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The Differential Response of Cortical and Trabecular Bone to Aluminum Administration in the Rat (42131)

WILLIAM G. GOODMAN

With the technical assistance of Jeanenne M. Gilligan

Medical and Research Services, Veterans Administration Medical Center, and Department of Medicine, University of California, Los Angeles, School of Medicine/San Fernanado Valley Program, Sepulveda, California 91343

Abstract. Osteomalacia has been noted following *in vivo* aluminum (Al) loading in the rat by some investigators but not by others. To determine whether the response of bone to Al differs as a function of the skeletal site examined, quantitative histology of cortical and trabecular bone was done in the tibiae from control (C, $n = 10$), Al-treated (AL, $n = 9$), nephrectomized control (NX-C, $n = 7$), and nephrectomized Al-treated (NX-AL, $n = 8$) rats given 2 mg/day of Al for 4 weeks. Bone Al content was determined by histochemical methods. In cortical bone, osteoid seam width, osteoid volume, and percent osteoid area were similar for all groups. In contrast, for trabecular bone, both forming surface ($\bar{x} \pm$ SD) (5.2 ± 3.4 vs 1.8 ± 1.1%, $P < 0.05$) and osteoid volume (1.7 ± 0.7 vs 1.0 ± 0.4%, $P < 0.05$) increased from control values in AL, although osteoid seam width did not differ. In NX-AL, trabecular forming surface (20.2 ± 6.7 vs 6.2 ± 2.4%, $P < 0.01$), osteoid area (13.2 ± 5.7 vs 3.5 ± 0.8%, $P < 0.01$), and osteoid width (18.7 ± 5.7 vs 9.7 ± 2.3 μm, $P < 0.01$) all were greater than in NX-C. Deposits of Al were undetectable in C and NX-C, were minimal in cortical bone in AL and NX-AL, but were present at 40.5 ± 11.5 and 71.1 ± 6.5% of trabecular surfaces in AL and NX-AL, respectively. Osteoid area and osteoid surface each correlated with trabecular bone Al. Thus, (a) osteoid accumulates in trabecular, but not in cortical, bone after 4 weeks of Al loading; (b) the extent of osteoid accumulation correlates with the bone Al content; and (c) the histologic response to Al in cortical and trabecular bone is related to local differences in the uptake of Al into bone. © 1985 Society for Experimental Biology and Medicine.

The accumulation of aluminum in bone has been associated with the development of osteomalacia in patients treated by hemodialysis (1–7). Aluminum deposition in the skeleton has also been documented in certain patients with normal renal function in whom clinical bone disease develops during the course of long-term, total parenteral nutrition (8). This latter group of individuals may also exhibit histologic osteomalacia. Despite such clinical observations, both the time course of the changes in bone histology and bone formation following exposure to aluminum and the various factors which contribute to the development of aluminum-associated osteomalacia have yet to be established.

Several investigators have presented data which indicate that rats develop osteomalacia in trabecular bone after receiving repeated parenteral injections of aluminum for 8 to 15 weeks (9–11). Renal insufficiency increases the severity of the histological changes of osteomalacia in aluminum-treated animals (9, 11). In contrast to these observations and as previously reported from this laboratory, osteomalacia was distinctly absent in the cortical bone of the tibia in rats given aluminum for 4 weeks by repeated intraperitoneal injection (12). This was true in intact as well as in subtotally nephrectomized animals (12). Subsequent confirmation of these histologic findings in cortical bone has been obtained in rats with normal renal function given repeated parenteral injections of aluminum for 6 weeks (13).

The current study was undertaken to evaluate the concurrent responses of cortical and trabecular bone to 4 weeks of aluminum administration in the rat. By studying both cortical and trabecular bone from the same animals, we sought to discern whether the conflicting results from previous studies of aluminum-induced osteomalacia in this species could be explained on the basis of differ-

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ences in either (a) the duration of study or (b) the skeletal site selected for histologic examination.

Methods. *Experimental protocol.* Forty weanling, male Holtzman rats were obtained at 21 days of age. One-half of the animals underwent a two-stage, subtotal nephrectomy before study. Two-thirds of the left kidney was removed after ligating both the upper and lower poles under pentobarbital anesthesia (20 mg/kg); this was followed 1 week later by a total right nephrectomy. After a 3-day recovery period, subtotally nephrectomized animals were randomly assigned to either a control (NX-C, $n = 10$) or an aluminum-treated (NX-AL, $n = 10$) group. Similarly, intact rats were assigned to a control (C, $n = 10$) or an aluminum-treated (AL, $n = 10$) group.

All animals were housed in individual metabolic cages, given free access to water, and maintained on standard laboratory rat chow (Ralston-Purina Co., St. Louis, Mo.) containing 0.6% calcium and 0.6% phosphorus for the duration of study (12, 13). To assure comparable weight gains among all four groups, animals within each group were ranked by weight at the beginning of the experimental period; rats of corresponding weight rank within each group were given an equal amount of food daily until termination at the conclusion of the study.

Rats in AL and NX-AL were given intraperitoneal (ip) injections of $AlCl_3$ in 0.9% saline vehicle for 5 days each week for a total of 4 weeks; animals in C and NX-C received ip injections of vehicle only. The daily dose of elemental aluminum (Al) was 2 mg/rat except during the first week of study. For the first 5 days of aluminum injections, incremental doses of 0.2, 0.4, 0.8, 1.2, and 1.6 mg/rat were given to avoid the development of chemical peritonitis. The cumulative dose of Al for rats in AL and NX-AL was 34.2 mg/rat. All rats were given ip injections of tetracycline (20 mg/kg) 14 days and 24 hr before termination on the final day of study (12–14). The daily injection of aluminum or vehicle was omitted on these 2 days in each of the four groups.

Twenty-four-hour urine collections were made for the measurement of creatinine clearances in one-half of the animals from each group 3 and 2 days, respectively, before termination (12). The animals were killed by cardiac puncture under anesthesia at the conclusion of the experiment. Serum was saved for subsequent determinations of the concentrations of calcium, phosphorus, 25-hydroxyvitamin D, 1,25-dihydroxyvitamin D, and immunoreactive parathyroid hormone (iPTH) by methods described previously (12, 15, 16). After terminating the animals, the left tibia was removed, stripped of adhering soft tissue, and placed in gauze saturated with 10% Formalin until sectioning (12, 13).

Bone measurements. Thirty-five micron sections of nondecalcified cortical bone were obtained from the tibial diaphysis at the fibular junction using a circular saw (Buehler Isomet, Buehler Ltd., Evanston, Ill.) as previously described (12, 13) and subsequently hand ground to a thickness of 10–15 μm. These sections were either stained with nuclear fast red, dehydrated in acetone, cleared in xylene, and mounted in ProTexx (Lerner Laboratories) for light microscopy (12–14), or stained for aluminum by the aurine tricarboxylic acid method (17).

Sections of undecalcified trabecular bone were obtained from plastic embedded specimens of the proximal tibial metaphysis. The proximal one-third of the tibia was infiltrated at 0°C for 6 days with a 15/85% mixture of glycol-methylmethacrylate (18). The infiltrating medium was changed twice, at 48 and 96 hr, and then polymerized at 0°C under 100% carbon dioxide. Five micron frontal sections of the embedded proximal tibia were obtained using a sledge microtome (Jung Autocut Model 1140, Reichert-Jung, Vienna, Austria) (19, 21). Light microscopy of trabecular bone was done on sections stained by the modified Goldner technique (20, 21); the histologic quantitation of aluminum was done on sections stained by the aurine tricarboxylic acid method (17).

All quantitative histological measurements were determined using a digitizer (Summagraphics Corp., Fairfield, Conn.) interfaced with a microcomputer (IBM PC, IBM Instruments, IBM Corp., Danbury, Conn.) and a series of measuring programs (12, 13, 21). The measurements of length, width, and area were determined directly from projected images for both cortical and trabecular bone as

previously described (12, 13, 21). The histologic quantitation of metaphyseal bone using this technique was done in an area measuring 1.8 × 0.6 mm within the secondary spongiosa where only fully calcified trabeculae were recognized.

Metaphyseal bone from the proximal tibia consists of both fully mineralized trabeculae and cores of cartilage undergoing calcification (19). Because of the complex structure of the proximal tibial metaphysis, surface and area measurements for quantitative histology of trabecular bone were also obtained using point-counting methods (22). Thus, a grid consisting of two sets of 13 parallel lines arranged at equal intervals and oriented perpendicular to one another was placed on the digitizer tablet. During image projection using a projection prism (243×), the grid occupied an area of measuring 0.62 × 0.62 mm, and the resolution of the line intercepts of the grid was 51 μm. Six contiguous grid areas covering a 1.8 × 1.2-mm region of the proximal tibial metaphysis were measured. The site used for histologic quantitation was located immediately adjacent to the primary spongiosa extending into the marrow space and was selected on the basis of work previously reported by Miller and Jee (19).

For the measurements of area, two points, separated by a distance of 5.1 μm, were evaluated at each of the 169 grid intercepts for the presence of mineralized bone, osteoid, or marrow space; a total of 2028 points per section were counted. Surface measurements were carried out by determining the type of bone surface, either osteoid or otherwise, at each point of intercept with the lines of the grid. A mean of 646 ± 60 points per section were evaluated for the quantitation of surface variables. The precision of the measurements using point counting methods, as estimated by the mean coefficient of variation in triplicate determinations from individual sections of bone from eight normal rats, were (in percentages) bone, 5.4; osteoid, 17.6; and osteoid surface, 31.2. The considerable variance in the values for the latter two variables in normal rats was due to the small percentages of area and surface represented by osteoid at this site in the tibial metaphysis. Both variables were determined with considerably greater precision when osteoid surface and osteoid area comprised a greater percentage of the trabecular bone sample (22) (see Results). There was good agreement between the histologic measurements done by direct tracing of projected images and by point-counting methods. The correlations between the values for osteoid area and osteoid surface obtained using these techniques were $r = 0.79$, $P < 0.005$, and $r = 0.87$, $P < 0.005$, respectively.

The histologic quantitation of aluminum at the tibial metaphysis was also done by point-counting methods and was expressed as the percentage of trabecular bone surface exhibiting a positive histochemical stain for aluminum (21).

Statistical analysis. All values are expressed as the means ± 1 standard deviation. Statistical analysis of the data was done using the *t* test for unpaired samples, analysis of variance, and linear regression analysis (23).

One aluminum-treated (AL), three nephrectomized control (NX-C), and two nephrectomized, aluminum-treated (NX-AL) rats died during the course of study. Preliminary biochemical determinations had also been done on bone obtained from the proximal tibia in two animals from each study group prior to histological examination. Thus, sections of metaphyseal bone were available for histologic quantitation in eight control, seven aluminum-treated, five nephrectomized control, and six nephrectomized aluminum-treated rats.

Results. The results for animal growth and the biochemical findings from each of the four study groups have been reported in detail previously (12). To summarize briefly, animal weights in C, AL, NX-C, and NX-AL were similar both at the beginning and at the conclusion of the experiment; thus, weight gains during the study did not differ among the four groups. There were no differences among groups in the serum levels of calcium, phosphorus, 25-hydroxyvitamin D, 1,25-dihydroxyvitamin D, or iPTH. Renal function was reduced in both groups of subtotally nephrectomized animals, but the values for creatinine clearance did not differ between C and AL (1.7 ± 0.5 vs 1.6 ± 0.6 ml/min) or between NX-C and NX-AL (0.7 ± 0.1 vs 0.7 ± 0.2 ml/min) (12).

At both the periosteal surface and the

endosteal surface in cortical bone, the width of the osteoid seam was similar in C, AL, NX-C, and NX-AL (Table I). Neither osteoid area nor the percentage of cortical bone occupied by osteoid differed among the four groups. The extent of the endosteal surface covered by osteoid was similar in C and AL, and was diminished in NX-AL when compared to NX-C (Table I). Thus, no evidence of osteoid accumulation was noted in cortical bone in either group of aluminum-treated animals.

In contrast to the histologic findings in cortical bone, the volume of osteoid in trabecular bone increased from control values in both groups of aluminum-treated animals. Total osteoid area was greater in AL than in C, but this change was primarily the result of an increase in the percentage of trabecular bone surface covered by osteoid (Table II). The width of osteoid seams in metaphyseal bone did not differ substantially between C and AL. However, osteoid area, percent osteoid surface, and osteoid seam width all increased in NX-AL from the values determined in NX-C (Table II). Of the four groups studied, the values for osteoid width, osteoid area, and percentage osteoid surface were greatest in nephrectomized, aluminum-treated rats. Each of these three histologic variables differed from its respective measurement in both NX-C and AL (Table II).

In neither group of saline-injected control rats was aluminum detected in trabecular bone by histochemical methods. However, all aluminum-treated animals had evidence of aluminum deposition at the tibial metaphysis (Figs. 1 and 2). The extent of aluminum deposition at the surface of trabecular bone was greatest in NX-AL (71.6 ± 6.5%) and exceeded the values in AL (40.5 ± 11.5%, $P < 0.01$). Moreover, the aluminum content of bone, expressed as the percentage of trabecular bone surface staining positive for aluminum, correlated with both percentage osteoid surface ($r = 0.74$, $P < 0.005$) and percentage osteoid area ($r = 0.68$, $P < 0.005$) when the data from all four groups were examined. To eliminate undue weighting of the correlation curve from the two control groups in which the individual values for bone aluminum content were invariably 0, both control groups were excluded, and only the data from aluminum-treated animals were considered. Similar relationships among these three variables were again evident. Thus, both osteoid area ($r = 0.69$, $P < 0.01$) and percentage osteoid surface ($r = 0.68$, $P < 0.05$) correlated with the bone aluminum content at the tibial metaphysis in animals given repeated injections of aluminum.

No evidence of aluminum deposition was noted in sections of cortical bone from either C or NX-C, and only occasional sites suggestive of aluminum deposition were noted in cortical bone in AL and NX-AL. When present, the intensity of the histochemical stain for aluminum was minimal when compared to that in trabecular bone. Given the thickness of the sections of cortical bone examined and concerns about the possibility of artifact, histologic quantitation of the aluminum content of cortical bone was not done.

The 14-day labeling interval failed to achieve double tetracycline labeling of bone

TABLE I. QUANTITATIVE HISTOLOGY OF CORTICAL BONE IN CONTROL AND ALUMINUM-TREATED RATS

	C ($n = 10$)	AL ($n = 9$)	NX-C ($n = 7$)	NX-AL ($n = 8$)
Osteoid seam width (μm)[a]				
Periosteal	5.5 ± 0.7	5.3 ± 0.5	4.7 ± 1.0	4.9 ± 0.6
Endosteal	3.8 ± 0.7	4.0 ± 0.8	3.6 ± 1.0	3.3 ± 0.7
Osteoid area (mm^2)	0.052 ± 0.008	0.050 ± 0.004	0.046 ± 0.011	0.045 ± 0.006
Percentage osteoid (% of tissue area)	1.74 ± 0.25	1.74 ± 0.16	1.48 ± 0.30	1.61 ± 0.37
Osteoid surface (% of endosteal surface)	66.5 ± 8.8	60.7 ± 8.7	71.4 ± 11.3	57.2 ± 13.2*

Note. Values are the means ± standard deviation.

[a] Presented with the permission of the Journal of Clinical Investigation.

* $P < 0.05$ vs NX-C.

TABLE II. QUANTITATIVE HISTOLOGY OF TRABECULAR BONE IN CONTROL AND ALUMINUM-TREATED RATS

	C (n = 8)	AL (n = 7)	NX-C (n = 5)	NX-AL (n = 6)
Osteoid seam width (μm)	7.9 ± 2.3	10.7 ± 3.5	9.7 ± 2.3	18.7 ± 5.7**
Osteoid area ($mm^2 \times 10^{-3}$)	4.56 ± 1.14	8.48 ± 3.49*	15.80 ± 3.61***	64.44 ± 27.83**
Percentage osteoid (% of tissue area)	1.0 ± 0.4	1.7 ± 0.7*	3.5 ± 0.8***	13.2 ± 5.7**
Osteoid surface (% of trabecular surface)	1.8 ± 1.1	5.2 ± 3.4*	6.2 ± 2.4***	20.2 ± 6.7**

Note. Values are the means ± standard deviation.
* $P < 0.05$ vs C.
** $P < 0.01$ vs NX-C.
*** $P < 0.005$ vs C.

at the tibial metaphysis, most probably due to the high rate of bone turnover at this skeletal site. Thus, direct comparisons between cortical and trabecular bone for the rates of bone formation and bone apposition, and for the mineralization lag time, could not be done.

Discussion. The results of the current investigation indicate that osteoid accumulates in trabecular bone in pair-fed rats given repeated intraperitoneal injections of aluminum for 4 weeks. As noted by other investigators (9, 11), renal insufficiency aggravates the severity of osteoid accumulation in the

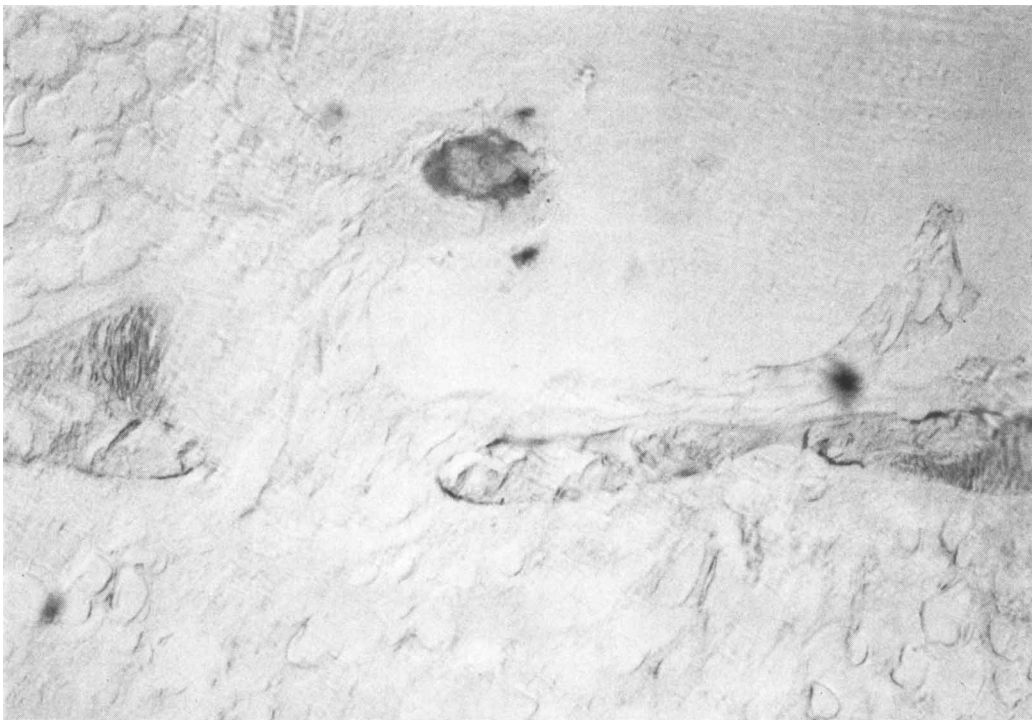

FIG. 1. A photomicrograph of a section of metaphyseal bone from an intact, aluminum-treated rat stained for aluminum by the aurine tricarboxylic acid method. The areas of aluminum deposition are indicated by the dark bands and are localized predominantly at the surface of trabecular bone. (Original magnification 250×.)

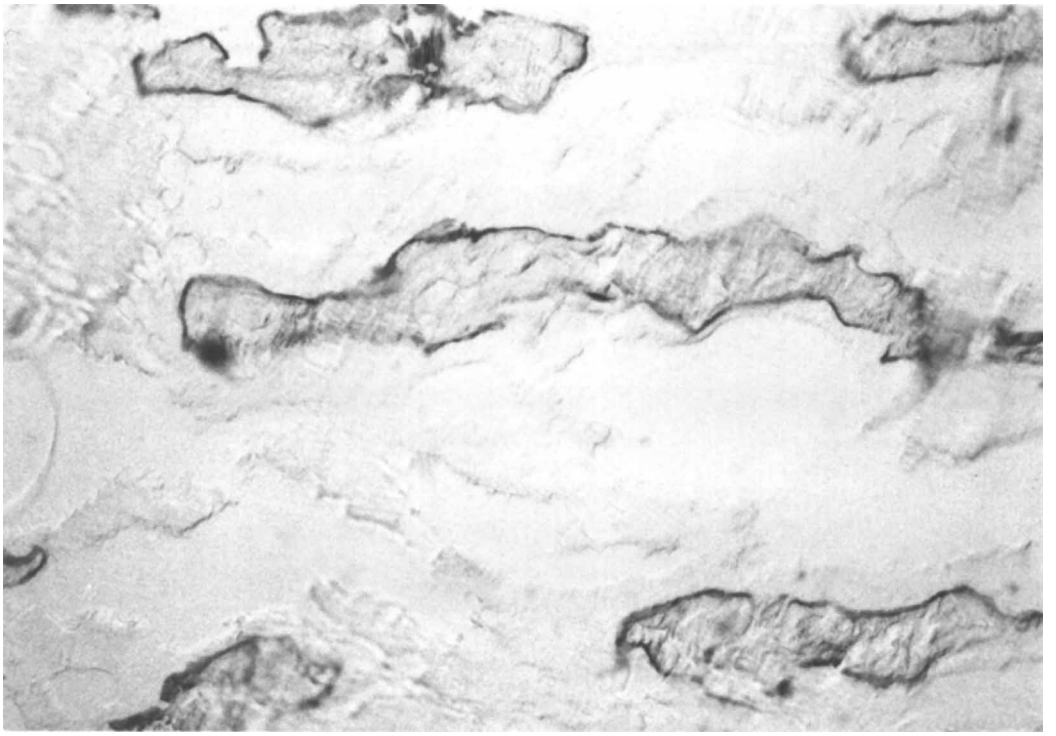

FIG. 2. The histochemical stain for aluminum in a section of metaphyseal bone from an aluminum-treated, nephrectomized rat. The method of preparation and photographic conditions are the same as for Fig. 1. (Original magnification 250×.)

proximal tibial metaphysis during aluminum administration. The degree of osteoid accumulation in the tibial metaphysis correlates with the extent of aluminum deposition at the surface of bone, and this observation is consistent with previous reports of aluminum-associated osteomalacia in both humans (3, 8) and the dog (21). In contrast to such findings in metaphyseal bone, neither osteoid accumulation nor deposits of aluminum were apparent in cortical bone in animals given aluminum. Thus, the histologic response to aluminum administration differs substantially as a function of the type of skeletal tissue examined in short-term experiments such as herein reported. These findings suggest that trabecular bone is more susceptible than cortical bone to the toxic effects of aluminum. This differential response between cortical and trabecular bone appears to be independent of factors such as the serum levels of calcium, phosphorus, iPTH, or metabolites of vitamin D.

Because of the absence of supporting tetracycline-based histomorphometric data, the increments in metaphyseal osteoid volume and osteoid seam width in aluminum-treated rats provide only indirect evidence of an osteomalacic lesion. Nevertheless, the histologic findings reported are consistent with previous histomorphometric observations following parenteral aluminum loading in the rat and in the dog (9–11, 21).

No evidence of osteoid accumulation was documented in the cortical bone of animals in the current 4-week investigation. This finding differs from previously published results which indicate that histologic osteomalacia does develop in cortical bone when rats are given aluminum parenterally for 9 weeks or longer (9, 10). However, as reported elsewhere and in agreement with the findings of the current investigation, cortical bone formation is reduced from control values in pair-fed rats given repeated injections of aluminum for 6 weeks without concomitant

increments in the volume of osteoid in cortical bone (13). The results of these short-term and long-term studies suggest that the initial response to aluminum administration in rat cortical bone is a reduction in bone formation which, with prolonged exposure to aluminum, may evolve into histologic osteomalacia.

The data presented indicate that the deposition of aluminum in trabecular bone is substantially greater than that in cortical bone after 4 weeks of aluminum loading. This observation, using histochemical methods, not only agrees with but also confirms previous findings using quantitative chemical analyses that demonstrated a higher aluminum content in trabecular bone than in cortical bone following sustained aluminum exposure in man and in the rat (9, 24). The correlation between the extent of osteoid accumulation and the aluminum content of bone also suggests that the localized deposition of aluminum at the surface of trabecular bone may be a critical factor that accounts for this histological change. The current data are consistent with evidence from clinical and experimental investigations and indicate that the histologic severity of osteomalacia corresponds to the degree of aluminum deposition in bone (2, 3, 25).

The differential response between cortical and trabecular bone during aluminum loading argues against a role for the concentration of aluminum in serum as a modifier of the histological changes in bone. Similarly, differences among groups in the plasma levels of calcium, phosphorus, iPTH, 25-hydroxyvitamin D, or 1,25-dihydroxyvitamin D were not appreciated. Such findings fail to support, but do not entirely exclude, alterations in the plasma levels of these systemic factors in the pathogenesis of aluminum-associated bone disease (11, 25, 26). The results of the current investigation are consistent with the hypothesis that the toxic effects of aluminum on bone are related to the localized deposition of aluminum in skeletal tissue. These changes predominate in trabecular bone.

The current findings indicate that the deposition of aluminum in trabecular bone is associated with osteoid accumulation at a time when only reductions in bone formation without increases in osteoid volume can be documented in cortical bone. It is possible, therefore, that aluminum has an early and direct inhibitory effect on the mineralization of osteoid in trabecular bone. Previous studies from this laboratory fail to support a primary role for aluminum as an inhibitor of mineralization. These data suggest that aluminum adversely affects either the function of osteoblasts (12, 13) or, as demonstrated by others (9), decreases the number of osteoblasts and, thus, reduces the rate of synthesis of osteoid. Such observations are based, however, upon *in vivo* studies of cortical, not trabecular, bone. Preliminary *in vitro* data do suggest that aluminum may interfere with the mineralization of newly formed bone matrix (27). Whether this effect on matrix calcification is a direct one or is mediated via cellular mechanisms remains to be determined. In support of the first possibility are the findings of Blumenthal and Posner which indicate that the formation and growth of hydroxyapatite crystals in a cell-free system *in vitro* are inhibited by aluminum (28). Additional work will be required to evaluate the chronology of the dynamic as well as the histologic response of trabecular bone to aluminum loading.

In summary, trabecular bone is more suseptible than cortical bone to the development of osteomalacia following exposure to aluminum. This differential response appears to be related to the greater tissue uptake of aluminum into trabecular, rather than cortical, sites in the skeleton. Osteoid accumulates in rat trabecular bone following aluminum administration in the absence of alterations in several systemic factors known to mediate the formation and mineralization of bone. The experimental findings presented support the concept that aluminum-associated osteomalacia represents the skeletal response to a localized accumulation of aluminum in bone.

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