Aluminum Inhibits Erythropoiesis In Vitro

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Abstract

Anemia has been associated with aluminum intoxication in patients on chronic dialysis and in animals. In studies presented here, in vitro human erythroid culture was used to delineate the effects of aluminum on normal hematopoiesis. Aluminum by itself in routine culture, even at very high levels (1,035 ng/ml), did not significantly affect erythroid colony growth. The addition of human transferrin to the culture, however, resulted in a marked dose-dependent inhibition of erythroid, but not myeloid colony growth. At all doses, CFU-E progenitors showed greater inhibition than burst-forming units (BFU-E). Aluminum inhibition was not overcome by increasing the dose of erythropoietin or adding additional burst-promoting activity to the culture. Inhibition by aluminum was directly related to the number of binding sites on transferrin in the culture, and was not observed in the presence of fully iron-saturated transferrin.

Introduction

Aluminum, a ubiquitous heavy metal, has been seen in markedly elevated levels in chronic dialysis patients, and was initially associated with dialysis-induced encephalopathy (1-4) and osteomalacia (5-10). More recently, aluminum loading has been implicated as a contributing factor in the anemia of chronic renal failure (11-18). An initial report by Elliot and MacDougall described a severe microcytic, hypochromic or normochromic, non-iron-deficient anemia in patients who were suffering from dialysis encephalopathy (11). Subsequently, other reports confirmed this clinical description of aluminum-induced anemia (12-18). All patients have elevated serum aluminum levels, and over half also have aluminum deposition in bone marrow macrophages (19, 20). In prospective and retrospective studies of these patients, reversal of the anemia occurs with chemical removal of aluminum from dialysate (14, 16, 17) or chelation with deferoxamine (18, 21), providing indirect evidence that aluminum is the etiologic agent in this process. More direct evidence comes from animal studies that show that prolonged parenteral injections of aluminum result in varying degrees of anemia in both normal and uremic animals (17, 22, 23). An early study in rabbits also suggested that anemia could be accompanied by basophilic stippling, circulating normoblasts, and red blood cells with decreased osmotic fragility (22). More recent studies in rats have provided conflicting data that characterize a hypoproliferative, microcytic anemia (23) or identify a slight reticulocytosis accompanied by decreased osmotic fragility of red blood cells (17). These differences are perhaps due to methods of aluminum administration.

Although aluminum apparently is capable of inducing anemia, the exact mechanism by which this occurs is unknown. Several possibilities exist. With a hypoproliferative, microcytic, and hypochromic anemia characteristically described, aluminum is postulated to decrease heme synthesis. This effect may be directly mediated through heme-synthetic enzymes that are thought to be inhibited by aluminum, such as ferrochelatase (24) or uroporphyrin decarboxylase (25, 26). Studies of levels of delta-levulinic acid dehydrogenase have been inconclusive (27-29). Alternatively, aluminum may interfere indirectly with the normal metabolism of iron (30). A decrease in osmotic fragility and a mild reticulocytosis suggest that increased red blood cell destruction may also contribute to anemia (17, 22). Therefore, the purpose of the studies reported here was to further elucidate the effect of aluminum (Al) on hematopoiesis, through the use of in vitro culture.

Methods

Clonal assays of hematopoietic progenitors. Bone marrow cells were obtained from normal human donors or from ribs of patients undergoing thoracotomy for isolated coin lesions, according to a protocol approved by the Human Subjects Subcommittee of the Minneapolis Veterans Administration. Bone marrow mononuclear cells were obtained by Ficoll-Hypaque sedimentation and cultured in methycellulose, as described previously for this laboratory (31), but with the following modifications. FCS (HyClone Laboratories, Sterile Systems, Inc., Ogden, UT) was limited to 20% of the final concentration. The total iron binding concentration (TIBC) of this FCS was 98% saturated, with the final concentration of TIBC in control cultures at 54 μg/dl (32). Cells were plated at a concentration of 1 × 10⁶/ml, and quadruplicate samples enumerated on day 7 for CFU-E and day 14 for burst-forming units (BFU-E) and CFU-C-derived colonies. 400 U/ml erythropoietin (EP; Terry Fox Cancer Institute, Vancouver, BC, Canada) was used; conditioned medium from 5637 bladder carcinoma cell line was used as a source of burst-promoting activity (BPA) and CSA.

Aluminum solutions and measurement. All glassware was acid washed and rinsed with deionized water to remove contaminating aluminum and iron. Aluminum stock solutions were prepared in deionized water, (aluminum < 1.5 ng/ml), filter sterilized, and stored in the dark at 22°C. Stock solutions were stored in tissue grade polyester tubes to avoid leaching of metals. For the citrate complex, equimolar volumes of 2 M AlCl₃·6H₂O and citric acid were stirred for 15 min at 22°C, the pH adjusted to 7.5 with NaOH, and diluted to final working concentrations with deionized water. Aluminum levels of...
stock solutions, as well as all culture constituents and the final culture itself were verified by flameless atomic absorption spectrophotometry (33, 34) (MetPath Laboratories, Teterboro, NJ). Aluminum levels were independently confirmed (courtesy of Dr. James McCarthy, Mayo Clinic and Laboratories, Rochester, MN) using flameless electrothermal atomization atomic absorption spectroscopy with a L'vov platform and Zeeman background correction, and reproducibility of ±5% (35).

Iron-saturated transferrins. Substantially iron-free (99%) human transferrin (a single lot found to be nontoxic in culture) was used for all experiments (Sigma Chemical Co., St. Louis, MO). It was diluted in sterile deionized water and added directly to the culture medium, or loaded with ferrous iron, to yield low (10–20%), medium (50–60%), or completely saturated (100%) transferrin (36, 37).

For iron-saturated transferrin, the appropriate amount of iron was prepared fresh in the form of the citrated complex. It was combined with transferrin and NaHCO3 (dry, final concentration 0.1 M), with the pH adjusted to 7.4 with 0.01 M citric acid. The solutions were diluted to 10 mg/ml transferrin with deionized water and incubated for 4 h at 22°C with shaking; they were then dialyzed overnight against excess phosphate buffer. Saturation levels were determined spectrophotometrically (DU; Beckman Instruments, Fullerton, CA), with 100% saturation indicated by a A465nm/A280nm ratio of 0.046 (36, 37).

Preparation of transferrin-depleted FCS. Bovine transferrin was removed by immunoprecipitation, using a rabbit antibovine transferrin IgG fraction, generously supplied by Dr. Helmut Huebers (University of Washington, Seattle, WA) (38).

Results

Aluminum, as either chloride (with and without a nonspecific chelator, EDTA) or citrate, had no significant effect on human hematopoietic progenitor growth, even at very high concentrations (Fig. 1). This observation was similar, whether aluminum was added directly to culture (FCS 98% iron-saturated TIBC), preincubated with marrow cells for 2 h before culture, or added daily to culture. When aluminum was added in the presence of unbound transferrin, however, significant inhibition of erythroid colony growth occurred. Greater inhibition of CFU-E- than BFU-E-derived progenitors was consistently seen, with BFU-E-derived inhibition unaffected by exogenous BPA. CFU-C-derived progenitors were not inhibited by the addition of aluminum and transferrin (118±12% SD control growth; n = 6). Inhibition occurred over a broad range of doses, including levels considered clinically toxic in serum (39) (Fig. 2). The dose response was steeper for CFU-E- than BFU-E-derived progenitors. Inhibition of erythroid colony growth in the presence of aluminum and transferrin could not be significantly overcome by increasing EP in the culture (Fig. 3).

Preliminary experiments showed that aluminum inhibition could, however, be overcome by the simultaneous addition of iron (as FeCl3) to the culture medium (data not shown). Therefore, to test the hypothesis that aluminum toxicity was dependent upon the amount of transferrin available for binding to the metal, varying amounts of iron-saturated transferrin were added to culture in the presence of a constant amount of aluminum. At two doses of aluminum, an inverse relationship was seen between the iron saturation and the aluminum-induced erythroid inhibition (Fig. 4). Similar experiments were also performed with FCS that was depleted of bovine transferrin, but to which variably saturated amounts of human transferrin were added. Growth in this depleted FCS was negligible, but was restored to 60 and 100% control growth (growth in untreated FCS) by the addition of 10 and 50% iron-saturated human transferrin, respectively. When aluminum was added to these preparations (superimposed bar graphs, Fig. 4), the
relationship of aluminum inhibition to the saturation of transferrin was confirmed.

In attempts to show the presence of aluminum intracellularly, two experimental approaches were used: colonies or cells grown in the presence of low to high doses of aluminum, or cells incubated with aluminum and transferrin for 2–24 h, were stained for aluminum (40); alternatively, these cells were washed in deferoxamine, lysed, and aluminum was measured by flameless atomic absorption spectrophotometry. In neither of these approaches were significantly greater amounts of aluminum found in aluminum-exposed cells when compared with the control population.

Discussion

These data showed that aluminum inhibited erythropoiesis in vitro through a mechanism dependent upon the availability of transferrin for binding to the heavy metal. Aluminum was not capable of interfering with the hematopoietic progenitor cell utilization of iron already bound to transferrin. These observations are compatible with previous evidence that demonstrates that transferrin is the major binding protein of aluminum in serum (41-43). Given the stability constants of aluminum and iron for transferrin (~10^{15} mol/liter and 10^{20} mol/liter, respectively) (43, 44), the presence of iron-bound transferrin or competition between iron and aluminum for transferrin would lead to diminished aluminum binding. This should decrease inhibition if aluminum toxicity were dependent upon transferrin binding. This prediction is consistent with data presented here: minimal inhibition of erythropoiesis was seen in the presence of 98% iron-saturated fetal calf TIBC or 100% iron-saturated human transferrin; iron added simultaneously with aluminum could prevent aluminum inhibition. CFU-E were more sensitive to aluminum inhibition than BFU-E, (and CFU-C not at all). This observation would also be expected if inhibition of progenitor cell proliferation depended upon delivery of the metal to the cell surface by transferrin. Transferrin receptors are known to increase with differentiation, and are thus present at a much greater density on CFU-E than BFU-E progenitors (45).

Although inhibition appears dependent upon transferrin, the exact mechanism of cellular damage is not known. Aluminum may work through accessory cells, may enter the cell to exert its effect on heme synthetic enzymes, and/or may provide direct toxicity at the proliferating cell membrane. Experiments not shown, removal of adherent cells or T lymphocytes did not decrease the inhibition seen with aluminum and transferrin, nor did the addition of exogenous BPA (Fig. 1), suggesting that accessory cells do not play a major role. Aluminum may enter the cell after binding to transferrin to exert its
toxic effect. However, increased intracellular presence of aluminum in erythroid progenitors could not be unequivocally demonstrated in these studies. These negative results could occur for technical reasons (33, 40); alternatively, aluminum-affected progenitors may not readily proliferate in vitro, or their progeny may be lost due to changes in membrane fragility (17, 22). Although aluminum has been detected in erythrocytes (46, 47), it has not been found in normoblasts of marrow from aluminum-intoxicated patients (20). Direct specific toxicity and/or interference at the cellular membrane remain possible mechanisms of aluminum-transferin inhibition, and require further study.

These in vitro studies demonstrated a very steep dose response for aluminum inhibition. Although measurement of aluminum in this system showed inhibition at clinically relevant levels, the toxic hematopoietic dose cannot be directly extrapolated. Aluminum is bound to other proteins in addition to transferrin (albumin in the culture) (42); furthermore, serum levels of aluminum may not accurately reflect the amount of tissue overload or toxicity (39, 48).

Aluminum inhibition was inversely proportional to the saturation of transferrin. Clinically, this finding suggests that iron deficiency would favor the development of aluminum-induced anemia. These data also showed that increased doses of EP did not significantly overcome the inhibition induced by aluminum. This observation would predict that treatment of patients with EP in the presence of aluminum overload may limit their marrow responsiveness, an effect that could become further exaggerated in the presence of iron deficiency (49). An awareness of, and improvement in the diagnostic techniques for aluminum-induced anemia would facilitate testing of these hypotheses. This becomes especially clinically relevant as therapeutic EP becomes available.

Therefore, in summary, one mechanism of aluminum-induced anemia is through the inhibition of erythropoiesis, an effect dependent upon transferrin binding of the metal. Whether inhibition of erythropoiesis is the only mechanism of hematopoietic toxicity, or whether shortened red blood cell survival plays an additional role in the aluminum-induced anemia of chronic renal failure remains to be determined.

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References


