Tissue Aluminum Accumulation and Toxic Consequences in Rats Chronically Fed Aluminum with and without Citrate

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Tissue aluminum accumulation and the potential toxic sequelae in rats fed moderate amounts of aluminum ($\approx$40 μmol of Al/g of diet) without citrate (Al diet) and with supplemental citrate (208 μmol of citrate/g of diet) (Al-Cit diet) for 6–7 months were examined. Rats fed the Al diet accumulated more aluminum in tibias and kidneys and rats fed the Al-Cit diet accumulated more aluminum in tibias, sera, and muscles than rats fed the basal diet. Rats fed the Al-Cit diet had 1.5-fold greater calculated body load of aluminum and excreted 1.5-fold more aluminum in urine after administration of desferrioxamine (DFO) than rats fed the Al diet. This suggests that DFO tests have some utility as indicators of body aluminum loads. Rats fed the Al-Cit diet exhibited increased renal stress and transitory increases in urinary excretion of hydroxyproline but no changes in bone calcium or tissue iron concentrations.

Although aluminum occurs naturally in most plant materials, the largest source of aluminum in the diets of most Americans is food additives (Greger, 1985, 1993; Pennington and Jones, 1988). Accordingly, consumption of aluminum from food by Americans varies from 1 to >100 mg daily (Greger, 1993). Ingestion of aluminum from pharmaceuticals, particularly antacids, is often 1–5 g daily (Lione, 1983).

The toxicological impact of orally administered aluminum is more debatable than that of parenterally delivered aluminum. Aluminum absorption is very low (probably <0.04%) (Ganrot, 1986; Greger and Powers, 1992). Many investigators examining the effects of oral exposure to aluminum have administered large gavage doses of aluminum (Domingo et al., 1991; Froment et al., 1989; Quartley et al., 1993; Slanina et al., 1985, 1986). A few have conducted short-term (<30 days) feeding studies (Brown and Schwartz, 1992; Ecelbarger and Greger, 1991; Greger and Donnaubauer, 1986; Greger and Powers, 1992). In practical situations, dietary exposure to aluminum is chronic.

Furthermore, the bioavailability of aluminum from diet, water, and pharmaceuticals is variable. Ingestion of supplemental calcium (Ecelbarger and Greger, 1991) or silicic acid (Quartley et al., 1993) has been found to reduce tissue retention of aluminum. Ingestion of citrate has been found to increase aluminum absorption and retention (Ecelbarger and Greger, 1991; Froment et al., 1989; Quartley et al., 1993; Slanina et al., 1985, 1986).

Previous work, especially with renal patients given parenteral doses of aluminum, has demonstrated that aluminum causes osteoblast dysfunction, decreases bone mineralization, and induces a microcytic anemia (Drüeke et al., 1986; Ganrot, 1986; Hewitt et al., 1990). The purpose of this study was to investigate the effects of

chronic exposure to dietary aluminum on tissue aluminum accumulation and consequences in mature, healthy animals.

A desferrioxamine test was used to assess body stores of aluminum (Milliner et al., 1984; Nebeker et al., 1986). It has been hypothesized that responses to DFO (i.e., changes in urinary aluminum concentrations after treatment with DFO) are proportional to the tissue aluminum stores.

METHODS

Two-month-old Sprague-Dawley rats (Harlan Sprague Dawley, Madison WI) were fed one of three diets ($n = 16$/treatment): basal diet (by analysis 0.40 μmol of Al/g of diet), Al diet [by analysis 40.2 μmol of Al/g of diet added as aluminum hydroxide (EM Science, Gibbstown, NJ)], or Al-Cit diet [by analysis 38.4 μmol of Al/g of diet and 208 μmol of citrate added as sodium citrate dihydrate (Mallinckrodt Specialty Chemical Co., Paris KY)]. Eight rats were fed each diet for 178 days (block A); eight rats were fed each diet for 208 days (block B).

Rats were fed lactalbumin-based, semipurified diets using AIN-76 formulation (American Institute of Nutrition, 1977) of mineral and vitamin mixtures as described previously (Greger and Powers, 1992). The aluminum in the basal diet was there naturally, a contaminant of mineral salts primarily. The test concentration of aluminum was fed because previously we found that young rats fed more than 40 μmol of Al/g of diet had depressed food intake and growth (Greger et al., 1986).

Rats were housed individually in stainless steel, wire-bottom cages in a room maintained at 23–24 °C with 12-h light/dark cycles. The facilities and protocol were approved by an institutional animal care and use committee. Deionized water was offered ad libitum. Feed intake by animals was not limited, but consumption was recorded daily. Rats were weighed weekly.

Desferrioxamine (DFO) Test. One day prior to sacrifice, half of the rats in all three treatments were injected intraperitoneally with 100 mg/kg desferrioxamine (Sigma Chemical Co., St. Louis MO) in a saline solution (pH 4.5); the rest were injected with saline alone (pH 4.5) (Greger and Powers, 1992). As part of the DFO test, urine was collected for 8 h after DFO or saline administration while rats were housed in Nalgene metabolic cages (Nalge Co., Rochester NY) which were acid-washed just prior to the collection period to reduce contamina-

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tion. Rats were not fed during the 8-h collection period to prevent any feed contamination of the urine. Urine was acidified (0.05% HNO_3, Ultrex II, Ultrapure reagent, J. T. Baker Co., Phillipsburg, NJ) and frozen until analysis.

Rats were anesthetized with CO_2 and killed by exsanguination. Serum, as opposed to plasma, was collected to avoid potential contamination from anticoagulants. Tibias, kidneys, livers, and gastronemius muscles were excised, cleaned of adhering matter, rinsed with deionized water, weighed immediately, and frozen in polystyrene test tubes (Sarstedt Inc., Arlington Heights, IL).

Chemical Analyses. Tissue and diet samples were digested overnight in acid-washed Teflon centrifuge tubes (Nalge) at 70 °C in a heating block with 0.2–5.0 mL of nitric acid (Ultrex II, Ultrapure reagent, J. T. Baker) and diluted. Diluted urine, tissue, and diet samples were analyzed for aluminum using an atomic absorption spectrophotometer with a graphite furnace atomizer (Hitachi Model 170–70 polarized Zeeman, Tokyo, Japan) by a standard addition technique (Ecelbarger and Greger, 1991). Spinach (SRM 1570) standards obtained from the National Institute of Standards and Technology were also analyzed by the same methods and determined to contain 805 ± 52 μg of Al/g ($n = 4$) (certified value = 870 ± 50 μg of Al/g).

Diets and tissue samples were heated at 450 °C overnight in a muffle furnace, dissolved in concentrated nitric acid, diluted and analyzed for iron and calcium by atomic absorption spectrophotometry (Greger and Snedecker, 1980). Lanthanum chloride (0.5% w/v) was added for calcium analysis.

Urine was collected for 2-day periods during the first week of the study when rats were 2 months of age and during the 19th and 20th weeks of the study when rats were 6.5 months of age. Food and water consumption continued normally during these collection periods. Urine was preserved with toluene, frozen, and analyzed for hydroxyproline (Goverde and Veenkamp, 1972) and protein (Peterson, 1977). Quality control was maintained by analyzing a pooled urine sample with each batch of experimental samples.

Statistical Analyses. All data collected before DFO administration (i.e., urine protein and hydroxyproline) were analyzed by two-way ANOVA (dietary treatment × time) (SAS Institute, 1985). Data collected after DFO administration were analyzed first by a three-way ANOVA (dietary treatment × DFO × block) The blocks (i.e., 178 vs 208 days of dietary treatment) did not significantly affect any measure of tissue aluminum accumulation, kidney function, or urinary excretion. DFO treatment only significantly affected serum aluminum concentrations and urinary aluminum and iron excretion. Thus, the statistical analysis was repeated with a two-way ANOVA (dietary treatment × DFO) to analyze serum aluminum and urinary aluminum and iron concentrations and a one-way ANOVA (dietary treatment) to analyze all other variables. Tests for least significance (lsd) were used to differentiate among means for variables that were significantly ($p < 0.05$) affected by treatments. The correlations (r) of urine and tissue aluminum concentrations to other variables were also determined (SAS Institute, 1985).

RESULTS

Rats grew rapidly from 2 to 4 months of age and then grew gradually from 4 to 8 months. Average weights at 2, 4, 6, and 8 months were 184, 443, 476, and 513 g, respectively. The dietary treatments had no significant effects on growth of rats. Feed intakes of animals averaged 18.3 ± 0.2 g/day throughout the study.

Aluminum Utilization. Rats fed aluminum with or without citrate and injected with saline excreted more aluminum in urine than similarly injected rats fed the basal diet (Figure 1). Rats excreted significantly more aluminum in urine when injected with DFO rather than saline if they were fed the Al and Al-Cit diets but not if they were fed the basal diet. Urinary excretion of aluminum was correlated with serum aluminum con-
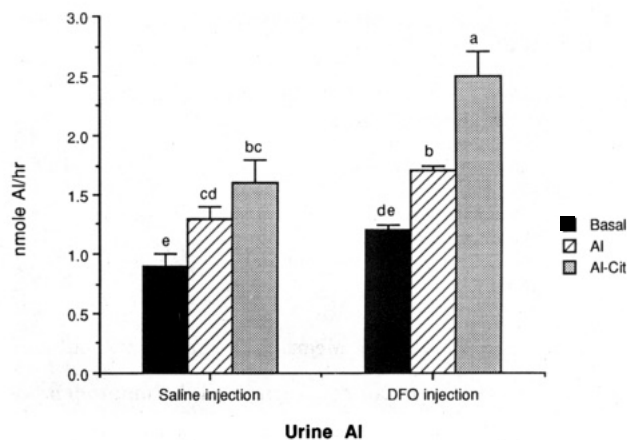
Figure 1. Urinary excretion of aluminum by rats fed semipurified diets supplemented with aluminum and citrate. Rats were injected (ip) with desferrioxamine (DFO) or saline, and urine was collected for 8 h. Values are means ± SEM ($n = 8$, except for Al-Cit with DFO injection, $n = 7$). Means without a common superscript letter differ significantly ($p < 0.05$).
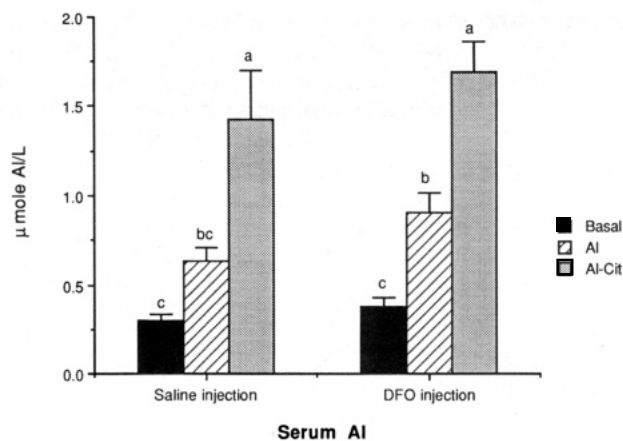
Figure 2. Serum aluminum concentrations of rats fed semipurified diets supplemented with aluminum and citrate and injected (ip) 24 h previously with desferrioxamine (DFO) or saline. Values are means ± SEM ($n = 8$, except for Al-Cit, $n = 7$). Means without a common superscript letter differ significantly ($p < 0.05$).

centration ($r = 0.699$, $p < 0.0005$), tibia aluminum concentrations ($r = 0.701$, $p < 0.0005$), and kidney aluminum concentrations ($r = 0.576$, $p < 0.005$) in rats treated with DFO but only with tibia aluminum concentrations ($r = 0.561$, $p < 0.01$) in rats treated with saline.

Serum aluminum concentrations were greater among rats fed the Al-Cit diet than among rats fed the other two diets after the saline injection (Figure 2). The DFO injections appeared to sharpen differences among dietary treatments. Twenty-four hours after the DFO injection, serum aluminum concentrations were significantly greater among rats fed the Al diet than among rats fed the basal diet and were significantly greater among rats fed the Al-Cit diet than among rats fed the basal or Al diet.

The effect of the dietary treatments on tissue aluminum concentrations varied with the tissue. Rats fed supplemental aluminum with or without citrate had higher concentrations of aluminum in bone than rats fed the basal diet (Figure 3). Rats fed the Al-Cit diet accumulated even more aluminum in bone than rats fed the Al diet.

The rats fed aluminum without citrate accumulated more aluminum in their kidneys than rats fed the basal

Table 1. Urinary Excretion of Protein and Hydroxyproline of Rats Fed Semipurified Diets Supplemented with Aluminum and Citrate

	basal diet	Al diet	Al-Cit diet
urine volume[a] (mL/day)			
2 months of age	11.5 ± 0.5^d	9.5 ± 0.7^d	21.4 ± 0.9^b
6.5 months of age	19.8 ± 1.5^{bc}	17.4 ± 1.1^{bc}	31.9 ± 1.3^a
urine protein[a] (mg/day)			
2 months of age	38 ± 2^c	39 ± 3^c	44 ± 2^c
6.5 months of age	128 ± 15^b	147 ± 15^b	206 ± 23^a
urine hydroxyproline[a] (μmol/day)			
2 months of age	4.77 ± 0.17^b	4.73 ± 0.18^b	6.11 ± 0.27^a
6.5 months of age	2.32 ± 0.10^c	2.11 ± 0.06^c	2.28 ± 0.21^c

[a] Means $\pm$ SEM ($n = 16$). Means for a variable without a common superscript letter differ significantly ($p < 0.05$).

Table 2. Tissue Iron Concentrations and Hematocrits of Rats Fed Semipurified Diets Supplemented with Aluminum and Citrate

	basal diet	Al diet	Al-Cit diet
serum Fe[a] (μmol/L)	124 ± 14	132 ± 14	109 ± 6
liver Fe[a] (μmol/g of wet wt)	2.95 ± 0.18	2.90 ± 0.16	2.79 ± 0.18
kidney Fe[a] (μmol/g of wet wt)	2.53 ± 0.11	2.69 ± 0.15	2.33 ± 0.09
ulna Fe[a] (μmol/g of wet wt)	0.47 ± 0.02	0.44 ± 0.01	0.42 ± 0.01
hematocrit[a] (%)	51.6 ± 0.9	51.4 ± 0.4	50.7 ± 1.0
urinary Fe[b] (nmol of Fe/h)			
DFO injection	52.5 ± 2.2^b	53.2 ± 2.1^{ab}	61.0 ± 5.0^a
saline injection	12.2 ± 2.4^c	11.8 ± 1.9^c	12.8 ± 2.1^c

[a] Means $\pm$ SEM ($n = 16$, except for Al-Cit, $n = 15$). No significant differences. [b] Means $\pm$ SEM ($n = 8$, except for Al-Cit with DFO injections, $n = 7$). Means for a variable without a common superscript letter differ significantly.
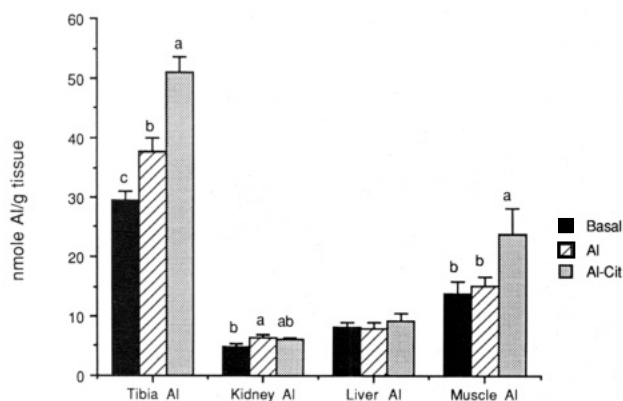
Figure 3. Tissue aluminum concentrations of rats fed semipurified diets supplemented with aluminum and citrate. Data from rats treated with saline and DFO are combined. Values are means $\pm$ SEM ($n = 16$, except for Al-Cit, $n = 15$). Means without a common superscript letter differ significantly ($p < 0.05$).

diet. The rats fed aluminum with citrate accumulated more aluminum in muscle than rats fed the basal or Al diet. DFO had no significant effects on tissue aluminum concentrations, so only average concentrations (i.e., saline and DFO treatments combined) are reported in Figure 3. Among the aluminum concentrations for the various tissues (tibia, kidney, liver, muscle), only tibia and muscle aluminum concentrations were correlated ($r = 0.457$, $p \leq 0.01$).

Rats in block A fed the basal, Al, and Al-Cit diets consumed 0.03, 3.35, and 3.43 g of aluminum, respectively, in 178 days and rats in block B consumed 0.04, 4.10, and 4.10 g of aluminum, respectively, in 208 days. Neither tissue nor urinary aluminum concentrations with and without DFO treatments differed between the two blocks. This indicates that the extra 30 days of exposure to oral aluminum had no measurable effects on the amount of aluminum accumulated in tissues.

Kidney Function. The ingestion of the Al-Cit diet had negative effects on the kidney function of the rats as judged by urinary volume and urinary protein excretion (Table 1). Urinary volume was affected by the Al-Cit diet very rapidly. Rats fed the Al-Cit diet excreted more urine than rats fed the basal and Al diets at 2 months of age as well as at 6.5 months of age. At 2 months of age, rats, after being fed the three diets for less than a week, excreted similar amounts of protein in urine. At 6.5 months of age, the rats fed Al-Cit diet excreted more protein in urine than rats fed the basal or Al diet. However, at 8 months of age, blood urea nitrogen concentrations of rats fed the basal, Al, and Al-Cit diets did not differ (10.7 ± 0.3, 10.7 ± 0.5, and 11.5 ± 0.4 mmol/L, respectively).

Bone Metabolism. The excretion of hydroxyproline in urine decreased as rats matured from 2 to 6 months of age (Table 1). At 2 months of age, rats fed the Al-Cit diet excreted significantly more hydroxyproline than rats fed the basal and Al diets, but the treatments did not significantly affect hydroxyproline excretion at 6.5 months.

At 8 months of age, the dietary treatments had no effect on the size of ulnas or tibias or the calcium concentration in ulnas. Rats fed the basal, Al, and Al-Cit diets had 4.81 ± 0.11, 4.95 ± 0.07, and 4.82 ± 0.08 mmol of Ca/g of bone, respectively. Indices of bone metabolism (i.e., calcium concentrations, hydroxyproline excretion) were not correlated to aluminum intake or any measure of aluminum accumulation.

Iron Utilization. When the rats were injected with DFO, they excreted 4-fold more iron in urine than when injected with saline (Table 2). The dietary treatments had no effect on urinary iron excretion after saline injections. However, when rats were injected with DFO, those fed the Al-Cit diet excreted more iron than those fed the basal diet. Ingestion of neither aluminum nor aluminum with citrate had any effect on the rats' serum, liver, kidney, or ulna iron concentrations or hematocrits. Urinary iron excretion was correlated to several indices of aluminum accumulation, i.e., urinary aluminum excretion ($r = 0.482$, $p < 0.05$), tibia aluminum concentrations ($r = 0.597$, $p < 0.005$), and serum aluminum concentration ($r = 0.419$, $p < 0.05$) in rats treated with DFO but not in rats treated with saline.

Tissue Aluminum Accumulation

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DISCUSSION

A man consuming 100 mg of aluminum daily in food (0.5 kg of dry weight/day) would ingest about 7.4 μmol of Al/g of dry diet; a man ingesting 1000 mg of aluminum in antacids daily would ingest about 74 μmol of Al/g of dry diet. Thus, the amount of aluminum (40 μmol of Al/g of dry diet) fed rats in this study was severalfold higher than typical human intakes of aluminum in food but consistent with moderate intakes of antacids (Greger, 1993; Lione, 1983).

Body Aluminum Loads and the DFO Test. We calculated the average body aluminum loads of rats using the analyzed aluminum concentrations in tibias, muscles, livers, and kidneys and the estimates of Pierson et al. (1978) that bone and muscle constituted 5% and 41%, respectively, of a healthy adult rat. On this basis we estimated that rats fed the basal, Al, and Al-Cit diets accumulated body loads of 3.95, 4.35, and 6.53 μmol of aluminum, respectively. Thus, rats fed the Al and Al-Cit diets appeared to accumulate 1.1- and 1.7-fold as much aluminum, respectively, as rats fed the basal diet.

Three-fourths of the calculated body aluminum loads of rats in this study was in muscle regardless of the treatment. Bone accounted for about one-fifth of the body aluminum load.

Urinary excretion of aluminum after DFO injection by rats fed the Al-Cit diet was 1.5-fold greater than of rats fed the Al diet (2.5 vs 1.7 nmol of Al/h); similarly estimated body aluminum loads among rats fed the Al-Cit diet were 1.5-fold greater than among rats fed the Al diet (6.53 vs 4.35 μmol of Al). This suggests that urinary excretion of aluminum after an injection of DFO can be used to compare relative body aluminum loads among treatments. This is consistent with the hypotheses of Milliner et al. (1984) and Nebeker et al. (1986) that DFO tests could be used to estimate tissue aluminum stores.

Toxic Sequelae to Oral Aluminum Administration. Many investigators have demonstrated that patients and animals with renal impairment accumulate aluminum more rapidly than those with normal renal function (Ecelbarger and Greger, 1991; Ganrot, 1986; Hewitt et al., 1990; Ittel et al., 1987). Less has been published on the effects of aluminum accumulation on kidney function. Previously, Ecelbarger and Greger (1991) and Bräunlich et al. (1986) have observed decreased kidney function in rats injected with aluminum. However, in short-term (<30 days) studies Ecelbarger and Greger (1991) noted that ingestion of aluminum did not affect kidney function.

The current study suggests that long-term (6 or 7 months) exposure to oral aluminum and citrate does lead to renal stress, i.e., larger urine volume and urinary protein excretion. However, renal stress was not correlated to kidney aluminum concentrations. The rats fed the Al diet had significantly elevated concentrations of aluminum in their kidneys and the rats fed the Al-Cit diet did not, but the latter group exhibited renal stress.

In this study, except for a transient increase in hydroxyproline excretion by rats fed the Al-Cit diet, no changes in bone metabolism were noted among rats fed the Al or Al-Cit diet. These animals were probably less prone to alterations in bone metabolism than dialysis patients for several reasons. Although bone aluminum concentrations were elevated in our rats fed aluminum, the aluminum concentrations were much lower than those observed in animals injected with aluminum

(Greger and Powers, 1992). Hewitt et al. (1990) suggested that bone aluminum concentrations of greater than 200 μg of Al/g (7 μmol of Al/g) are associated with osteomalacia in dialysis patients. Although these rats experienced renal stress, their kidney function was still adequate, as judged by BUN concentrations. Thus, renal synthesis of 1,25-dihydroxy-vitamin D was probably adequate to maintain bone mineralization.

Anemia is a symptom of aluminum toxicity among renal patients (Drüeke et al., 1986; Hewitt et al., 1990). However, rats in this study fed aluminum for 6–7 months were not anemic and did not have reduced tissue iron stores. This may be because the rats were past their period of most rapid growth and were fed $\approx$0.85 μmol of Fe/g of diet, i.e., the amount suggested by the American Institute of Nutrition (1977). Previously, Greger and Powers (1992) noted that weanling rats fed similar amounts of aluminum and iron had reduced hematocrits but no reductions in tissue iron concentrations.

In summary, we predicted on the basis of DFO tests that rats fed aluminum with citrate accumulated 1.5-fold greater body loads of aluminum than rats fed similar amounts of aluminum without citrate. The calculated body loads of aluminum based on tissue aluminum analyses were fairly consistent with the DFO estimates. Rats fed moderate aluminum (40 μmol of Al/g of diet) with citrate for 6–7 months exhibited few side effects except for increased renal stress.

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