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ALUMINUM SALTS STIMULATE LUMINOL-ENHANCED CHEMILUMINESCENCE PRODUCTION BY HUMAN NEUTROPHILS

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Aluminum intoxication is currently thought to play a major role in the development of Alzheimer's disease and in certain pathologic manifestations seen during long-term hemodialysis and aging. The hypothesis that aluminum toxicity is mediated via an increased free radical production was tested by studying the effects of two aluminum and five other metallic compounds on the production of luminol-enhanced chemiluminescence (LECL) by human neutrophils.

$AlCl_3$, $Al_2(SO_4)_3$ and $FeCl_3$ were found to stimulate LECL production by human neutrophils whereas $FeCl_2$, CuCl, $CuCl_2$, $AuCl_3$ were inactive. Metal chelators such as Desferal, EDTA and DETAPA suppressed aluminum-induced stimulation and depressed cell-dependent LECL below basal levels. Sodium azide and Cytochalasin B greatly depressed both basal and aluminum-induced stimulation of LECL production, suggesting that, in this system, most of this stimulation was due to myeloperoxidase.

These results suggest that high tissue aluminum concentrations may induce cell-tissue lesions by stimulating local production or release of mediators of tissue damage.

KEY WORDS: Aluminum toxicity, polymorphonuclear leukocytes, chemiluminescence, mechanism of stimulation.

Patients suffering from severe renal failure and maintained on long-term dialysis often suffer serious health problems, such as myoclonic encephalopathy,[1,2] osteomalacia,[2,3] accelerated joint destructions[4] and amyloidosis.[5] Aluminum intoxication may play a role in the development of these disorders, since increased aluminum concentrations were found in the tissues and body fluids of patients on chronic hemodialysis, including in the brain,[1] joint tissues[3,6] and bone.[7,8] Furthermore, high brain tissue Al concentrations were observed in Alzheimer's encephalopathy[9] and in aging.[10] Although, $AlPO_4$ has been shown to cause inflammation when injected in the rat paw,[11] the role of aluminum in joint destruction of patients on chronic dialysis is not known.

In order to further analyze the possible mechanisms of aluminum toxicity, we investigated the effect of two aluminum and five other metallic compounds on the production of LECL by human polymorphonuclear (PMN) cells.[12] It has already been shown that aluminosilicates activate phagocytes[13] and aluminum chloride stimulates Fe-dependent lipid peroxidation.[14] Certain metals (Fe and Cu) are known to catalyze lipid peroxidation and decomposition[15] and, as transition metals, they also catalyze hydroxyl radical formation from H_2O_2 and $O_2^{\cdot-}$ radicals.[16,17]

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MATERIALS AND METHODS

Human peripheral PMN leukocytes were isolated under sterile conditions from heparinized blood of apparently healthy donors, by centrifugation on Ficoll-Hypaque gradients (Mono-poly resolving medium, Eurobio, France), according to Ferrante and Thong's[18] method recommended by the manufacturer. The cell pellet corresponding to the PMN leukocytes (2nd from the top of a gradient) was aspirated with a Pasteur pipette, transferred to another culture tube and rinsed twice by centrifugation (5 min., 600 rpm, + 4°C) using Hanks' sterile salt solution. The cells were finally resuspended in Ham's F-12 culture medium at a density of 10^6 cells/ml and immediately incubated at 37°C. The following metallic compounds: $AlCl_3$ $Al_2(SO_4)_3$, $FeCl_2$, $FeCl_3$, $AuCl_3$, $CuCl_2$ and CuCl were tested. All compounds were purchased from Prolabo Co., Paris, and were of analytical grade. The salts were dissolved in distilled water and immediately used for experiments. 20 μl of concentrated solution were added to 2 ml of cell suspension to give the final concentrations mentioned in the text and figures. In some experiments, prior to or after the addition of aluminum salts, the following compounds were added in 5–100 μl to 2 ml of cell suspension: metal chelators, Desferal (Ciba-Geigy, stock solution 100 mg/ml), ethylenediaminetetraacetic acid, EDTA (Sigma, stock sol. 6.7 mg/ml) and diethylenetriaminepentaacetic acid, DETAPA, (Sigma, stock sol. 4 mg/ml) and sodium azide, NaN_3 (Sigma, stock sol. 1.6 mg/ml), Horseradish peroxidase, HHP (Sigma, stock sol. 170 U/ml), Cytochalasin B (Sigma, stock sol. 1 mg/ml dimethylsulfoxide) and superoxide dismutase, SOD (Sigma, stock sol. 3000 U/ml), and the LECL was being measured.

The production of reactive molecules was measured as light emission amplified by the addition of 50 μg of luminol (19, 20) per ml of cell suspension. LECL was measured at 37°C using a luminometer (Eurobio, model 107). The 30″ integral CL was recorded every minute, for 5–10 min., following the addition of luminol. In a preliminary experiment, the cells were also stimulated by incubation for 1 hour at 37°C with 1 mg/ml of pre-swollen, serum-coated (1 h incubation at 37°C in 3 volumes of normal human serum, followed by rinsing in Dulbecco's phosphate buffered saline, PBS) zymosan A (Sigma, 10 mg/ml stock suspension in Ham's F-12 medium) or by adding phorbol-12-myristate-13-acetate, PMA (Sigma, stock sol. 0.01 mg/ml in DMSO) in 5–10 μl, prior to the addition of luminol.

RESULTS

In preliminary experiments, the effect of zymosan A and PMA on the production of LECL by PMN cells was tested. Incubation of cells for 1 h with 1 mg/ml of opsonized zymosan A resulted in a 5-fold increase in basal LECL, whereas addition to the cultures of 100 ng of PMA gave an immediate and much greater increase in LECL production. These experiments were carried out to assess the functional properties of the cells.

The effects of $AlCl_3$ and $Al_2(SO_4)_3$ on the basal LECL of PMN cells are shown in Figure 1. Compared to the control basal levels, $AlCl_3$, at a concentration of 20 μM, induced approximately eleven-fold stimulation, and $Al_2(SO_4)_3$ a six-fold stimulation. At the same concentration of other metallic compounds, only $FeCl_3$ enhanced the production of LECL by PMN leukocytes 2–3 fold (not shown). At a concentration equal or higher than 1 mM no stimulation was induced. The range of active con-

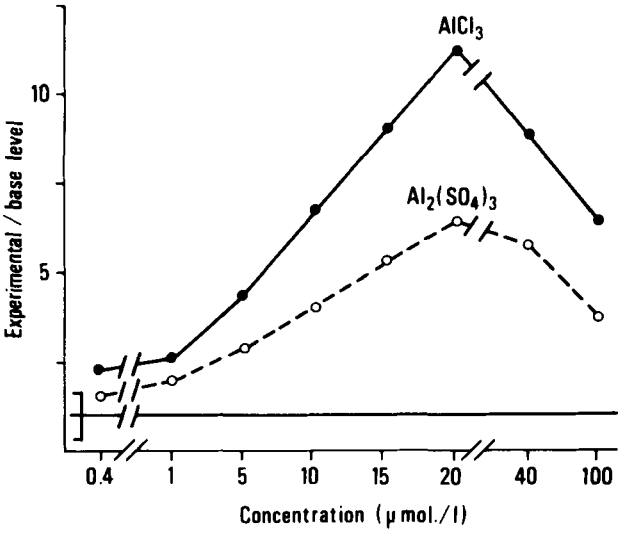

FIGURE 1 Aluminum induced stimulation of cell-dependent, luminol-enhanced chemiluminescence by human neutrophils. Measurements were performed 20 min after the addition of metal salts. Integral chemiluminescence production was recorded every 30 sec and the results calculated as 5 min cumulative production at each concentration. The points represent the ratio of means (two cultures) of treated, over untreated cultures. The horizontal and vertical lines indicate base levels (mean ± SD) of untreated cultures recorded at 1 hour intervals during the experiment.

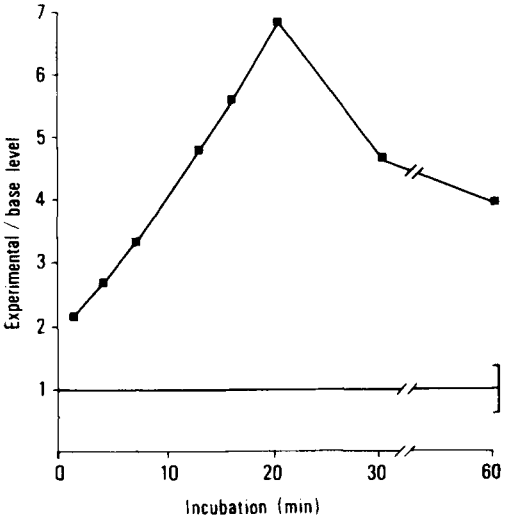

FIGURE 2 Time-course of aluminum-stimulated production of cell-dependent, luminol-enhanced chemiluminescence by human neutrophils. Chemiluminescence production was recorded at indicated times every 30 seconds for 2 minutes after the addition of 20 μM/l of $AlCl_3$. Each point represents the ratio of values (cumulative production for 2 minutes) obtained for one treated and one untreated culture. Horizontal and vertical lines represent base levels (mean ± SD) of untreated cultures recorded during the experiment.

centrations was relatively narrow and the test compounds were active at concentrations between 10^{-4} M and 10^{-6} M. A dose-dependent linear stimulation was seen with both aluminum compounds between 1 and 20 μM. Above 20 μM a dose-dependent suppression of simulation was noticed. Time course studies showed the effect to be a rather early event, since enhancement of LECL production was apparent 1 min. after incubation of the cells with $AlCl_3$, although maximum stimulation was attained only after 20 min. of incubation (Figure 2). When cells were omitted, or cell extracts were used, aluminum salts had no effect on background chemiluminescence (not shown). Also, aluminum elicited only a small stimulation of Cl production by mononuclear cells isolated from peripheral blood (not shown).

The addition of 1 mg/ml of Desferal to the cultures decreased the LECL of both unstimulated and $AlCl_3$-stimulated cells (Figure 3a). Similar results were obtained when 0.67 mg/ml EDTA or 0.4 mg/ml DETAPA was added to the cells prior to the addition of luminol (Figure 3b). This resulted in a decrease in basal LECL production. $AlCl_3$ was added 6 min after the start of the experiment and this induced marked stimulation of LECL production in control cultures and only slight stimulation in those treated with 0.67 mg/ml EDTA or 0.4 mg/ml DETAPA. In the same system, NaF added alone or in combination with $AlCl_3$ had no or only small effect on LECL.

The addition of 90 μM NaN_3 and/or 2.5 μg of Cytochalasin B to the cultures dramatically reduced LECL production by both unstimulated and $AlCl_3$-stimulated cultures (Figure 4). Aluminum salts were unable to reactivate cells following treatment of the cultures with sodium azide or Cytochalasin B. However, the addition of SOD and HRP to NaN_3 – and/or Cytochalasin B-suppressed cultures, stimulated LECL production in both cases, particularly if a PMA stimulus was given (Figure 4). However, this stimulation remained lower than that of control-unsuppressed cultures (not shown). Also, in cultures initially treated with HRP and SOD, NaN_3 induced a sgnificant decrease in LECL production by aluminum-stimulated cultures and only a small decrease in unstimulated cultures (Figure 5).

DISCUSSION

There is a considerable increase in the O_2 consumption of PMN leukocytes when they are stimulated during phagocytosis of particulate material (bacteria, latex, zymosan) or soluble agents (PMA).[19,20] This results in light production when luminol is added to cultures of stimulated PMN cells,[21,22] and LECL production was frequently used to assess functional state of PMN cells.[23] The main mechanism of light emission from luminol[24,25] is thought to be a release of varying amounts of myeloperoxidase from azurophilic granules, leading to the formation of oxidized haloids at the expense of H_2O_2.

Since aluminum has fixed oxidation numbers and its salts have no direct effect on light emission from luminol (not shown), the aluminum-induced stimulation of LECL production by PMN leukocytes is likely to be a cellular effect. Metals seem to be required for both aluminum-stimulated and basal LECL production by PMN cells. Metal dependency for light production by PMN cells is unlikly to be an oxidation-reduction catalysis of the Fenton reaction[16] since Fe^{2+} salts have no effect in this system. Aluminum may activate some other cellular mechanisms that increase CL production. For instance, it may stimulate myeloperoxidase or its release from azurophilic granules, thus increasing a production of oxidized halides[21,22] or activate

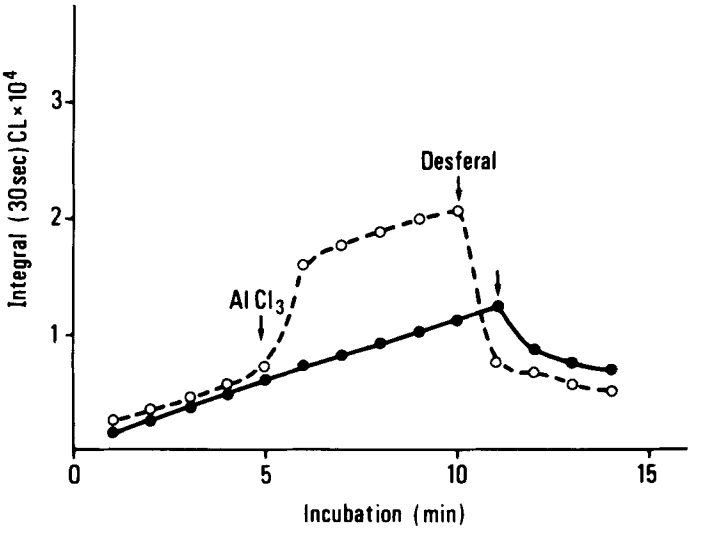

FIGURE 3a The effect of Desferal on cell-dependent, luminol-enhanced chemiluminescence by human neutrophils. Cultures were untreated (●) and treated (5 min after the addition of luminol) with 20 μM/l $AlCl_3$ (○). Desferal (1.0 mg/ml) was added after 10 and 11 min, respectively for aluminum-treated and untreated cultures, and chemiluminescence production measured every 30 sec, for an additional 4 minutes.

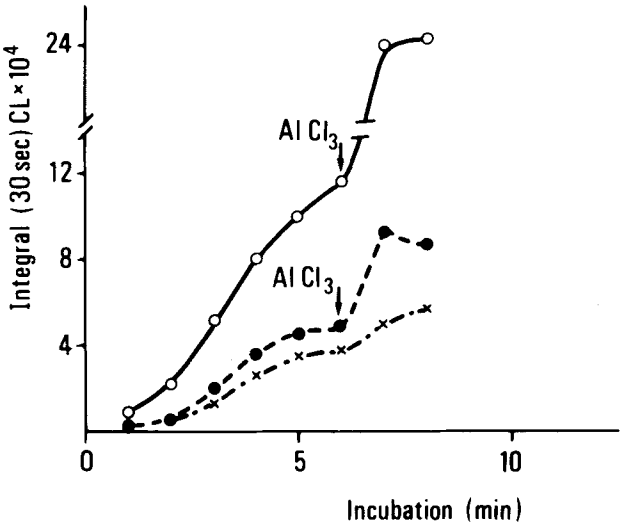

FIGURE 3b Time-course of production of cell-dependent, luminol-enhanced chemiluminescence by human neutrophils. Cells were incubated with: 0.4 mg/ml DETAPA, (●); or 0.67 mg/ml EDTA (x); control cultures (○). 6 min after the addition of luminol, $AlCl_3$ (20 μM/l) was added, and chemiluminescence production recorded every 30 seconds for an additional 3 minutes.

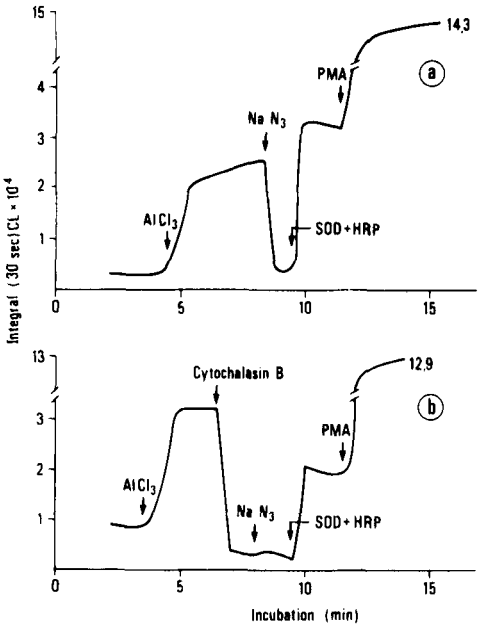

FIGURE 4 Inhibitory effect of sodium azide and cytochalasin B on aluminum-stimulated luminol-enhanced chemiluminescence production by human PMN leukocytes and stimulation by SOD, HRP and PMA. Final concentrations: $AlCl_3$, 20 μM/l; Sodium azide, 90 μM/l; Cytochalasin B, 2.5 μg/ml; SOD, 7.5 U/ml; HRP, 0.9 U/ml; PMA, 50 ng/ml. Integral (30 sec) chemiluminescence was recorded every minute for 15 minutes.

membrane-bound NADPH oxidase which is responsible for O_2^- production by phagocytes.[19,20] In order to answer this question we attempted to evaluate the importance of both routes of luminol oxidation in this system. NADPH oxidase activity was evaluated following the addition of 90 μM NaN_3 which inhibits cell-derived myeloperoxidase.[24] Exogenous SOD was added to accelerate conversion of O_2^- to H_2O_2, and also HRP, which is not inhibited by NaN_3, to catalytically remove H_2O_2 preventing it from interacting with other molecules and ensuring optimal oxidation of luminol.[25] Under these conditions, the addition to cultures of NaN_3 invariably caused a dramatic reduction of LECL in both aluminum-stimulated and unstimulated cultures, suggesting that the main cause of light production in the system was the endogenous myeloperoxidase. Further, cultures were treated with Cytochalasin B which inhibits the release of myeloperoxidase from azurophilic granuales, causing consistently a profound inhibition in luminol oxidation. As the NaN_3 and Cytochalasin B-induced reduction in light emission could be partially overcome by adding SOD and HRP, in particular after PMA stimulation, it was postulated that NADPH oxidase may have had some effect in relation to luminol oxidation. A small increase of LECL production by aluminum-stimulated human blood mononuclear cells (not shown) also favors hypothesis of NADPH oxidase involvement in this system, although discrete contamination of the cell population with PMN leukocytes cannot be ruled out. Nevertheless, aluminum stimulation of LECL production by PMN cells

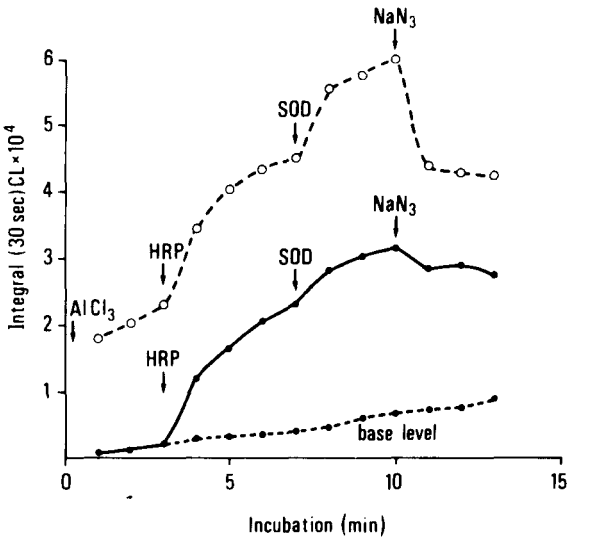

FIGURE 5 The effect of HRP, SOD and sodium azide on base and aluminum-stimulated luminol-enhanced chemiluminescence production by human neutrophils. Final concentrations: $AlCl_3$, 20 μM/l; sodium azide, 90 μM/l; HRP, 0.9 U/ml; and SOD, 7.5 U/ml. Integral (30 sec) chemiluminescence was recorded every minute for 13 minutes.

seems to be due mainly to myeloperoxidase. This is also supported by the inability of aluminum salts to induce a significant stimulation of LECL production in the extracts of PMN leukocytes, in which diffusion of the enzyme into extracellular space may have already occured (not shown).

Several pieces of evidence suggest that aluminum is toxic for cells and tissues. The toxic role of aluminum in the development of encephalopathy in patients on chronic dialysis is generally accepted;[1] it has been partly ascribed to its enhancing effect on iron-induced peroxidation of membrane phospholipids in the brain.[14] The role of aluminum in dialysis-induced osteomalacia is controversial, though aluminum deposition in bone seems to be correlated with impaired bone mineralization in rats,[26,27] and with a decrease in the number of osteoblasts[8] and bone phosphatase activity.[28]

Clear-cut evidence for the toxicity of aluminum in hemodialysis arthropathies is also lacking, in spite of the fact that increased aluminum concentrations were found in the synovium, synovial fluid and articular cartilage of these patients[6] and despite the phlogogenic activity of aluminum phosphate crystals injected in the rat-paw[11] and rabbit knee joint.[29] To what extent our findings are relevant to aluminum toxicity in vivo is also unknown. Nevertheless, they shed some light into mechanisms that may be of pathophysiological significance.

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