells with CHX prevented the activation of caspase 3 (Figure 5.9). The activation of caspase 3 by Al-malt did not increase significantly beyond 250 μM (Figure 5.6 and 5.9). Pretreatment with CHX completely abolished caspase 3 activation induced by 250 μM Al-malt but only reduced the activation in cells treated with 500 μM Al-malt (Figure 5.9). The same trend is evident in Figure 5.8 showing complete inhibition of cell death by CHX in cells treated with 250 μM but only partial inhibition in cells treated with 500 μM Al-malt. It is possible that 0.5 μg/ml of CHX does not completely inhibit transcription in N2a cells thus accounting for these results. Higher doses of CHX need to be investigated to confirm this hypothesis.

Al-malt-induced cell death is independent of oxidative stress and major kinase pathways

Al is a known to exacerbate and induce oxidative stress and therefore, the role of reactive oxygen species in Al-malt-induced cell death was examined using inhibitors of lipid peroxidation and free radical scavengers. Vitamin E is known to inhibit lipid peroxidation and prevents Al-induced oxidative stress in amyloid precursor protein transgenic mice (Tg2576).[35] Similarly, silymarin, a mixture of natural flavonoids has been shown to scavenge free radicals and inhibit lipid peroxidation.[36] Pretreatment with both antioxidants did not afford any protection to N2a cells against Al-malt-induced cell death (Table 5.1). Ascorbic acid, another antioxidant vitamin was also used and

pretreatment subtly but significantly increased cell death in response to Al-malt. We can concluded from these findings that oxidative stress is not the major factor driving cell death in N2a cells treated with Al-malt.

We also used agonists and antagonists of major kinase signaling pathways to determine the involvement of signal transduction in Al-malt neurotoxicity. Pretreatment of N2a cells with two inhibitors of mitogen activated protein kinase pathways, PD 98059 to inhibit extracellular regulated kinase and SB 203580 to inhibit p38 MAPK did not influence the cytotoxicity observed following treatment with Al-malt (Table 5.2). Inhibition or activation of protein kinase C using calphostin C and phorbol 12-myristate 13-acetate [37], respectively, did not prevent or exacerbate the toxicity of Al-malt (Table 5.2).

Al-malt-induced neurotoxicity may involve alterations in Ca^{2+} homeostasis

The results of the present study indicate that apoptosis is a major form of cell death induced by Al-malt and increases in intracellular Ca^{2+} are often implicated in apoptosis. We examined the involvement of the extracellular Ca^{2+} pool on toxicity in N2a cells. Pretreatment with 3 mM EGTA to chelate all extracellular Ca^{2+} did not influence death induced by Al-malt but did significantly increase cell death in cells not treated with Al-malt (Table 5.3). Microscopic examination indicated that all the cells were floating when treated with EGTA. Therefore, the effect on cell death may be related to the lack of substrate contact in the normally adherent N2a cells. For this reason we decided to block Ca^{2+} influx from the extracellular milieu using the store operated Ca^{2+}-channel blocker lanthanum (La^{3+}).[38] Pretreatment with 100 μM La^{3+} resulted in significant protection of N2a cells from Al-malt toxicity since LDH release was reduced

from 36% to 31% (Table 5.3). This small but significant decrease in cell death indicates that Ca^{2+} regulation may play an important role in Al-malt neurotoxicity and warrants further investigation.

Discussion

Apoptosis has been implicated as a prominent form of cell death in many human neurodegenerative diseases including AD.[39,40] Understanding the role of environmental factors in the induction of apoptosis in the brain or alterations in natural apoptotic processes is vital to defining the etiology of neurodegenerative diseases. Recent studies have generated a strong body of evidence indicating the potential of Al-malt to induce apoptosis and neurodegeneration in the brain[20,23,11] as well as *in vitro*.[41,25] *In vitro* models represent an effective system for determining the mechanism by which Al-malt induces neurodegeneration. The present study focused on characterizing the mode of cell death in N2a cells treated with Al-malt. The results clearly show that apoptosis is induced by Al-malt and that necrosis also plays a role in the neurotoxicity. We also determined the effects of pharmacological intervention on the toxicity of Al-malt. Al-malt-induced apoptosis was dependent upon *de novo* protein synthesis as evidenced by a marked inhibition of toxicity in the presence of CHX. Necrosis induced by Al-malt was not inhibited by CHX. In addition, the toxicity of Al-malt appears to be independent of oxidative stress and major cellular kinase signaling pathways.

The manner in which a cell dies can have a great impact on the resulting response in the surrounding tissue. Death by necrosis has been shown to act as a natural adjuvant inducing oxidative stress and the production of numerous pro-inflammatory cytokines.[30]

This is due to the indiscriminant release of the cytoplasmic contents from the dying cells. The result is an area of inflammation and immune-mediated cell damage in innocent cells surrounding the initial insult. In contrast, death by apoptosis is a controlled event usually with minimal loss of membrane integrity until the later stages termed secondary necrosis. Instead, the cytoplasmic contents are systematically degraded from within. This type of cell death often involves pahgocytosis by resident tissue macrophages and the release of anti-inflammatory cytokines.[42] A recent study suggested that this contention may not always apply. Jemmerson *et al*.[32] showed that cytochrome *c*, active caspase 3 and LDH were all released from apoptotic cells and that release began within 2 h of the initiating stimulus. Previously, it was thought that all of these proteins were degraded in the cytoplasm of apoptotic cells. Therefore, apoptotic cell death may also substantially contribute to death-induced secondary inflammation and damage to surrounding tissues. The findings of this study confirm that LDH and active caspase 3 are released from apoptotic cells since inhibition of apoptosis with CHX reduced LDH release and caspase 3 activity in the media. These findings suggest that the apoptosis seen *in vivo* following treatment with Al-malt could contribute to secondary immune-mediated damage and further neurodegeneration.

The involvement of *de novo* protein synthesis in toxicant-induced apoptosis has been demonstrated previously.[33] Our finding of a marked decrease in the neurotoxicity of Al-malt in the presence of the protein synthesis inhibitor CHX suggests that Al-malt-induced apoptosis is at least partially dependent on *de novo* protein synthesis. Several candidate proteins have been identified from *in vivo* studies in the rabbit brain. The pro-apoptotic protein Bax has been implicated in Al-malt-induced neurodegeneration in the

rabbit.[20,23,11] It is possible that new synthesis of Bax is required to induce apoptosis in response to Al-malt treatment. Recently it was shown that apoptosis induced by nitric oxide (NO) treatment in osteoblast cells was accompanied by an increase in Bax protein synthesis.[43] Interestingly, inhibition of protein synthesis partially inhibited the apoptosis suggesting that *de novo* synthesis of Bax may be responsible for the apoptosis induced by NO. The demonstration of increased Bax immunoreactivity in the CA1 region of the hippocampus[11,23] of rabbits treated with Al-malt strongly supports a role for new Bax synthesis in the induction of apoptosis in this model.

Caspases are important mediators of apoptosis and caspase activation has been demonstrated in Al-malt-induced neurodegeneration.[21,20,23] Our *in vitro* N2a cell model also showed strong caspase 3 activation in response to Al-malt treatment. Surprisingly, inhibition of protein synthesis using CHX inhibited the activation of caspase 3 in Al-malt treated cells implicating *de novo* protein synthesis in the activation of this death effector. Protein synthesis could affect the regulation of caspase 3 in two ways; 1) new pro-caspase 3 synthesis and 2) synthesis of other proteins required for caspase 3 activation. New synthesis of pro-caspase 3 and 7 was required for apoptosis in TSU-Pr1 prostate carcinoma cells.[44] Ghribi *et al*.[45] demonstrated that caspase 12 synthesis was induced in the hippocampus of rabbits treated with Al-malt. This finding supports the involvement of new synthesis of caspase family members in the activation of apoptosis by Al-malt. In addition, our finding of a decrease in Al-malt-mediated caspase 3 activation following inhibition of protein synthesis supports this conclusion.

Oxidative stress is another important mediator of the neurodegeneration seen following exposure to Al.[11] In the present study, pretreatment with antioxidants did not

reduce the cell death observed in N2a cells treated with Al-malt. In fact, cell death was significantly increased in cells pretreated with ascorbate [46]. It has been reported that ascorbate can enhance oxidative damage induced by iron-mediated ROS production.[47] Studies have demonstrated that Al can disrupt iron metabolism and increase iron-uptake in N2a cells[27] thus supporting an interaction between ascorbate and iron. Therefore the increase in cell death observed in cells pretreated with ascorbate may be through a different mechanism than that induced by Al-malt. Likewise, Al-malt-induced cell death in N2a cells was shown to be independent of major kinase signaling pathways. A similar independence of cell death from kinase pathways has been demonstrated for zinc, another neurotoxic metal.[48]

An increase in cytoplasmic levels of Ca^{2+} can lead to the activation of caspase-mediated apoptosis. This effect is through the release of cytochrome *c* from mitochondria following an increase in mitochondrial Ca^{2+} uptake.[49] Release of cytochrome *c* can lead to caspase 3 activation. Recently it was demonstrated that Ca^{2+}-mediated stress in the endoplasmic reticulum can induce apoptosis via activation of caspase 12 independently of cytochrome *c* release.[50] Both of these caspases have been implicated in Al-malt neurotoxicity[45,20] and a role for Ca^{2+} deregulation has also been suggested.[51] The present study supports the involvement of influx of Ca^{2+} from the extracellular environment. Pretreatment of N2a cells with La^{3+} significantly reduced Al-malt neurotoxicity. Although this effect was not observed when extracellular Ca^{2+} was chelated using EGTA, the findings still indicate that this ion may be involved in the toxicity. Recent evidence shows that prevention of Ca^{2+} release from intracellular stores using dantrolene reduced $AlCl_3$-induced cell death in primary hippocampal neurons.[52]

Therefore, further studies examining intracellular stores as well as other antagonists of Ca^{2+} channels to prevent influx from extracellular stores are warranted.

In summary, the results of the present study strongly suggest that apoptosis is a prominent form of cell death in N2a cells treated with Al-malt thus supporting previous *in vivo* findings. The substantial release of intracellular enzymes observed in these cells even when dying by apoptosis has implications for the involvement of Al-malt in secondary damage induced by immune activation and inflammation. Furthermore, the inhibitory effect of CHX on Al-malt-induced cell death indicates that *de novo* protein synthesis is intimately involved in the resulting neurotoxicity. The identification of proteins that are up-regulated in response to treatment with Al-malt will greatly improve our understanding of the mechanisms involved in Al neurotoxicity and may provide insight into the etiology of neurodegenerative diseases including AD. Such research may also lead to effective strategies for therapeutic intervention in human neurodegenerative diseases.

Materials and Methods

Materials

Cell culture reagents were obtained from GIBCO Life Technologies, Inc. (Grand Island, NY, USA). Annexin V-FITC was procured from Pharmingen (BD Biosciences Pharmingen, San Diego, CA). The CaspACE™ assay system was purchased from Promega Corporation (Madison, WI). Propidium iodide, PMA, calphostin c, ascorbate [46], α-tocopherol acetate [46], lanthinium chloride (source of La^{3+}), EGTA, silymarin and CHX were purchased from Sigma Chemical Company (St. Louis, MO). PD 98059 and SB

203580 were obtained form Calbiochem-Novabiochem Corporation (San Diego, CA). All other reagents used were purchased from Sigma and were tissue culture grade.

Cell culture and treatment

Neuro-2a (N2a; CCL-131, American Type Culture Collection, Manassas, VA) murine neuroblastoma cells were grown in DMEM supplemented with 10% heat inactivated fetal calf serum (FCS) and 1% penicillin-streptomycin (Pen-Strep®, GIBCO, Grand Island, NY). Cultures were maintained in plastic tissue culture vessels in a humidified atmosphere at 37°C with 5% CO_2. Cells were seeded at 2 x 10^5/ml for each experiment and allowed to grow for 18 h prior to treatment with Al-malt. All treatments were performed in DMEM with 10% FCS unless stated otherwise. Treatment durations were all 24 h unless stated otherwise.

The speciation chemistry of Al is very complex and aqueous solutions of Al^{3+} undergo extensive hydrolysis over time yielding colloidal complexes that may influence the bioavailability of Al.[53] To control for this, Al^{3+} stocks were made immediately prior to treatment by dissolving $AlCl_3$ in millipore water at a concentration of 10 mM and this stock was used to make the treatment solutions (1-1000 μM $AlCl_3$). The pH of the treatment solutions was adjusted to 7 prior to addition to the cells. Several dietary ligands including citrate, lactate and maltolate have a high affinity for Al and can influence both the solubility of the metal as well as its toxicity. Numerous studies have demonstrated that maltolate greatly enhances the toxicity of Al both *in vitro*[54,25,24] and *in vivo*.[11] The toxicity of Al^{3+} and Al-malt were investigated in the present study. Al-malt was prepared as previously described in detail[55] and a 20 mM stock solution was prepared immediately prior to use. Al-malt forms a meta-stable solution with a pH of 7.

Each molecule of Al binds 3 molecules of maltolate and for this reason the maltolate control was added to cells at 3X molar concentrations. All solutions were sterile filtered using 0.22-μM syringe filters immediately after preparation.

Determination of Al cytotoxicity in N2a cells

Mitochondrial enzyme activity, an indirect measure of the number of viable respiring cells, was determined using the 3(4,5-dimethyl thiazolyl-2)2,5-diphenyl tetrazolium bromide (MTT) assay. N2a cells were seeded in 96-well culture plates at a density of 20,000 cells/well in 100 μl and allowed to grow for 18 h before treatment. Cells were treated by adding 100 μl of a 2X concentration of the respective reagent ($AlCl_3$, Al-malt or maltolate as detailed in figures) and 24 h later, MTT was added to each well at a final concentration of 0.5 mg/ml for 4 h. Media was removed and the purple formazan crystals were dissolved in 100% DMSO. Absorbance at 570 nm was determined using a PowerWaveX™ absorbance microplate reader (Bio-Tek Instruments, Inc., Winooski, VT). Data are expressed as relative absorbance at 570 nm.

Cytotoxicity was determined using the lactate dehydrogenase (LDH) release assay as previously described.[56] Briefly, N2a cells were seeded as described above for the MTT assay. Treatments were added after 18 hours as 2X concentrations. Plates were then incubated for 24 h following which 40 μl of the supernatent was added to a new 96 well to determine LDH release and 40 μl of 6% triton X-100 was added to the original plate for determination of total LDH. 100 μl of 4.6 mM pyruvic acid in 0.1 M potassium phosphate buffer (pH 7.5) was added to the supernatent samples using repeater pipette. Then 100 μl of 0.4 mg/ml reduced β-NADH in 0.1 M potassium phosphate buffer (pH 7.5) was added to the wells and the kinetic change in absorbance at 340 nm was read for

a duration of 1 min using a PowerWaveX™ absorbance microplate reader. This procedure was repeated with 40 µl of the total cell lysate to determine total LDH U/well. A change of 0.001 absorbance units/min is equivalent to 1 U/l of LDH activity.[32] The percentage of LDH release was determined by dividing the LDH released into the media by the total LDH following cell lysis in the same well. All pharmacologic agents used in tables 1-3 were added 30 min prior to treatment with Al-malt.

Determination of DNA synthesis as an index of cell proliferation

The effects of Al treatment on N2a cell proliferation and DNA synthesis was determined using a modification of the thymidine incorporation assay previously described.[57] Briefly, cells were treated with Al-malt for 24 h, following which 25 µCi/ml of [methyl-^{3}H]thymidine was added for an additional six h. Cells were then harvested onto glass fiber filter paper (Cambridge Technology, Watertown, MA) using a cell harvester (PHD, Cambridge Technology). The harvested cells were lysed with deionized water and dried with 95% ethanol. The filter papers were placed in scintillation vials containing 2 ml of liquid scintillation cocktail (Ready-Solv, Beckman, Fullerton, CA) and counted in a liquid scintillation counter (Pharmacia, Turku, Finland). Proliferative responses (uptake of ^{3}[H]thymidine) were expressed as stimulation index [disintegrations per minute $(DPM)_{treated}/DPM_{control}$].

Determination of apoptosis using the acridine orange/ethidium bromide assay

The ability of acridine orange and ethidium bromide dual staining to differentiate live from apoptotic from necrotic cells make this simple assay a promising tool in cell death research. The assay was performed as previously described.[58] N2a cells were treated with Al-malt for 24 hours following which the cells were harvested by

trypsinization. The floating and adherent cells were collected and combined for this assay. Cells (10^6) were centrifuged at 200×*g* for 10 min at 4 °C. The cells were resuspended in 1 ml PBS and 2 µl of acridine orange and 2 µl of ethidium bromide (final concentration 200 ng/ml each) were added to each tube and the cells were stained for 5 min. Two-parameter fluorescence was acquired from 20,000 individual cells per sample using an EPICS XL/MCL flow cytometer with a 488 nm argon-ion laser. Green emissions from acridine orange and red emissions from ethidium bromide were captured at 525 and 620 nm, respectively. Live, apoptotic and necrotic populations were analyzed using WinMDI™ flow analysis software. Discrimination between cell populations was based on previously published diagrams and descriptions.[58,59]

Determination of phosphatidyl serine externalization: Annexin V binding assay

The externalization of phosphatidyl serine is an early event in apoptosis and serves as a signal for phagocytosis by macrophages.[30] Flow cytometry analysis was performed to determine this event using the Annexin V binding assay (BD Biosciences Pharmingen, San Diego, CA). Briefly, cells were treated with Al-malt for 24 h following which 100,000 cells were combined washed 2X in cold PBS and resuspended in 100 µl 1X binding buffer. Five µl of Annexin V-FITC and 5 µl of propidium iodide (final concentration 2.5 µg/ml) were added and gently mixed followed by incubation protected from light for 15 min. Cells were acquired within 60 min using an EPICS XL/MCL (Coulter Cytometry, Hialeah, FL, USA) flow cytometer. The fluorochrome was excited using the 488 nm line of an argon-ion laser and Annexin V and PI emissions were collected at 525 and 620 nm, respectively. A total of 20000 events were acquired for

each sample. The following controls were run at the time of assay 1) unstained cells, 2) Annexin V only and 3) PI only.

Determination of caspase 3 activation

Caspase 3-like activity was determined using the CaspACE™ fluormetric activity assay (Promega Corporation, Madison, WI) with modifications as noted. Recently it was demonstrated that within 4 and 24 h approximately 15% and 58%, respectively, of the active caspase 3 enzyme had been released into the media.[32] Traditional assays require a wash step prior to lysis of the cells that may result in the loss of the active enzyme and a false negative result. For this reason, we decided to lyse the cells in the treatment media using 1% triton X-100. Briefly, cells were treated in 24 well plates at a density of 200,000/ml for 24 h following which triton X-100 was added to a final concentration of 1%. The cells were lysed by trituration and centrifuged at 10,000 x *g* for 10 min to remove cell debris. The supernatent was assayed for active caspase 3-like activity using the CaspACE™ system according to instructions supplied. The fluorescence of cleaved substrate was determined using a Spectramax Gemini fluorescent plate reader (Molecular Devices, Irvine, CA, USA). The fluorescence signal was digitized and analyzed using SoftMax Pro™ (version 3.1.1, Molecular Devices, Irvine, CA, USA) and the concentration of caspase 3-like activity determined from an APC standard curve.

Cell cycle analysis

The cell cycle distribution of N2a cells treated with Al-malt was determined using flow cytometery. Cells were treated with Al-malt for 24 h and then trypsinzed and resuspended in nuclear isolation media (NIM, 50 μg/ml propidium iodide, 1 mg/ml RNase A, 0.1% Triton X100). Nuclear DNA content from 50000 cells was determined

using an EPICS XL/MCL (Coulter Cytometry, Hialeah, FL, USA) flow cytometer with a 488 nm argon-ion laser and an emission wavelength of 620 nm. DNA histograms were analysed using WinMDI™ flow analysis software.

Analysis of cellular and nuclear morphology

Cellular morphology was examined using phase and scanning electron microscopy and nuclear morphology was examined using an epifluorescence microscope following staining with Hoechst 33258 (H33258). Cells were seeded (2 x 10^5/ml) in 6 well plates for phase contrast and nuclear morphology imaging. Following 24 h treatment with Al-malt, cells were stained with H33258 (1 μg/ml) for 5 min and phase and fluorescence microscopy was performed using an IX71 inverted microscope (Olympus America, Inc., Melville, NY). Digital images were captured using a MagnaFire SP® digital camera.

Scanning electron microscopy was performed cells seeded on 13 mm Thermanox™ coverslips (Nalge Nuc International, Rochester, NY) in 24 well plates and treated with Al-malt for 24 hours. Following treatment the coverslips were removed from the well and rinsed with ice cold PBS for 5 seconds and immediately plunge-frozen in liquid nitrogen. The frozen monolayers were freeze-dried for 18 hours at -50°C and carbon coated using Denton DV502A vacuum evaporator (Denton Vacuum, Moorsetown, NJ). This fixation method did not affect cellular morphology as shown in Figure 6 and by others.[60] Imaging was conducted using a LEO 982 field emission scanning electron microscope (Leo Electron Miscoscopy Inc., Thornwood, NY).

Statistical analysis

All statistical analysis was performed using Minitab statistical software (Minitab Inc., State College, PA). Treatment effects were determined using one-way analysis of variance followed by Tukey's post-hoc analysis. A value of $P < 0.05$ was considered significant unless indicated otherwise.

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Table 5.1. The effects of pretreatment with antioxidants on Al-malt-induced cell death in N2a cells.

Treatment	LDH release[a] (% of total)	
	Control (no Al)	Al-malt (500 μM)
Media only	10.1 ± 0.6	40.8 ± 0.8
Plus pharmacological agent[b]		
Ascorbate (1 mM)	7.8 ± 0.6	49.1 ± 0.8*
α-tocopherol	8.8 ± 2.3	42.9 ± 0.7
Silymarin (25 μM)	12.1 ± 2.0	40.4 ± 0.4

[a] LDH release was calculated by dividing the LDH units in the supernatent by the LDH units in the lysate and supernatent combined and is expressed as % of total in the respective well.

[b] All pharmacological agents were added to the cultures 30 min prior to the addition of Al-malt.

* indicates significant difference from the control group at $P < 0.01$.

Table 5.2. The effects of pharmacological manipulations of major cellular kinase pathways on cell death induced by Al-malt in N2a cells.

	LDH release[a] (% of total)	
Treatment	Control (no Al)	Al-malt (500 μM)
Media only	8.9 ± 0.9	41.9 ± 1.3
Plus pharmacological agent[b]		
PD 98059 (10 μM)	9.7 ± 0.8	43.3 ± 0.7
SB 203580 (5 μM)	9.8 ± 0.9	44.2 ± 0.4
Calphostin C (100 nM)	7.5 ± 0.9	43.1 ± 0.6
PMA (100 nM)	6.5 ± 1.0	38.1 ± 1.3
PMA (500 nM)	8.7 ± 1.8	41.6 ± 1.8

[a] LDH release was calculated by dividing the LDH units in the supernatent by the LDH units in the lysate and supernatent combined and is expressed as % of total in the respective well.

[b] All pharmacological agents were added to the cultures 30 min prior to the addition of Al-malt.

Table 5.3. The effects of pharmacological manipulation of extracellular Ca^{2+} on Al-malt-induced cell death in N2a cells.

	LDH release[a] (% of total)	
Treatment	Control (no Al)	Al-malt (500 μM)
Media only	4.8 ± 0.4	35.7 ± 0.3
Plus pharmacological agent[b]		
EGTA (3 mM)	12.5 ± 1.6*	35.5 ± 2.0
La^{3+}	2.4 ± 0.8	30.7 ± 1.1*

[a] LDH release was calculated by dividing the LDH units in the supernatent by the LDH units in the lysate and supernatent combined and is expressed as % of total in the respective well.

[b] All pharmacological agents were added to the cultures 30 min prior to the addition of Al-malt.

* indicates significant difference from respective control group at $P < 0.05$.

Figure 5.1 The effects of two different aluminum compounds on the viability of N2a neuroblastoma cells. Cells were treated with $AlCl_3$ (1-1000 μM), Al-malt (62.5-500 μM) and maltolate (1500 μM) at the indicated concentrations for 24 h. The relative number of live cells was then determined using the MTT assay. The dotted line represents the LDH release (5 of total) from cells treated with Al-malt (62.5-500 μM). Mean ± SEM (n = 5 wells/treatment). Data are representative of three independent experiments with similar results. Significantly different from the control group at * $P < 0.01$, ** $P < 0.001$.

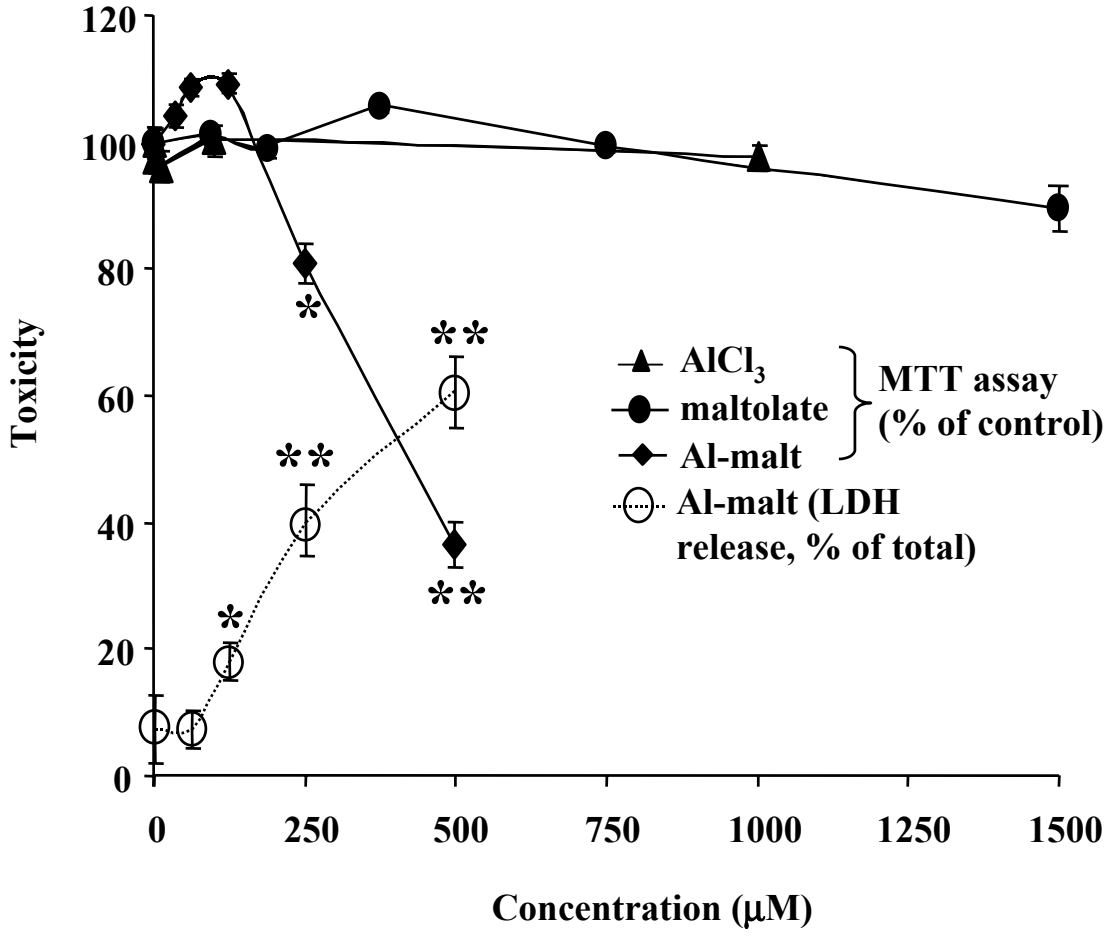

120
100
80
60
40
20
0
Toxicity
0
250
500
750
1000
1250
1500
Concentration (μM)
AlCl3
maltolate
Al-malt
MTT assay
(% of control)
Al-malt (LDH
release, % of total)
*
**
**
**
*

Figure 5.2 Treatment of N2a cells with Al-malt reduced DNA synthesis, an index of proliferation. Cells were treated with Al-malt (62.5-500 μM) and maltolate (1500 μM) at the indicated concentrations for 24 h. ^{3}H-thymidine was then added for an additional 4 h and DNA synthesis was determined according to the Materials and Methods. Mean ± SEM (n = 5 wells/treatment). Data are representative of three independent experiments with similar results. Significantly different from the control group at * $P < 0.05$, ** $P < 0.001$.

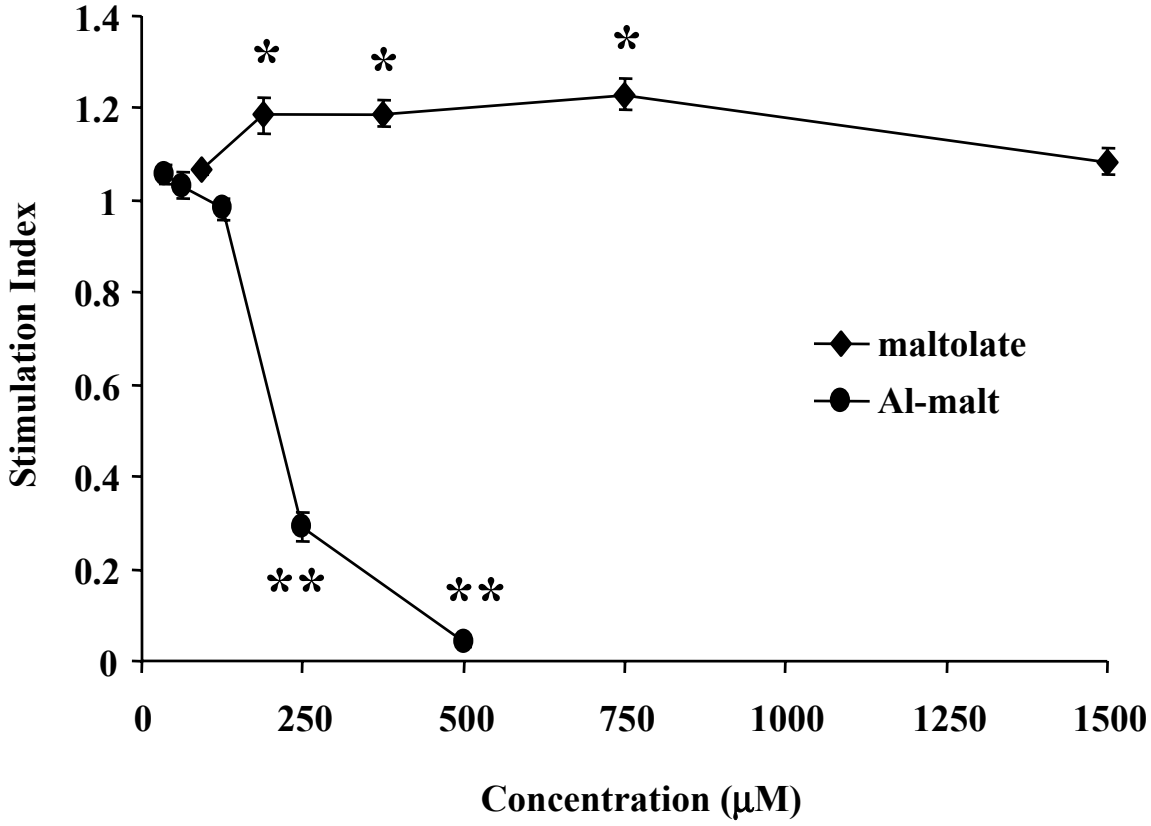

1.4
1.2
1
0.8
0.6
0.4
0.2
0
Stimulation Index
0
250
500
750
1000
1250
1500
Concentration (μM)
maltolate
Al-malt
*
*
*
**
**

Figure 5.3 Al-malt induces apoptosis and necrosis in N2a cells. Cells were treated with Al-malt and maltolate at the indicated concentrations for 24 h. Discrimination of live, apoptotic and necrotic cell populations was determined using the acridine orange/ethidium bromide flow cytometry assay as outlined in the Materials and Methods. Live cells are defined by the oval labeled L and apoptotic and necrotic populations are labeled Ap and N, respectively. (a-control, b-1500 μM maltolate, c-62.5 μM Al-malt, d-125 μM Al-malt, e-250 μM Al-malt, f-500 μM Al-malt). Data are representative of three independent experiments with similar results.

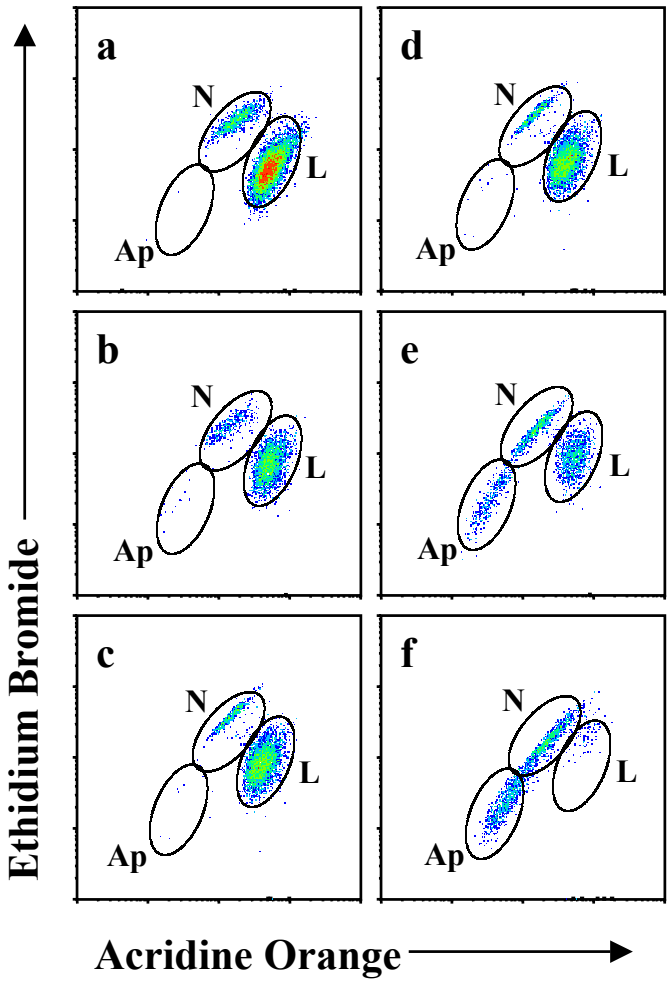

a
N
L
Ap
d
N
L
Ap
b
N
L
Ap
e
N
L
Ap
c
N
L
Ap
f
N
L
Ap
Ethidium Bromide
Acridine Orange

Figure 5.4 Externalization of phosphatidyl serine is induced in cells treated with Al-malt. Cells were treated with Al-malt and maltolate at the indicated concentrations for 24 h following which Annexin V binding was performed according to the Materials and Methods. Live cells are in the lower left quadrant. The upper right quadrants represents necrotic and late apoptotic cells and the lower right quadrants represents early apoptotic cells. (a-control, b-1500 μM maltolate, c-62.5 μM Al-malt, d-125 μM Al-malt, e-250 μM Al-malt, f-500 μM Al-malt). Data are representative of three independent experiments with similar results.

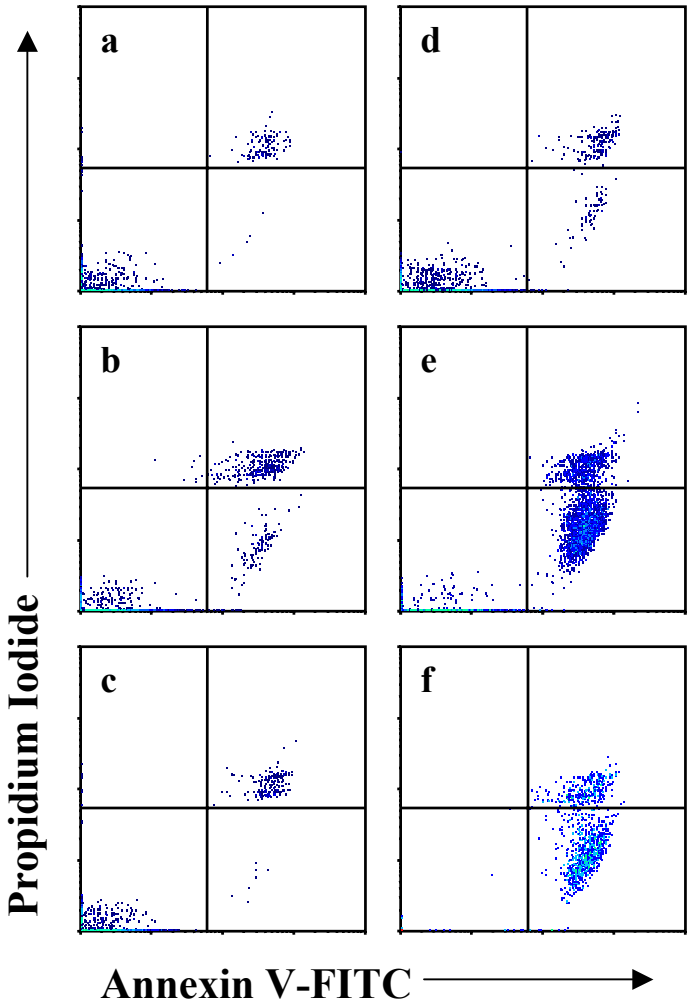

a
d
b
e
c
f
Propidium Iodide
Annexin V-FITC

Figure 5.5 Cellular and nuclear morphology indicative of apoptosis and necrosis can be seen in N2a cells treated with Al-malt. Cells were treated with Al-malt and maltolate at the indicated concentrations for 24 h and then examined by light microscopy (all 200x) and scanning electron microscopy. (a-control phase contrast; b-control H33258; c-500 μM Al-malt phase contrast; d-500 μM Al-malt H33258; e-CHX phase contrast; f-CHX H33258; g-500 μM Al-malt+CHX phase contrast; h-500 μM Al-malt+CHX H33258; i-control SEM; j-500 μM Al-malt SEM) Micrographs are representative of two independent experiments with similar results.

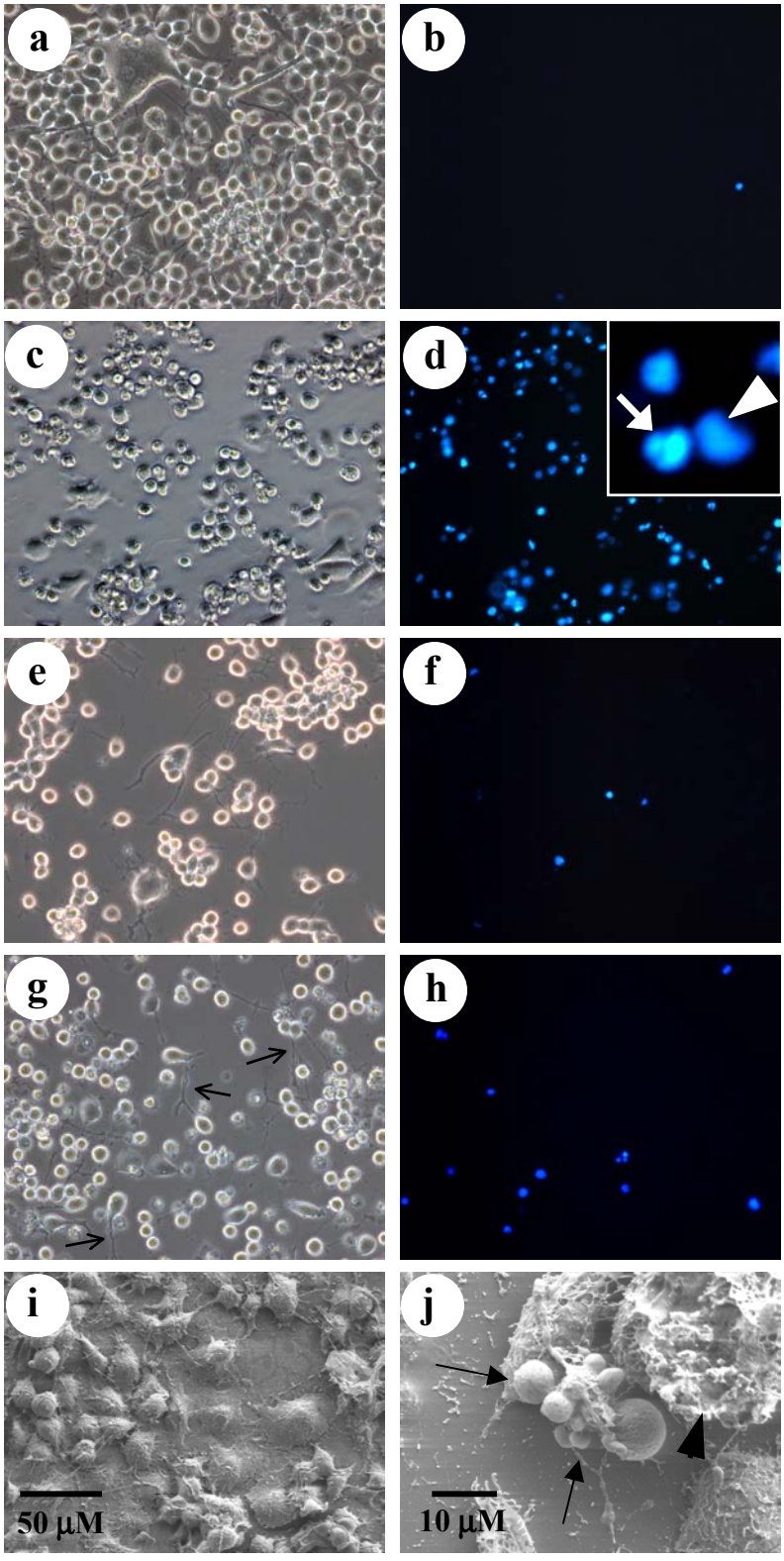

a
b
c
d
e
f
g
h
i
50 µM
j
10 µM

Figure 5.6 Treatment of N2a cells with Al-malt results in activation of caspase 3. Cells were treated with Al-malt (62.5-500 μM) and maltolate (1500 μM, open bar) at the indicated concentrations for 24 h and then caspase 3-like activity was determined using the CaspAce® system as outlined in the Materials and Methods. Mean ± SEM (n = 3 wells/treatment). The experiment was repeated three times with similar results. Significantly different from the control group at * $P < 0.001$.

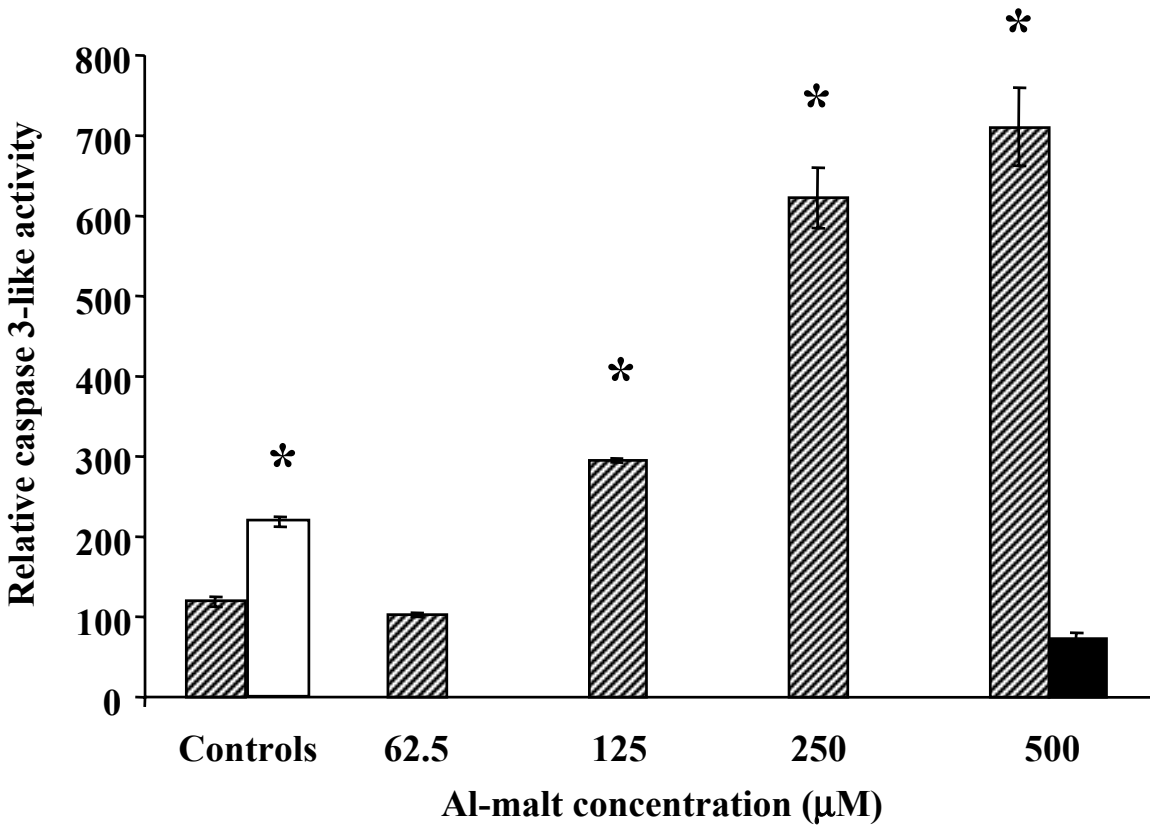

Relative caspase 3-like activity
800
700
600
500
400
300
200
100
0
*
*
*
*
Controls
62.5
125
250
500
Al-malt concentration (μM)

Figure 5.7 Al-malt-induced cell death in N2a cells is inhibited by pretreatment with the protein synthesis inhibitor cyclohexamide. Cells were treated with Al-malt (62.5-500 μM) for 24 h in the presence (solid bars) or absence (hatched bars) of a 30 min pretreatment with CHX (0.5 μg/ml). The release of LDH from the cells was then determined as outlined in the Materials and Methods. Mean ± SEM (n = 5 wells/treatment). Data are representative of three independent experiments with similar results. Significantly different from the control group at * $P < 0.05$, ** $P < 0.001$. § Significantly different from the respective group treated with Al-malt only at $P < 0.005$.

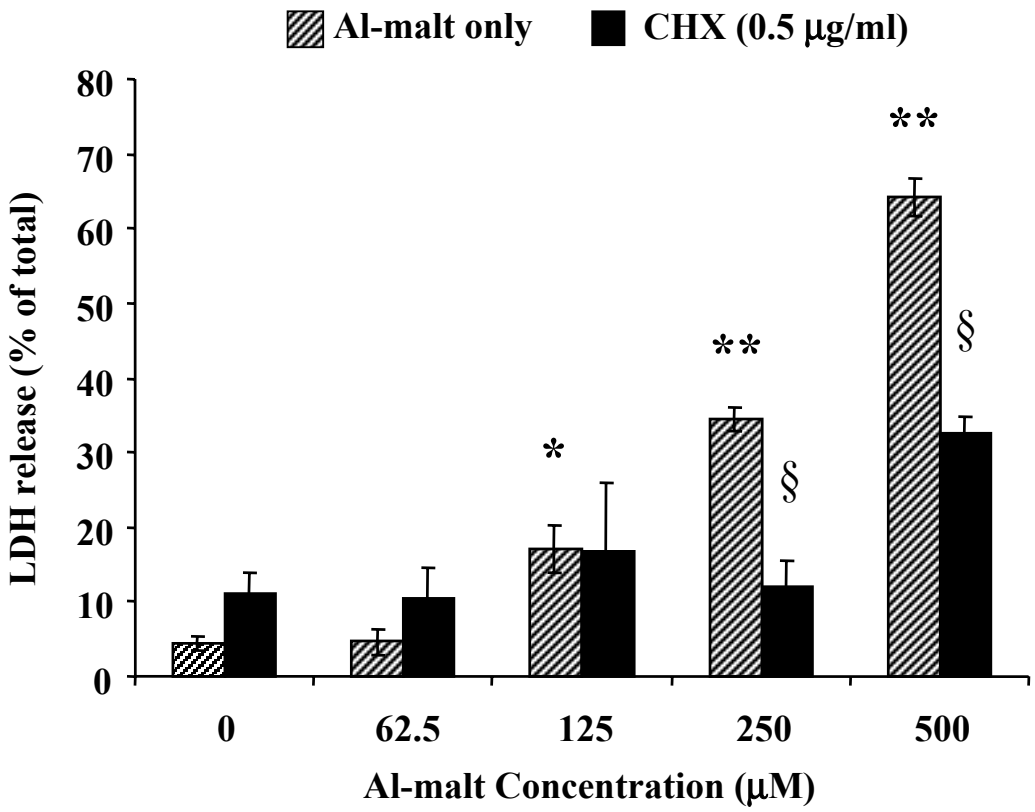

Al-malt only
CHX (0.5 μg/ml)
LDH release (% of total)
80
70
60
50
40
30
20
10
0
*
**
**
§
§
0
62.5
125
250
500
Al-malt Concentration (μM)

Figure 5.8 Al-malt-induced apoptosis is inhibited by cyclohexamide. Cells were treated with Al-malt (62.5-500 μM) at the indicated concentrations for 24 h in the presence or absence of CHX (0.5 μg/ml). Discrimination of live, apoptotic and necrotic cell populations was determined using the acridine orange/ethidium bromide flow cytometry assay as outlined in the Materials and Methods. Live cells are defined by the oval labeled L and apoptotic and necrotic populations are labeled Ap and N, respectively. The protection of N2a by pretreatment with CHX was confirmed by examining the cell cycle in response to treatment. CHX pretreatment inhibited the appearance of a hypodiploid DNA peak. Data are representative of three independent experiments with similar results.

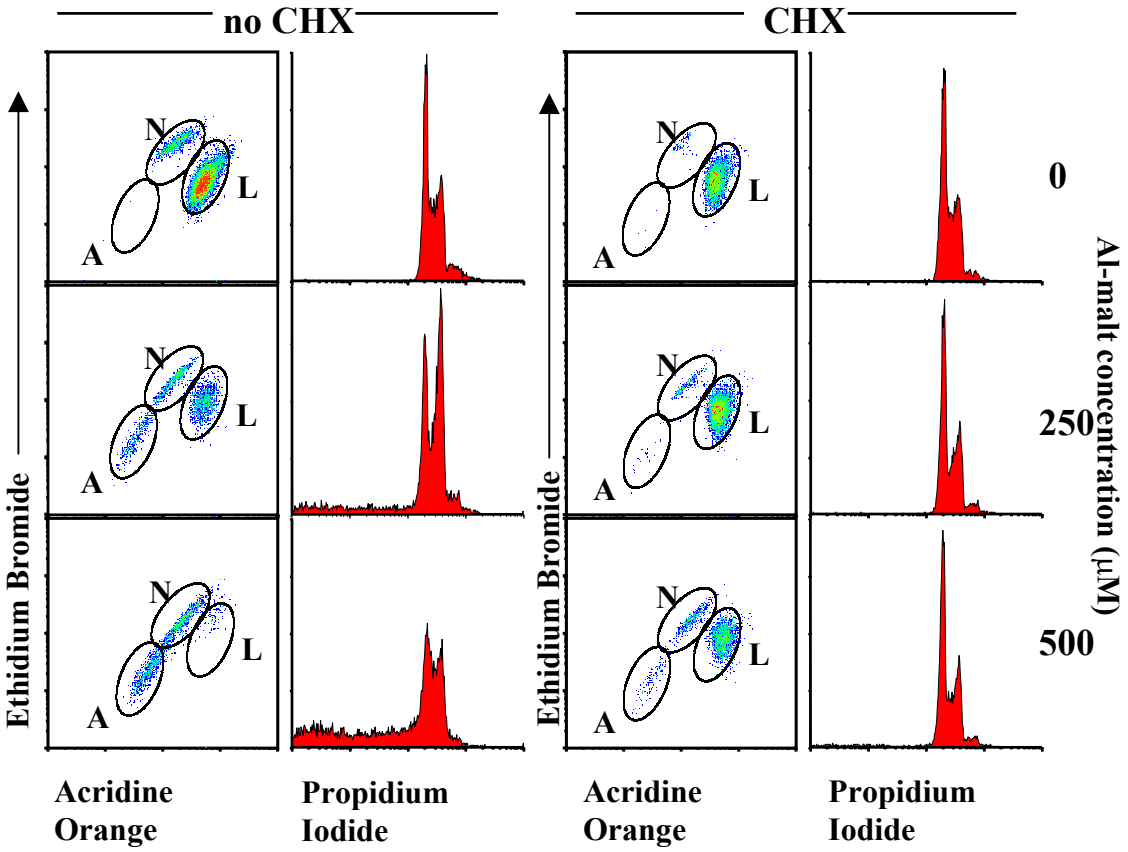
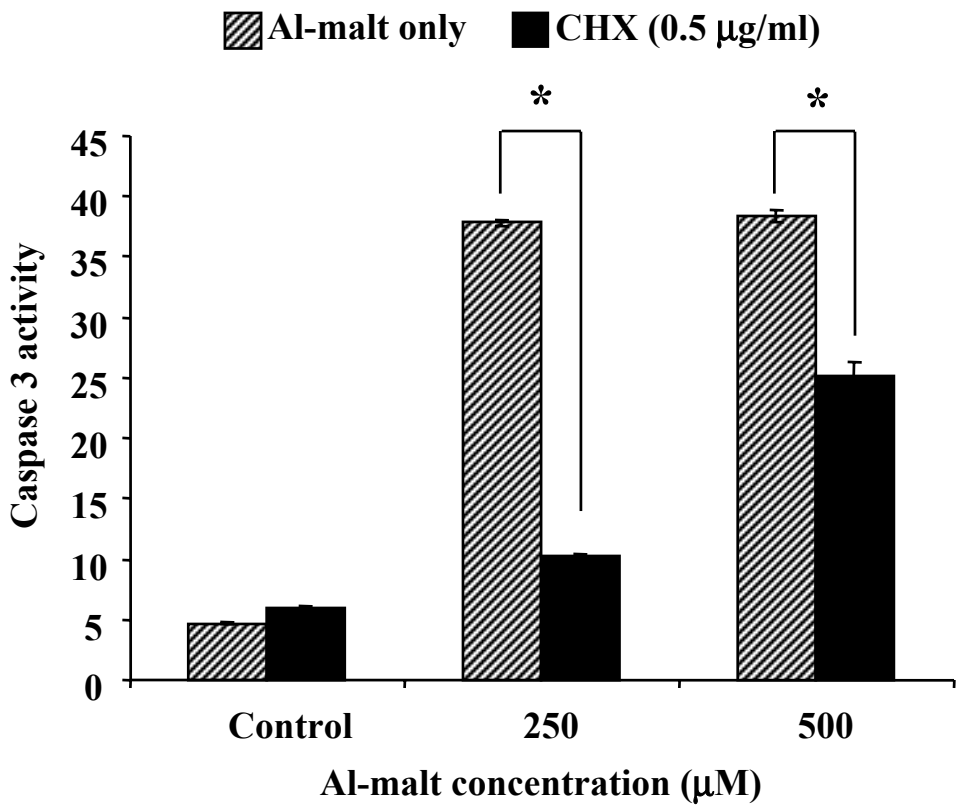

no CHX
CHX
N
L
A
Ethidium Bromide
Acridine Orange
Propidium Iodide
0
250
500
Al-malt concentration (μM)

Figure 5.9 Inhibition of protein synthesis inhibits caspase 3 activation by Al-malt. Cells were treated with Al-malt (62.5-500 μM) at the indicated concentrations for 24 h in the presence or absence of CHX (0.5 μg/ml) and then caspase 3-like activity was determined using the CaspACE® system as outlined in the Materials and Methods. Mean ± SEM (n = 3 wells/treatment). The experiment was repeated three times with similar results. Significantly different from the control group at * $P < 0.001$.


Al-malt only
CHX (0.5 μg/ml)
*
*
45
40
35
30
25
20
15
10
5
0
Caspase 3 activity
Control
250
500
Al-malt concentration (μM)

CHAPTER 6

ALUMINUM DISRUPTS THE PRO-INFLAMMATORY CYTOKINE/NEUROTROPHIN BALANCE IN PRIMARY BRAIN ROTATION-MEDIATED AGGREGATE CULTURES: POSSIBLE ROLE IN NEURODEGENERATION[1]

[1]Johnson, V.J. and Sharma, R.P. To be submitted as a Brief Communication to NeuroToxicology.

Abstract

The etiology of human neurodegenerative diseases including Alzheimer's disease (AD) are exceedingly complex and our understanding of the mechanisms involved is far from complete. The experimental neurotoxicology of aluminum has been shown to recapitulate virtually every pathophysiological feature of AD and therefore represents a useful model to study the mechanisms involved in neurodegeneration. The present study investigated the effects of aluminum maltolate (Al-malt) on the delicate balance that exists between pro-inflammatory cytokines and neurotrophins using primary brain rotation-mediated aggregate cultures. Aggregates were treated with Al-malt (5-150 μ*M) on day 15* in vitro *for 72 h. Cell death increased in a time- and concentration-dependent manner reaching significance in aggregates treated with 150* μ*M Al-malt in 48 h and 50* μ*M by 72 h. Analysis of gene expression at 72 h revealed a concentration-dependent increase in tumor necrosis factor* α *and macrophage inflammatory protein-1*α *suggestive of a state of inflammation. In contrast, a dramatic concentration-dependent decrease in the expression of nerve growth factor (NGF) and brain derived neurotrophic factor was observed. In fact, NGF expression could not be detected in aggregates treated with 50 and 150* μ*M Al-malt. The results indicated a differential regulation of pro-inflammatory cytokines and neurotrophins in brain tissue following treatment with Al-malt. Such findings provide insight into the possible involvement of deregulation of the cytokine/neurotrophin balance in the etiology of neurodegeneration.*

Keywords: Aluminum; aggregate culture; TNFα**; MIP-1**α**; NGF; BDNF; Cytokine/neurotrophin balance; Neurodegeneration**

INTRODUCTION

Current understanding of the mechanisms involved in the development and progression of neurodegeneration is far from complete. Even less is known about the role environmental factors play in the initiation and progression of human neurodegenerative disorders including Alzheimer's disease (AD) and Parkinson's disease. Numerous studies have indicated a role for oxidative stress and pro-inflammatory cytokine production in neurodegeneration (Akiyama et al., 2000; McGeer et al., 2001). Disruption of normal levels of neurotrophic factors has also been linked to neurodegeneration (Connor and Dragunow, 1998). Recent evidence strongly implicates disruption of the pro-inflammatory/neurotrophin balance as a causative factor in neurodegeneration seen in Parkinson's disease (Nagatsu et al., 2000), central nervous system (CNS) *Schistosoma mansoni* infection (Fiore and Aloe, 2001) and autoimmune encephalitis (Muhallab et al., 2002). Additionally, Alzheimer's-like neurodegeneration was demonstrated in anti-nerve growth factor (NGF) transgenic mice (Capsoni et al., 2000). The interaction between pro-inflammatory cytokines and neurotrophins is further supported by the observations that overexpression of tumor necrosis factor α (TNFα) in the brain decreases NGF levels in the hippocampus (Aloe et al., 1999) and low non-cytotoxic levels of TNFα markedly reduced signaling from neurotrophic factors (Venters et al., 2000; Venters et al., 1999).

Direct intracisternal administration of aluminum maltolate (Al-malt) has been shown to induce neurodegeneration similar to that observed in human AD and has been proposed as a model to study the mechanisms of neurodegeneration (Ghribi et al., 2002). Studies indicate that Al can also induce the production of TNFα in human T98G

glioblastoma cells (Campbell et al., 2002). Alterations in the production of NGF have also been reported in mice treated with Al (Alleva et al., 1998). The purpose of the present study was to determine the effect of Al-malt on the pro-inflammatory cytokine/neurotrophin balance in the central nervous system. We employed the rotation-mediated neural cell aggregate culture system and found that Al-malt concentration-dependently increased the expression of TNFα and macrophage inflammatory protein-1α (MIP-1α) concomitant with marked decreases in the expression of NGF and brain derived neurotrophic factor (BDNF). These alterations in gene expression correlated with a concentration-dependent increase in cell death. Results of the present study implicate a shift towards increased inflammatory mediators and decrease trophic factors in the etiopathogenesis of Al-malt-induced neurodegeneration and extend current knowledge of the mechanisms involved.

MATERIALS AND METHODS

Materials

Aluminum chloride was purchased from J.T. Baker (Mallinckrodt-Baker Inc., Phillipsburg, NJ). Maltolate was obtained from TCI America (Portland, OR) and the aluminum maltolate (Al-malt) complex was made as previously described in detail (Bertholf et al., 1989). Delbecco's Modified Eagle's Medium (DMEM) and Albumax II were purchased from Gibco Life Technologies (Invitrogen, Carlsbad, CA). All other cell culture reagents were procured from Sigma Chemical Co. (St. Louis, MO). The primers used for expression analysis were synthesized by Intergrated DNA Technologies

(Coralville, IA). All other reagents were obtained from Sigma and were of cell culture grade when available.

Cell culture system

The rotation-mediated aggregate neural culture system has been described in detail by Honegger and Monnet-Tschudi (1997). This culture system has been used previously to investigate the neurotoxicity of Al (Atterwill et al., 1992). Briefly, the telencephalon was asceptically removed from gestation day 15 Swiss Webster mice (Harlan, Indianapolis, IN). The tissue was mechanically dissociated in Puck's D (Honegger and Monnet-Tschudi, 1997) by gently teasing through a 200 μM nylon mesh (Sefar America Inc., Depew, NY) followed by filtration through a 120 μM nylon mesh to remove debris. The single cell suspension was counted using a hemocytometer and the viable cell concentration was adjusted to 10 x 10^6 cells/ml in serum free media (Honegger and Monnet-Tschudi, 1997). Immediately, a total of 40 x 10^6 cells were added to each 25 ml Delong flask, 4 ml of media was added and the flasks were placed on an orbital shaker at 68 RPM in a humidified incubator at 37°C with 10% CO_2. The speed of rotation was progressively increased to 74 RPM by day 1 and then the aggregates were transferred to 50 ml Delong flasks. Rotation speed was increased by 1 RPM/day until a final speed of 80 RPM for the rest of the culture period. Aggregates were treated on day 15 *in vitro* by removing 4 ml of media and adding 4 ml of fresh media containing 2X concentrations of Al-malt (5-150 μM). Since each molecule of Al is bound by 3 molecules of maltolate, a control group was treated with 450 μM maltolate. Media samples were taken every 24 h until 72 h when the aggregates were harvested for gene expression analysis.

Cytotoxicity assay

Cell death induced by Al-malt was determined using the lactate dehydrogenase (LDH) release assay as previously described (He et al., 2002). Briefly, 40 μl of culture media was added to a 96 well plate and then 100 μl of 4.6 mM pyruvic acid in 0.1 M potassium phosphate buffer (pH 7.5) was added using a repeater pipette. One hundred μl of 0.4 mg/ml reduced β-NADH in 0.1 M potassium phosphate buffer (pH 7.5) was added to the wells and the kinetic change in absorbance at 340 nm was read for a duration of 1 min using a PowerWaveX™ absorbance microplate reader (Bio-Tek Instruments, Inc., Winooski, VT). Data are presented as % of control (no treatment) LDH release.

Analysis of gene expression

Cell aggregates were washed with ice cold PBS and then disrupted in TRI® reagent (Molecular Research Center, Cincinnati, OH) according to manufacturer's protocol. Reverse-transcriptase polymerase chain reaction (RT-PCR) was used to analyze the expression of mRNA for TNFα, MIP-1α, NGF, BDNF and β-actin (internal control) as previously described (Johnson and Sharma, 2001). The sense and antisense primers used were 5'CTCTTCAAgggACAAggCTg3' and 5'CggACTCCgCAAAgTCTAAg3' for TNFα, 5'CTgCCTgCTgCTTCTCCTAC3' and 5'CTgCCTCCAAgACTCTCAgg3' for MIP-1α, 5'TCAgCATTCCCTTgACACAg3' and 5'TAATACgACTCACTATAgggAgCACTgAgAACTCCCCCATgT3' for NGF, 5'gCggCAgATAAAAAgACTgC3' and 5'TAATACgACTCACTATAgggAgCTTATgA-ATCgCCAgCCAAT3' for BDNF and 5'ATGGATGACGATATCGCT3' and 5'ATGAGGTAGTCTGTCAGGT3' for β-actin. The NGF and BDNF primers contain a T7 promoter consensus sequence on the antisence primer to facilitate use in *in vitro*

transcription. The thermal cycles consisted of denaturation at 94°C for 15 sec, annealing at 54°C for 15 sec and extension at 72°C for 30 sec followed by a final extension at 72°C for 5 min. The number of cycles optimized within the linear range of amplification for each primer set were 25 cycles for TNFα and MIP-1α, 30 cycles for NGF and BDNF and 35 cycles for β-actin.

The amplification products were fractionated on 2% agarose gel and documented using a Kodac DC290 digital camera. The resulting images were digitized and quantified using UN-SCAN-IT software (Silk Scientific, Inc., Orem, UT) and the pixel values for each cytokine were normalized to that of β-actin.

Aggregate size analysis

Aggregates were treated with Al-malt (5-150 mM) or maltolate (450 mM) for 72 h and then collected and washed once in ice cold phosphate buffered saline. The aggregates were then fixed in 4% paraformaldehyde for 1 h and then examined using an Olympus IX71 inverted microscope (Olympus America, Inc., Melville, NY). Digital images were captured using a MagnaFire SP® digital camera and size analysis was performed using Image Pro Express software.

Statistical analysis

All statistical analysis was performed using Minitab statistical software (Minitab Inc., State College, PA). Treatment effects were determined using analysis of variance followed by Fisher's PLSD post-hoc test. Effects were considered significant at $P < 0.05$.

RESULTS

Treatment of fetal aggregate cultures with Al-malt resulted in a concentration- and time-dependent increase in LDH release indicating cell death (Fig. 6.1). At 24 h an increase in cell death was observed in aggregates treated with 150 μM Al-malt although statistical significance was not reached. LDH release in this group was significantly increased over control by 48 h and remained significantly elevated at 72 h. At 72 h a concentration-dependent increase in cell death was evident in aggregates treated with Al-malt. Unexpectedly, aggregates treated with a 450 μM Al-malt (3x molar concentration of highest Al-malt group) showed increased cell death but only at 72 h (Fig. 6.1).

Analysis of gene expression showed that Al-malt induced a concentration-dependent increase in the expression of TNFα (Fig. 6.2). A concomitant increase in MIP-1α was observed suggesting a chemotactic response in microglial cells to sites of cell death. The expression of pro-inflammatory genes in aggregates treated with 150 μM Al-malt was significantly increased by not higher than the 50 μM Al-malt group. The expression of β-actin was also decreased (Fig. 6.2) in this group over the control indicating a deficit in total gene expression at this dose likely due to the high toxicity at this concentration. The increase in pro-inflammatory gene expression was accompanied by a dramatic concentration-dependent loss in the expression of neurotrophic factors (Fig. 6.3). NGF mRNA was not detectable in aggregates treated with 50 and 150 μM Al-malt. Treatment with 450 μM maltolate also increased TNFα and MIP-1α gene expression (Fig. 6.2) correlating with the cell death (LDH release) seen at 72 h. In contrast to the

effect of Al-malt, no changes in the expression of NGF or BDNF were evident in aggregates treated with maltolate (Fig. 6.3).

Morphometric analysis using digital imaging software revealed that Al-malt decreased the size of aggregates (Fig. 6.4). The decrease in size may reflect an inhibition of stem cell proliferation concomitent with the loss of cells due to toxicity. Aggregates treated with maltolate alone showed an increased size when compared to untreated aggregates (Fig. 6.4) suggesting a proliferative effect of maltolate on the stem cells in the aggregate culture.

DISCUSSION

Neurodegeneration is a term coined to define the cell death that is observed in many diseases of the CNS including AD and Parkinson's disease. The series of pathophysiological events that culminate in neurodegeneration are poorly understood. A promising hypothesis is that oxidative stress and inflammation play a central role in the cell death observed (Akiyama et al., 2000). Studies demonstrate that oxidative stress and inflammation are key factors in the neurotoxicity of Al (Campbell et al., 2002) and this metal has been considered a risk factor for the development of AD (Perl and Brody, 1980) and other human neurodegenerative syndromes (Perl et al., 1982). Therefore we tested the hypothesis that Al causes a shift towards a state of inflammation in the CNS that is involved in cell lose and neurodegeneration. We found that pro-inflammatory gene expression was increased in neural tissue treated with Al-malt. This inflammation correlated in time and concentration with Al-malt-induced cell death. Interestingly, a

decrease in trophic support appears to play a role in the neurodegeneration observed since the expression of NGF and BDNF was markedly reduced.

Previous studies have demonstrated a reciprocal relationship between pro-inflammatory cytokines and neurotrophic factors in the CNS. It has been proposed that there is a cytokine/neurotrophin balance that is central to the maintenance of homeostasis in the brain and disruption of this balance can lead to detrimental changes in the CNS (Aloe et al., 1999) and play an important role in neurodegeneration associated with disease (Nagatsu et al., 2000) and with normal aging (Macdonald et al., 2000). In the present investigation Al-malt, an environmental factor involved in neurodegeneration, caused a shift in this balance towards increased pro-inflammatory cytokine production. In addition to the increase in TNFα and MIP-1α, a dramatic decrease in NGF and BDNF was evident. This finding indicates that Al-malt is causing neurodegeneration by two potentially interrelated mechanisms, immune-mediated neuronal death and neuronal death due to diminished trophic support. The increase in inflammatory gene expression may also be caused by Al-malt-induced necrosis since necrotic neurons have been shown to increase TNFα production in primary glial cells (Viviani et al., 2000).

Strong evidence supports an interaction between cytokines and neurotrophins. Aloe et al. (1999) showed that the overexpression of TNFα in Tg6074 transgenic mice resulted in a significant decrease in NGF in the hippocampus. The physiological consequence of this decrease was a loss of septal cholinergic neurons that depend on NGF for survival. Cholinergic neurons are intimately involved in learning and memory and Tg6074 transgenic mice show neurobehavioral alterations including decreased learning ability (Fiore et al., 2000; Fiore et al., 1996). Therefore, the increase in TNFα

observed in the present study may be responsible for the down regulation of NGF and BDNF expression both of which may cause neuronal loss. Since the removal of NGF from PC-12 cells and primary neurons in culture has been shown to induce apoptosis (Chang and Johnson, 2002), the observed decrease in neurotrophin gene expression may play an important role in the apoptosis seen in Al-malt-treated rabbits (Savory et al., 1999; Ghribi et al., 2001a). Interestingly, co-administration of glial-derived neurotrophic factor prevented Al-malt-induced neurodegeneration in the rabbit brain (Ghribi et al., 2001b) suggesting a protective role for neurotrophic factors in Al-malt neurotoxicity. The results of the present investigation offer the possibility that Al-malt may decrease neurotrophic factor expression in the CNS and that supplementation with GDNF may be counteracting the decrease, thus protecting against Al-malt neurotoxicity in the rabbit.

Paradoxically, we observed a similar increase in TNFα and MIP-1α in aggregates treated with maltolate alone and this correlated with increased LDH release at 72 h. One possible explanation for this effect is the direct induction of cell death as previously observed in neurons treated with maltolate (Hironishi et al., 1996). This could lead to secondary inflammation and an increase in TNFα expression. The lack of effect of maltolate on NGF and BDNF expression indicates that the mechanisms responsible for the cell death are different than for the cell death caused by Al-malt. Alternatively, the observation of an increase in the size of the aggregates following treatment with maltolate may be a critical factor. The aggregates proliferate in the initial stages of the culture until they reach a terminal diameter that is dictated by the limit of diffusion of nutrients, oxygen and waste (Honegger and Monnet-Tschudi, 1997). An increase in diameter as observe in the present study would result in an increased core of necrosis in

the center of the aggregate due to the lack of nutrients and oxygen and waste removal. Therefore, it is possible that the increase in TNFα is secondary to maltolate-induced proliferation and aggregate growth. We recently found that maltolate stimulated proliferation in Neuro-2a cells (Johnson and Sharma, submitted) thus supporting this hypothesis.

In summary, Al-malt caused cell death in a 3-dimensional model of brain tissue. A disruption in the pro-inflammatory cytokine/neurotrophin balance was observed and is likely responsible for the cell death. These findings provide insight into etiology of Al neurotoxicity and afford support for a role of Al in neurodegeneration. The current *in vitro* neural model will be useful for investigating the mechanisms involved in neurodegeneration. Differential regulation of cytokine and neurotrophin genes may play a prominent role in neurodegenerative diseases. Pharmacological alterations of these pathways may give promise for possible prevention and therapeutic intervention in neurodegenerative disease states as has been demonstrated for neurotrophic factors and Al neurotoxicity (Ghribi et al., 2001b; Ohyashiki et al., 2002).

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Fig. 6.1. Treatment of aggregating neural cell cultures with Al-malt induce cell death. Aggregate cultures were treated on day 15 *in vitro* with Al-malt (5-150 μM) or maltolate (450 μM) for 72 h. Samples of the culture media were taken every 24 h for the determination of LDH release. Data are expressed as % of control LDH release at the respective time point. (C = Control). Mean ± SEM (n = 3 flasks/group). Data are representative of three independent experiments with similar trends. * Significantly different from control group at $P < 0.05$.

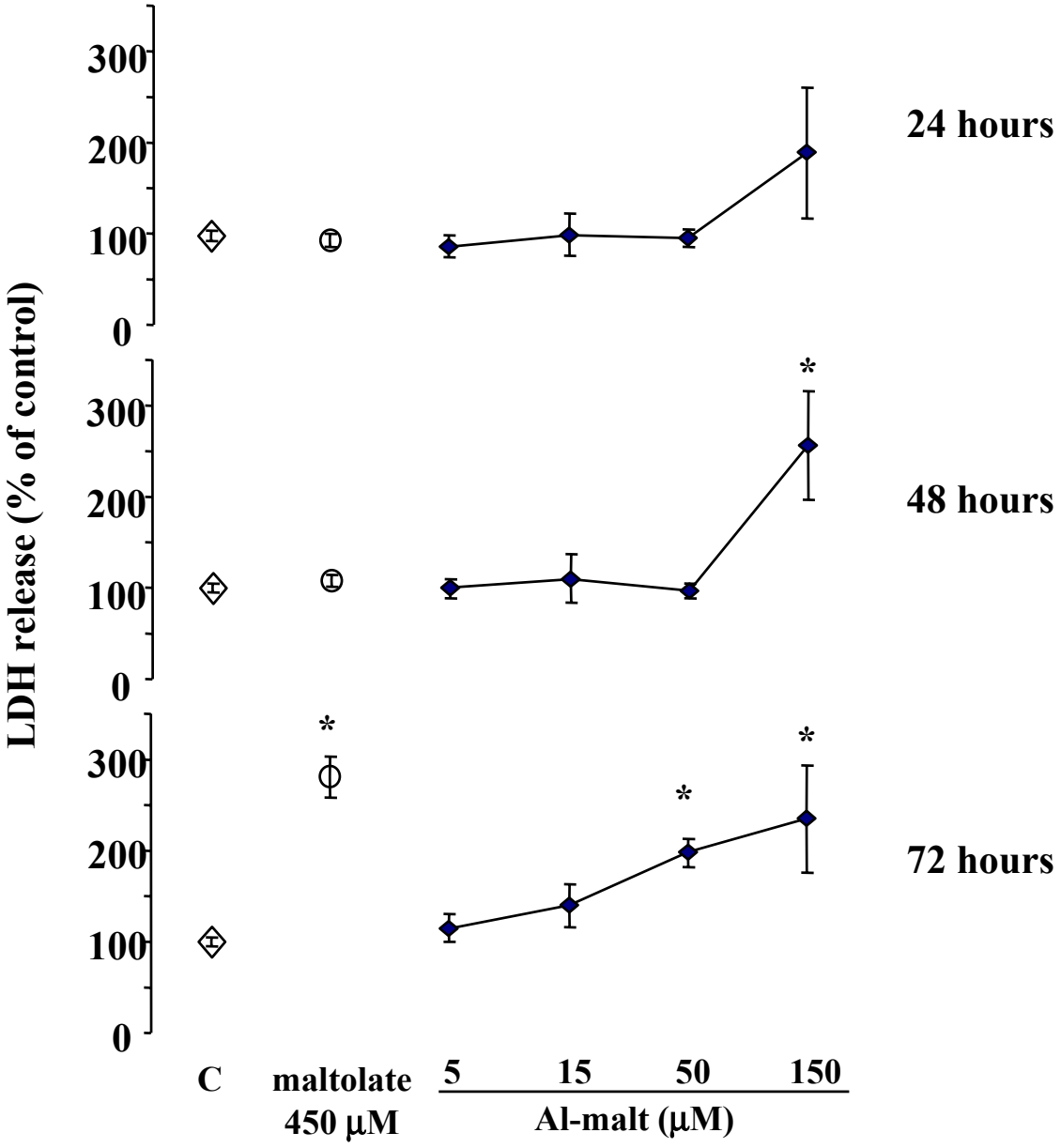

LDH release (% of control)
300
200
100
0
24 hours
48 hours
72 hours
*
C
maltolate
450 μM
5
15
50
150
Al-malt (μM)

Fig. 6.2. Al-malt increases pro-inflammatory gene expression in rotation-mediated neural aggregate cultures. Aggregate cultures were treated on day 15 *in vitro* with Al-malt (5-150 μM) or maltolate (450 μM) for 72 h. Following treatment, aggregates were washed in ice cold PBS and disrupted in TRI® reagent and total RNA was extracted. RT-PCR was used to examine the expression of TNFα and MIP-1α. The order of the bands in the representative gels (A) is the same as for the bars. Densiometric analysis of the gels for TNFα (B) and MIP-1α (C) was performed. Mean ± SEM (n = 3). Data are representative of three independent experiments with similar trends. * Significantly different from control group at $P < 0.05$.

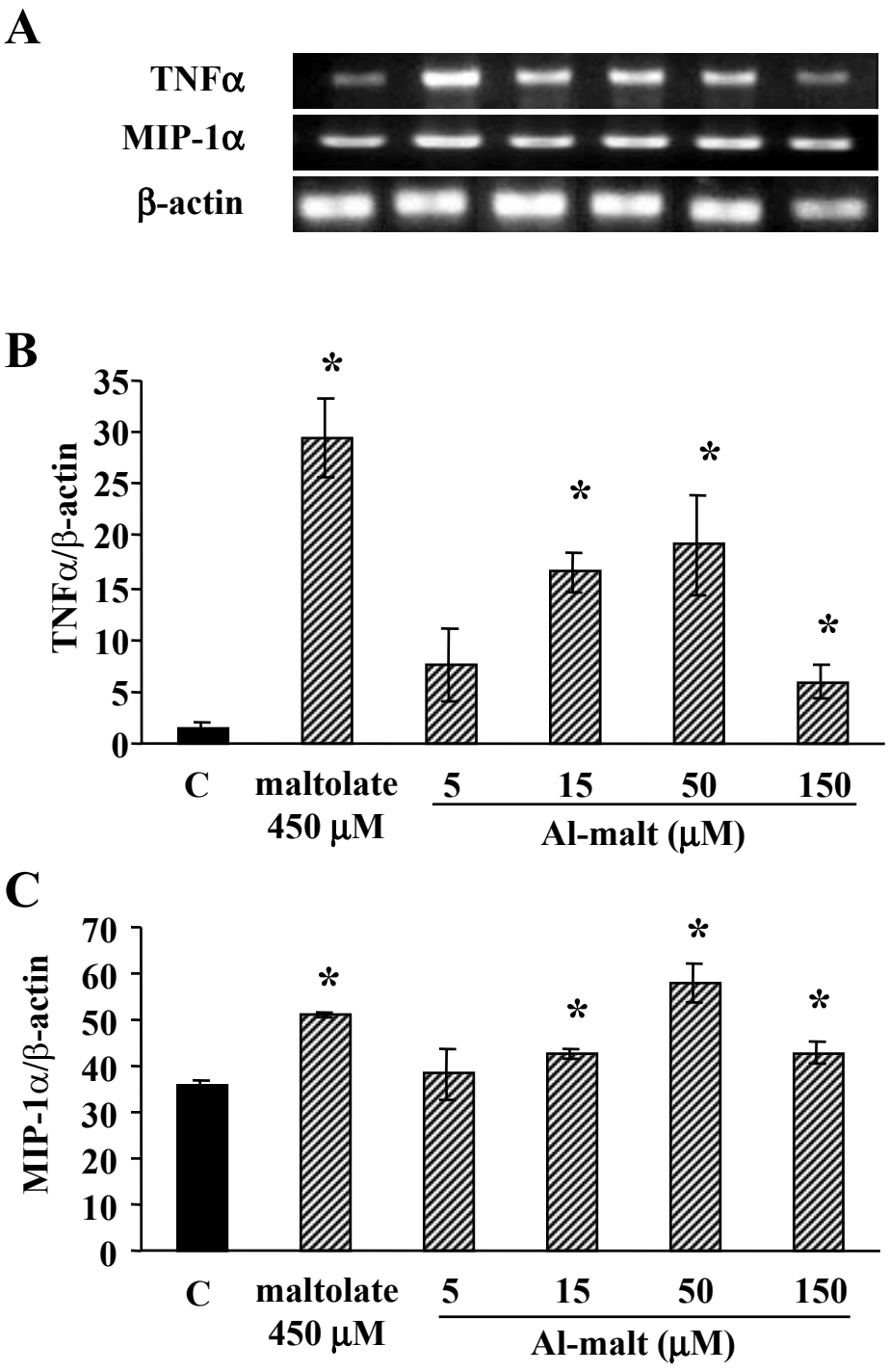

A
TNFα
MIP-1α
β-actin
B
TNFα/β-actin
35
30
25
20
15
10
5
0
*
C
maltolate
450 μM
5
15
50
150
Al-malt (μM)
C
MIP-1α/β-actin
70
60
50
40
30
20
10
0
*
C
maltolate
450 μM
5
15
50
150
Al-malt (μM)

Fig. 6.3. Al-malt decreases neurotrophin gene expression in rotation-mediated neural aggregate cultures. Aggregate cultures were treated on day 15 *in vitro* with Al-malt (5-150 μM) or maltolate (450 μM) for 72 h. Following treatment, aggregates were washed in ice cold PBS and disrupted in TRI® reagent and total RNA was extracted. RT-PCR was used to examine the expression of NGF and BDNF. The order of the bands in the representative gels (A) is the same as for the bars. Densiometric analysis of the gels for NGF (B) and BDNF (C) was performed. Mean ± SEM (n = 3). Data are representative of three independent experiments with similar trends. * Significantly different from control group at $P < 0.05$.

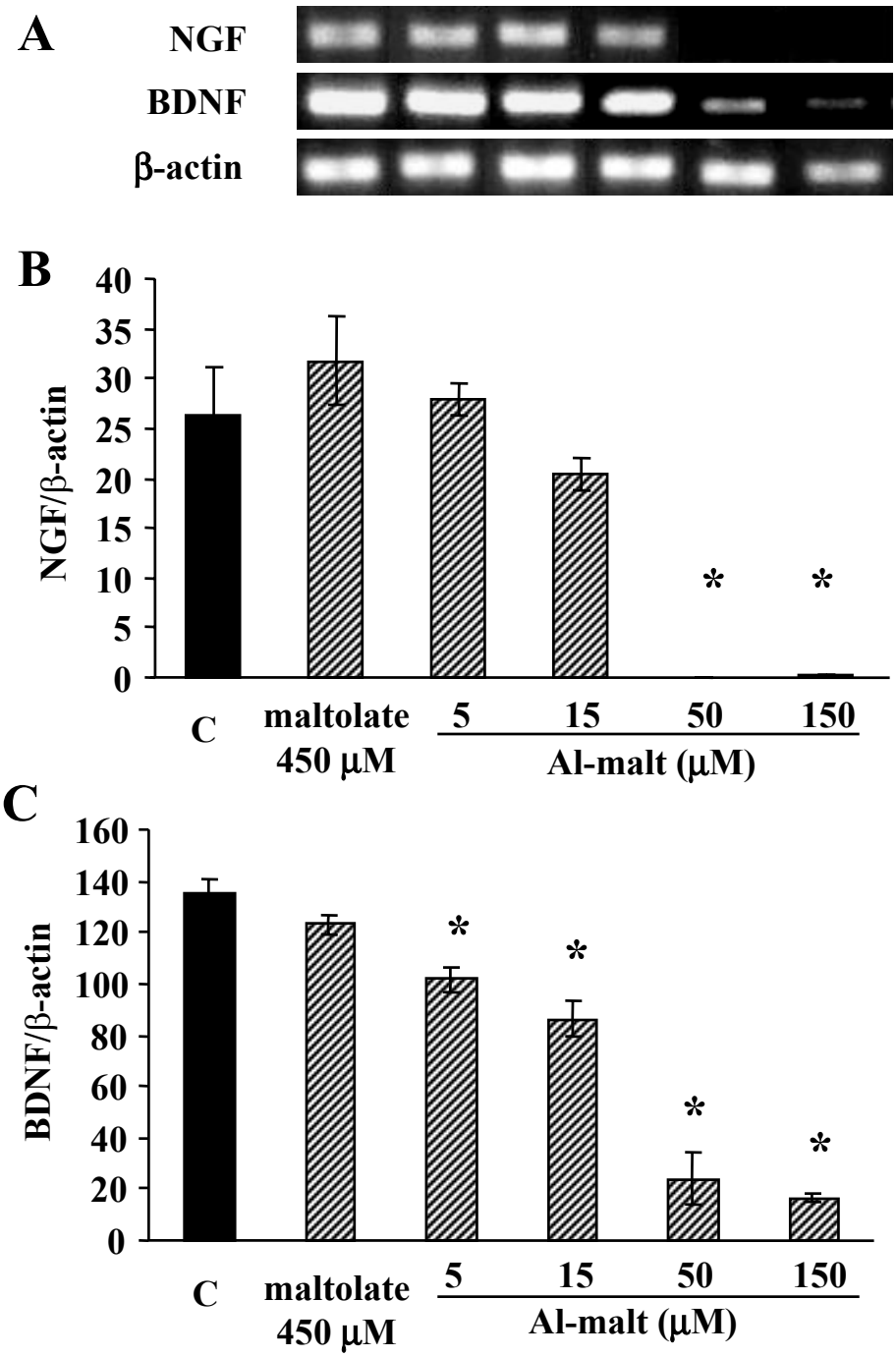
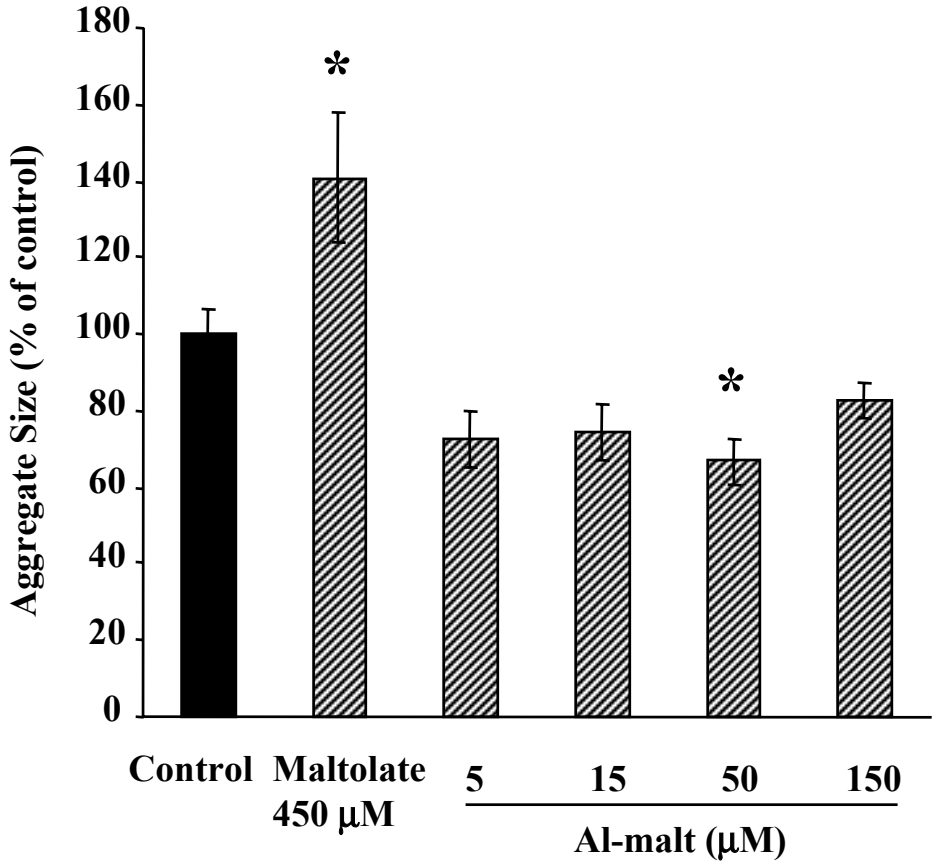

A
NGF
BDNF
β-actin
B
NGF/β-actin
40
35
30
25
20
15
10
5
0
*
*
C
maltolate
450 μM
5
15
50
150
Al-malt (μM)
C
BDNF/β-actin
160
140
120
100
80
60
40
20
0
*
*
*
*
C
maltolate
450 μM
5
15
50
150
Al-malt (μM)

Fig. 6.4. Al-malt decreased the size of aggregates whereas maltolate increased aggregate size. Aggregate cultures were treated on day 15 *in vitro* with Al-malt (5-150 mM) or maltolate (450 mM) for 72 h following which aggregates were fixed in 4% paraformaldehyde for 1 h. Fixed aggregates were allowed to settle in wells of a 96-well plate and digital micrographs were captured and used to determine aggregate size. Data are presented as mean ± SEM of 25 randomly selected aggregates. * Significantly different from the control group at $P < 0.05$.


Aggregate Size (% of control)
180
160
140
120
100
80
60
40
20
0
*
*
Control
Maltolate 450 μM
5
15
50
150
Al-malt (μM)

CHAPTER 7

SUMMARY AND CONCLUSIONS

The objectives of the present study were to 1) investigate the effect of aluminum on reactive oxygen species (ROS) and tumor necrosis factor α (TNFα) production in murine microglial cells, 2) determine the effects of aluminum on neural cell membrane physiology, 3) define the mode of cell death induced by lipophilic aluminum maltolate and 4) determine the impact of aluminum maltolate on the pro-inflammatory cytokine/neurotrophin balance in a 3-dimensional brain culture system and how this correlates with aluminum neurotoxicity.

In the first study we demonstrated that treatment of BV-2 microglial cells with aluminum as $AlCl_3$ resulted in a marked decrease in mRNA expression for TNFα and the production of TNFα protein. A decrease was also observed in cells that were stimulated with lipopolysaccharide, a powerful inducer of TNFα production. The decrease in TNFα production correlated with a decrease in activation of nuclear factor κB (NFκB) thus indicating that the impact of aluminum on this transcription factor is likely responsible for the observed effects on TNFα production. Further mechanistic studies revealed that aluminum induced ROS production and oxidative stress. Lipid peroxidation byproducts are known inhibitors of NFκB and the addition of antioxidants to BV-2 cells abrogated the effects of aluminum on TNFα production. These findings implicate oxidative stress as a primary mechanism involved in aluminum neurotoxicity. A decrease in glial function following exposure to aluminum may adversely affect homeostasis in the brain. Without trophic support from glial cells or the scavenging and cleaning function they perform, inflammation may increase and lead to neurodegenerative changes.

The second study investigated the effects of aluminum as $AlCl_3$ on viability and membrane function in PC-12 neuron-like cells. Aluminum did not cause cytotoxicity in

this cell line even at concentrations as high as 3 mM. Nevertheless, dramatic membrane alterations were observed. Membrane fluidity was reduced in cells treated with aluminum and this correlated with an increase in proton extrusion. It is likely that a reduction in membrane fluidity altered the function of membrane ion channels that culminated in alteration in proton flux across the membrane. A dose-dependent hyperpolarization of the membrane was also observed which may be related to the decrease in membrane fluidity and proton extrusion. Aluminum also increased ROS production in PC-12 cell further implicating oxidative stress in aluminum neurotoxicity. Lipid peroxidation is known to decrease membrane fluidity and therefore, aluminum may be causing the observed membrane alteration via an increase in oxidative stress.

The third study delineated the mode of cell death, apoptosis versus necrosis induced by aluminum treatment in Neuro-2a (N2a) cells. It was clearly evident that cells were dying by a mix of apoptosis and necrosis in response to aluminum maltolate (Al-malt) treatment. Al-malt induced the translocation of phosphatidyl serine from the inner leaflet to the outer leaflet of the plasma membrane and also potently increased caspase 3-like enzyme activity thus supporting apoptosis as a mechanism of cell death. Visual confirmation of cell shrinkage and nuclear condensation and fragmentation was obtained upon microscopic examination of cells treated with Al-malt. The apoptotic cell death but not the necrotic cell death was dependent on *de novo* protein synthesis. This was determined by the decrease in lactate dehydrogenase (LDH) release in cell pretreated with cyclohexamide (CHX), a potent translational inhibitor. CHX also dramatically reduced the apoptotic population and the activation of caspase 3. Since LDH release was not inhibited completely in the presence of CHX, the remaining cell death is likely by

necrosis. Cell death caused by Al-malt was independent of major kinase signaling pathways and oxidative stress. The lack of dependence on oxidative stress may be due to cell line differences or more likely due to the lipophilic nature of the Al-malt complex.

The final study determined the effect of Al-malt on the pro-inflammatory cytokine/neurotrophin balance in rotation-mediated brain aggregate cultures. Treatment of this culture system resulted in an up-regulation of pro-inflammatory gene expression. TNFα and MIP-1α expressions were increased indicating inflammation and chemotaxis of inflammatory cells to the site of injury. These changes correlated concentration-dependently with increased cell death. Interestingly, a dramatic decrease in the expression of neurotrophin genes was observed following treatment with Al-malt. BNDF expression decreased dose-dependently and NGF expression was not detectable in aggregates treated with 50 and 150 μM Al-malt. These results strongly implicate disruption of the cytokine/neurotrophin balance as a potential mechanism in aluminum neurotoxicity.

Overall, the data presented in this dissertation indicate that oxidative stress plays an important role in aluminum neurotoxicity. In addition, the interaction between neuron and glial cells types can define the toxicity of this metal and treatment of isolated cell populations may provide results that conflict with that observed *in vivo*. A new mechanism potentially driving the neurotoxicity of aluminum *in vivo* has been identified, alteration of the cytokine/neurotrophin balance. Importantly, similar mechanisms may be initiating and maintaining the neurodegeneration observed in many human dementing diseases including Alzheimer's disease and Parkinson's disease. The aggregate culture

model and Al-malt represents an applicable model to investigate the mechanisms of neurodegeneration and further research is warranted.