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Aluminum Ions Stimulate Mitosis in Murine Cells in Tissue Culture

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Addition of aluminum to the culture medium of Nakano mouse lens epithelial (NMLE) cells and Swiss 3T3K cells induced both ^{3}H-thymidine incorporation and mitosis. This is in contrast to other metal ions such as vanadium, which, at concentrations high enough to increase ^{3}H-thymidine incorporation, actually inhibits mitosis (Jones and Reid, J Cell Physiol 121:199, 1984 [1]). Aluminum concentrations between 20 μM and 50 μM were most effective. The 3T3 cells respond to aluminum with a 7.6-fold increase, and NMLE cells respond with a 21-fold increase in ^{3}H-thymidine incorporation. DNA synthesis in NMLE cells was also found to be synergistically stimulated by aluminum and low concentrations of insulin (4.5×10^{-8} M). A 3.25-hr incubation with 50 μM aluminum was sufficient to induce 50% of maximum ^{3}H-thymidine incorporation during the 40-hr assay. Aluminum-stimulated ^{3}H-thymidine incorporation is inhibited by hydroxyurea, and aluminum causes an increase in cell number. Also, by sedimentation equilibrium analysis of the product of aluminum-stimulated DNA synthesis it was found that a single copy of DNA was synthesized following addition of aluminum to quiescent cells. These facts indicate that aluminum induces both S-phase DNA synthesis and mitosis. However, only 48% of the NMLE cells found to be labeled with DNA went on to divide. In contrast, although only a small percentage of 3T3 cells were found to be labeled after aluminum treatment, all of these cells appeared to go through mitosis.

Key words: aluminum, insulin, mitogenic agent, cell growth

Aluminum is the most widely distributed metallic element in the earth's crust, but it is not found in the metallic state. It is most commonly found in combination with such elements as oxygen, fluorine, and silicon and is, following oxygen and silicon, third in abundance in the lithosphere, hydrosphere, and atmosphere [2]. Although soil concentrations can range between 150 and 250,000 ppm aluminum, concentrations in vegetation and foodstuffs are in the 0.1 to 1.0 ppm range [2].

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Nonetheless, humans consume and absorb substantial amounts of dietary aluminum, and resulting human serum levels are calculated at 1.6 μg/liter [3]. Although ubiquitous in the environment and present in human tissue, aluminum, unlike many other metals, has no known physiological function and is not considered an essential component in the diet [4]. It does, however, have biological effects. Sternweis and Gilman [5] have shown that the activation of the guanine nucleotide-binding regulatory component of adenylate cyclase by fluoride requires aluminum at levels of 5–10 μM, and recently it was reported that aluminum will increase ^{3}H-thymidine incorporation into DNA in the presence of other mitogens [6], and by itself will cause an increase in cell growth [7]. Aluminum has also been implicated in several types of dementia. Increased amounts of aluminum have been found in the brain tissue of Alzheimer's disease patients [8], and studies employing x-ray spectrometry have shown that the increased aluminum levels are only in neurons containing neurofibrillary tangles [9]. Other CNS disorders exhibiting increased aluminum accumulation include amylotrophic lateral sclerosis, parkinsonism-dementia of Guam [10], and dialysis encephalopathy [11,12]. Although CNS lesions reminiscent of those seen in Alzheimer's disease can be induced in experimental animals by the intracerebral or intrathecal injection of aluminum salts, there is no proof that the association between aluminum and human neurofibrillary tangle formation is a casual one.

The part played by trace metals ions in the stimulation and maintenance of growth of mammalian cells in vitro has emerged in recent years as an important one. Selenium, zinc, copper, and vanadium [13] have been demonstrated to increase clonal growth in some cell types. Selenium is, in fact, an essential nutrient [14]. Although aluminum is found in greater amounts in the environment than any of these elements, little is known about its ability to influence cellular metabolism.

In this study, the effect of aluminum upon DNA synthesis and cell growth in Nakano mouse lens epithelial (NMLE) cells and Swiss mouse 3T3K cells was examined.

MATERIALS AND METHODS

Cell Culture

Nakano mouse lens epithelial cells, explanted as previously described [15], were plated in 24-well plates at a concentration of 1.5×10^5 cells/well, incubated for 48 hr in RPMI 1640 (Flow) with 5% fetal calf serum (FCS) (Hyclone, Logan, UT), washed, then serum-starved for 48 hr to bring them to quiescence. The cells were then washed, fed, and exposed to the experimental reagents. 3T3K cells (kindly supplied by Dr. E.A. Adelberg, Yale University) were plated at 2×10^4 cells/well in 24-well plates in 45% Waymouth's medium (Flow, McLean, VA), 45% minimum essential medium (Dulbecco's modication) (Flow), and 10% FCS. After a 12-day incubation without refeeding, the cells were washed and refed with serum-free medium containing the experimental reagents. Aluminum chloride (Fisher, Springfield, NJ), aluminum sulfate (Fisher), and two different lots of aluminum potassium sulfate (Fisher) were tested. In some experiments Lilly Ultrapure Insulin was added.

^{3}H-Thymidine Incorporation

Sixteen hrs after the experimental reagents were added to the wells, 0.2 μCi/well ^{3}H-thymidine (Amersham, Arlington Heights, IL) was inoculated into the wells

and incubated for an additional 24 hr. At the end of this 40-hr period, the cells were washed twice in ice-cold 0.2 M NaCl, incubated at 5°C in 5% trichloroacetic acid (TCA) for 20 min, washed twice with ice-cold 5% TCA, rinsed in absolute ethanol, digested in 0.5 M NaOH, and counted by means of scintillation cocktail.

Autoradiography

Cells used in autoradiographic experiments were prepared in an identical manner to those used in ^{3}H-thymidine assays. Sixteen hrs after the test reagents were added to the wells, each well received 10 μCi/well ^{3}H-thymidine. After a 24-hr incubation, the cells were fixed in 10% buffered formalin, washed twice in 0.2 M NaCl, and precipitated with two washes of 5% TCA. After coating the plates with a 0.5% gelatin, 0.5% chromium potassium sulfate solution, AR10 autoradiographic-stripping plate film (Kodak) was applied to the cells and incubated in the dark for 1 wk, then developed by conventional techniques.

Cell Counts

Cells to be counted were treated in a manner identical to those used in the ^{3}H-thymidine incorporation studies except that the ^{3}H-thymidine was deleted. At the end of the 40-hr incubation period, the cells were detached from the wells employing 0.125% trypsin in 25 mM ethylenediamine tetraacetic acid (EDTA) and counted in a Coulter counter.

Sedimentation Equilibrium

The sedimentation equilibrium experiments were performed following the method of Smith and colleagues [16]. Briefly, the lens cells were prelabeled with 0.4 Ci/ml ^{32}P, serum-starved to quiescence, and incubated with medium alone, with 20 μM aluminum, or with 10% dialyzed FCS. Twenty-four hrs later, each well was inoculated with bromodeoxyuridine (BrdU) 10 M) and ^{3}H-thymidine (0.8 Ci/ml). All manipulations with BrdU were performed in the dark to prevent photolytic decomposition. After incubating for 24 hr, the cells were harvested, and the DNA was extracted and placed on a cesium chloride gradient of 1.72 g/ml density. Following centrifugation at 36,000 RPM in a type 40 fixed angle Beckman rotor, the gradients were fractionated and counted in a scintillation counter.

RESULTS

^{3}H-thymidine incorporation levels measured at the end of the 40-hr incubation demonstrated that aluminum induced substantial and statistically significant increases in DNA synthesis in both cell types (Figs. 1 and 2). In both the NMLE cells and the 3T3K cells, the maximum response occurred at 50 μM aluminum. In the NMLE cells, increased DNA synthetic activity at 20 μM aluminum was observed, whereas 10 μM aluminum produced ^{3}H-thymidine incorporation levels equal to the unstimulated controls (Fig. 1). When 1 mM hydroxyurea was added to wells containing 20 μM aluminum, the aluminum-stimulated increase in DNA synthesis was ablated.

For sedimentation equilibrium studies, fractions harvested from cesium chloride gradients containing BrdU-labeled extracts from aluminum-stimulated cells revealed two peaks (Fig. 3). The peak at fraction 25 (five fractions from the top of the gradient) represents light-light DNA labeled by ^{32}P incorporation. The peak at fraction 15 is

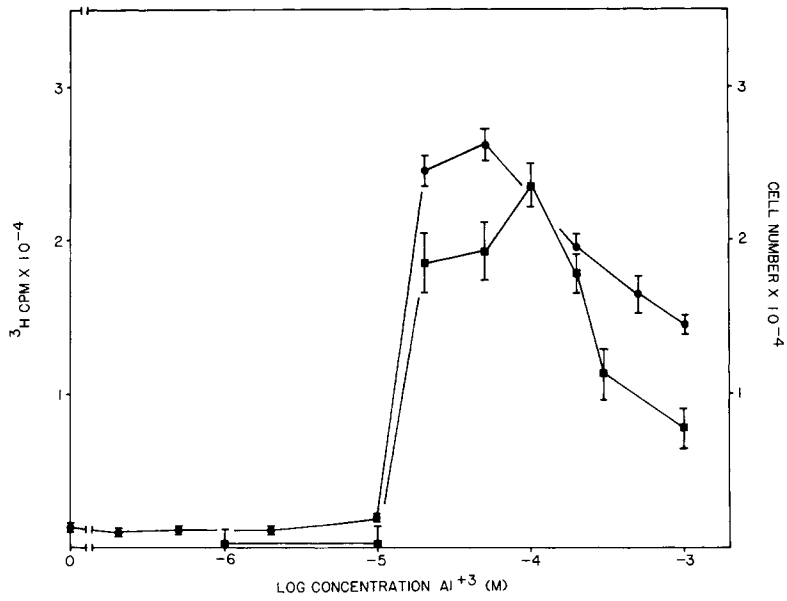

Fig. 1. Effect of aluminum on ^{3}H-thymidine incorporation (●) and cell number (■) in Nakano mouse lens epithelial cells in culture. Each point represents three wells tested. Vertical bars represent one standard deviation. The cell number data are presented with control values (1.4×10^5) subtracted. Each point represents eight wells counted. Error bars represent standard error of the means. For comparison, FCS-stimulated cells experience approximately a doubling in number during the 40-hr incubation.

hybrid light-heavy DNA containing BrdU and labeled by ^{3}H-thymidine incorporation. The identity of these peaks was established by comparison with appropriate controls. Cells stimulated by 10% FCS in the presence of ^{32}P and in the absence of BrdU produced a peak only at fraction 25, whereas cells stimulated with 10% dialyzed FCS in the presence of ^{3}H-thymidine and BrdU produced a peak in the denser fractions centered on fraction 15. By stimulating cells in the presence of both ^{3}H-thymidine and BrdU for more than one cell cycle, labeled material was found in fractions centered on fraction 8.

In order to determine if this increase in ^{3}H-thymidine incorporation and S-phase DNA synthesis accompanied an increase in mitotic activity, cells incubated in the presence and absence of aluminum were counted. The increases in both NMLE and 3T3K cell numbers occurred at aluminum concentrations that corresponded well with those that induced increased levels of ^{3}H-thymidine incorporation (Figs. 1,2). Autoradiographic nuclear labeling appeared in approximately 52% of the NMLE cells and 10% of the 3T3K cells treated with aluminum (20 μM). FCS (10%) induced nuclear labeling in about 98% of both cell types, whereas unstimulated cultures had fewer than 1% labeled nuclei. A comparison between autoradiography and mitosis is seen in Table I. Although fewer 3T3 cells than NMLE cells show nuclear labeling, essentially all the labeled 3T3 cells must go through mitosis, whereas less than half of the NMLE cells divide.

NMLE cells were exposed to 50 μM aluminum for varying periods of time at the beginning of the 40-hr ^{3}H-thymidine incorporation assay. At that concentration,

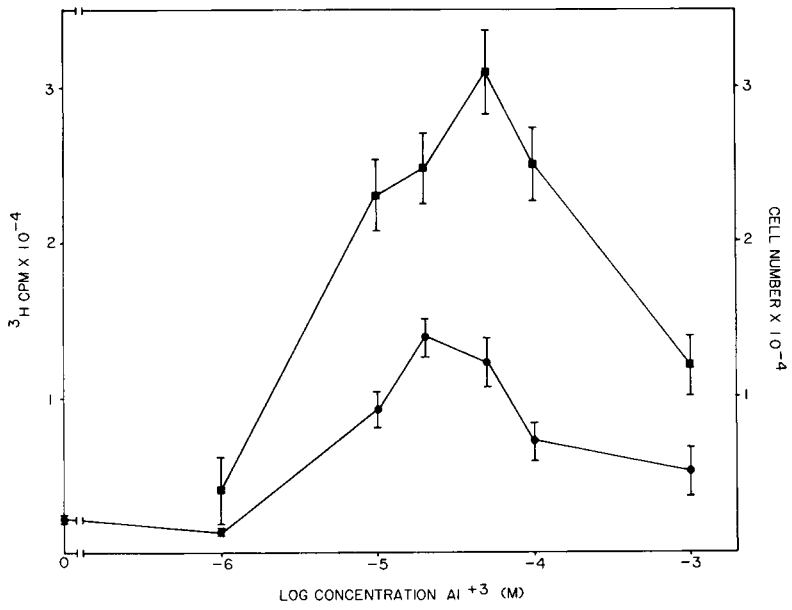

Fig. 2. Effect of aluminum on ^{3}H-thymidine incorporation (●) and cell number (■) in Swiss 3T3K cells. Each point represents three wells tested. Vertical bars represent one standard deviation. In the cell counting data, each point represents the mean of six wells counted with a control value of 1.1×10^5 subtracted. Error bars represent standard error of the means. For comparison, FCS-stimulated cells experience approximately a doubling in cell number during the 40-hr incubation.

an incubation of 3.25 hr was sufficient to induce 50% of maximum ^{3}H-thymidine incorporation by the end of the 40-hr assay (Fig. 4).

Two different lots of aluminum potassium sulfate and two other aluminum compounds, aluminum chloride and aluminum sulfate, were tested in ^{3}H-thymidine incorporation assays, and all four induced identical responses in the cells tested.

In order to test for the synergistic effect of other factors, aluminum was tested with varying concentrations of insulin on the NMLE cells (Fig. 5). Aluminum (2×10^{-4} M) was found to be synergistic with insulin especially at the lowest concentration tested (1.5×10^{-8}). A similar synergistic effect can be seen in the cell count data from Table II. Experiments were performed with epidermal growth factor (EGF) (data not shown), and no synergism was observed. All of these experiments were carried out for the normal 40-hr assay.

DISCUSSION

The ability of aluminum to stimulate ^{3}H-thymidine incorporation and also to stimulate an increase in cell number supports the thesis that aluminum is driving the cells into S-phase and then into mitoses. Hydroxyurea (1 mM), a selective blocker of S-phase DNA synthesis that functions by blocking the synthesis of dATP but that does not block DNA repair [17,18], completely blocked the aluminum-induced increase in ^{3}H-thymidine incorporation. The autoradiographs of aluminum-stimulated NMLE cells show an all-or-none effect. Nuclei are either thoroughly labeled or not labeled

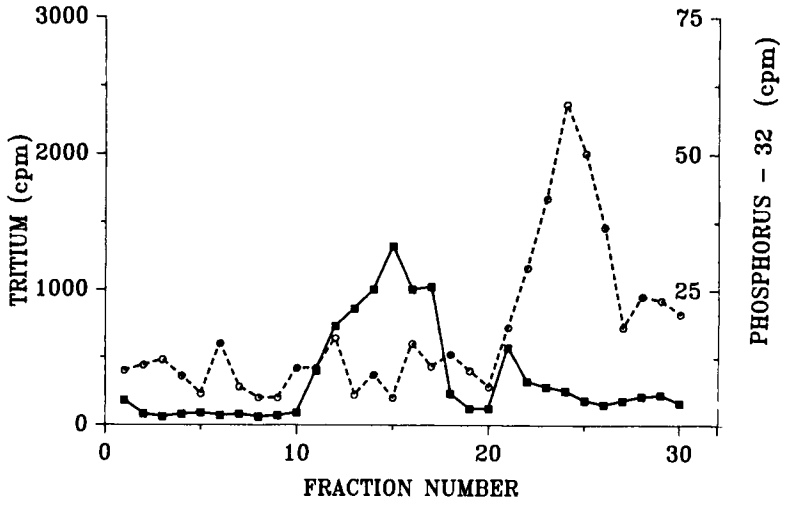

Fig. 3. Cesium chloride gradients of DNA extracts from cells grown in the presence and absence of BrdU. The light-light DNA peak is detected by prelabeling the cells with ^{32}P in the absence of BrdU (○). The hybrid light-heavy DNA appears in a denser fraction from cells stimulated with aluminum followed later by the additions of BrdU and ^{3}H-thymidine (■).

TABLE I. Comparison Between Autoradiography and Mitosis in NMLE Cells and 3T3 Cells

	Percent increase cell no.	Percent labeled nuclei	Percent increase cell no./ percent labeled nuclei
Aluminum (20 μm, NMLE)	24 ± 4	52 ± 3.8	0.46
FCS (10%, NMLE)	78 ± 8	99 ± 0.3	0.78
Aluminum (20 μM, 3T3)	11 ± 2	9.7 ± 0.4	1.1
FCS (10%, 3T3)	93 ± 6	98 ± 0.6	0.94

at all. This phenomenon is compatible with initiation of S-phase DNA synthesis but not DNA repair [19]. The demonstration of mitotic activity, the all-or-none labeling of the nuclei in autoradiographs, and the effect of hydroxyurea all support the thesis that the increased rate of ^{3}H-thymidine incorporation is the result of aluminum-induced S-phase DNA synthesis. This was substantiated by the sedimentation equilibrium results, which showed that aluminum initiated one round of DNA transcription in the first 20 hr after addition, in those cells that it was able to stimulate. The ability of three different aluminum-containing compounds to elicit identical responses from the cells at the same concentration emphasize the unlikelihood that these responses are due to an unidentified contaminant.

In a recent paper, Smith [6] used aluminum to augment the ^{3}H-thymidine incorporation response of 3T3 and 3T6 cells in the presence of FCS and insulin. He found that aluminum alone induced a two times increase in 3T3 and a five times increase in 3T6 cells. Using different cell lines, we find that aluminum alone causes 3T3K cells to respond with a 7.6 times increase, and NMLE cells respond with a 21 times increase in ^{3}H-thymidine incorporation. Smith [6] also found a correspondingly lower radiolabeling rate in 3T3 cells, whereas we found approximately 10% nuclear labeling in the 3T3K cells. Smith [6] did not, however, perform cell counts. Because,

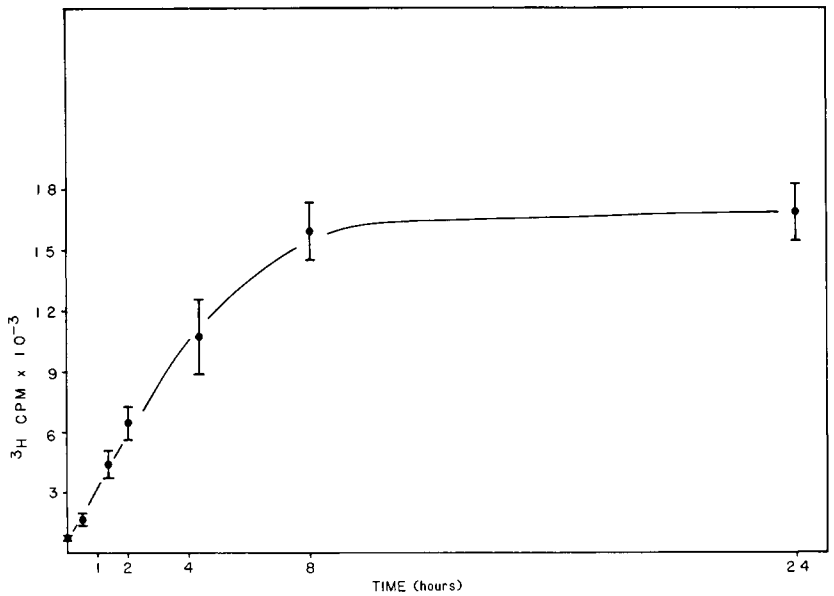

Fig. 4. Effect of incubation time on aluminum-induced ^{3}H-thymidine incorporation in Nakano mouse lens epithelial (NMLE) cells. NMLE cells were plated in a 24-well plate. Each set of wells (three per data point) was exposed to aluminum (50 μM) in serum-free RPMI 1640 medium beginning at time zero. The medium from each set of cells was removed, and the cells were washed and replaced with medium to which no aluminum had been added. The time points on the X-axis represent the number of hours beginning at zime zero that the cells from the corresponding data point were exposed to aluminum. Twenty-four hrs after time zero, ^{3}H-thymidine (0.2 μCi/ml) was added, and the cells were harvested for counting 40 hr after time zero.

as we found in the case of vanadium, ^{3}H-thymidine incorporation is not an accurate indicator of mitosis [1], cell counts are required to prove the mitogenicity of aluminum. As can be seen in Figures 1 and 2, aluminum is a mitogenic agent. Interestingly, all of the 3T3 cells that are labeled appear to pass through mitosis, whereas a much lower percentage of the NMLE cells do. The fact that fewer stimulated NMLE cells divide might be due to the fact that a second mitotic agent is required. The fact that the addition of insulin along with aluminum increases the number of cells that divide would fit with this idea; however, the results would also seem to imply that the presence of the second agent is required after the initiation of S phase DNA synthesis.

Although aluminum is present in the blood and serum and is excreted in the urine, no physiological function for aluminum is known, and it is not considered essential [4]. Recent values for aluminum concentrations in the blood vary. Gorsky and Dietz [20] reported human serum levels as determined by atomic absorption spectroscopy (AAS) of 1 μM, whereas Allain and Mauras [21] used plasma emission spectroscopy and found a titer of 0.5 μM in blood. Frech and colleagues [3], on the other hand, used AAS to measure aluminum titers in human blood drawn in a controlled class 100 atmosphere. They received a value of 60 nM. If this figure is correct, it means that the effective concentration of aluminum in tissue culture is three orders of magnitude greater than the concentration of aluminum is in normal serum. The significance of this apparent disparity is difficult to determine. Blood aluminum

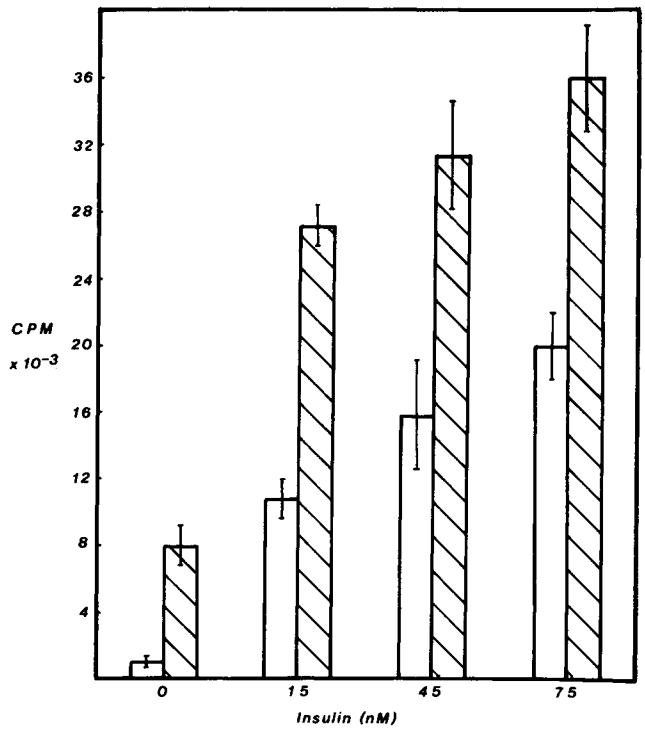

Fig. 5. Effect of aluminum on the insulin-induced ^{3}H-thymidine incorporation in NMLE cells. Cells plated in 24-well plates were taken to quiescence. They were then treated with insulin (open bars, concentration shown) or insulin plus aluminum (hatched bars, Al = 5×10^{-5} M). The ^{3}H-thymidine (0.2 μCi/ml) was added 24 hr after the addition of insulin and aluminum, and the cells were harvested for counting 16 hr later (total time 40 hr).

TABLE II. Effect of Aluminum and Insulin on Growth of NMLE Cells

	Percent increase cell counts
Aluminum (50 μM)	24 ± 4
Insulin (10^{-7} M)	6 ± 2
Aluminum plus insulin	34 ± 4

titers may not accurately reflect tissue aluminum levels, either intracytoplasmic or extracellular. Campbell et al [2], for example, reported aluminum levels in some tissues that were greater than those we used in vitro. Because of the assay methods used, however, these values should be viewed with caution. Another important factor is cell-type sensitivity. The data presented in this paper, for example, demonstrate that one cell type (NMLE) responds more vigorously to aluminum than does the other (3T3K).

Also, for these same NMLE cells we find that aluminum is synergistic with insulin at low concentrations in stimulating DNA synthesis and cell mitosis. This, coupled with our previous results that show that insulin also synergistically stimulates

these same cells in the presence of other growth factors [22], indicates that lens cells may be a possible physiological target cell for aluminum in vitro. Further experiments are required to determine the concentration of aluminum in normal NMLE cells and to determine whether these cells can concentrate aluminum and whether aluminum at lower concentrations is synergistic with multigrowth factor systems.

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