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Reversible Inhibition of Osteoclastic Activity by Bone-Bound Gallium (III)

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Abstract Gallium(III) is a new therapeutic agent for hypercalcemia. Ga^{3+} reduces osteoclast action, but how it inhibits the cell's physiology is unknown. In vivo, 7–12 μM Ga(III) reduces calcium release from bone, but surprisingly, 10–100 μM Ga^{3+} added to isolated avian osteoclasts did not reduce their degradation of L-(5-^{3}H)-proline bone. ^{3}H-proline labels bone collagen specifically, and collagenolysis is an excellent indicator of bone dissolution because collagen is the least soluble component of bone. Ga(III) > 100 μM inhibited osteoclasts in vitro, but also killed the cells. To resolve this apparent conflict, we measured ^{67}Ga distribution between bone, cells, and media. Gallium binds avidly but slowly to bone fragments. One hundred micrograms of bone clears 60% of 1 μM gallium from 500 μl of tissue culture medium, with steady state at > 24 h. Osteoclasts on bone inhibited gallium binding capacity ~40%, indicating a difference in available binding area and suggesting that osteoclasts protect their substrate from Ga binding. Less gallium binds to bone in serum-containing medium than in phosphate-buffered saline; 30% reduction of the affinity constant suggests that the serum containing medium competes with bone binding. Consequently, the effect of [Ga] on bone degradation was studied using accurately controlled amounts of Ga(III) pre-bound to the bone. Under these conditions, gallium sensitivity of osteoclasts is striking. At 2 days, 100 μg of bone pre-incubated with 1 ml of 1 μM Ga^{3+}, with 10 pmoles Ga^{3+}/μg bone, was degraded at 50% the rate of control bone; over 50 pM Ga^{3+}/μg bone, resorption was essentially zero. In contrast, pre-treatment of bone with [Ga^{3+}] as high as 15 μM had no significant effect on bone resorption rate beyond 3 days, indicating that gallium below ~150 pg/μg bone acts for a limited time and does not permanently damage the cells. We conclude that bone-bound Ga(III) from medium concentrations <15 μM inhibits osteoclasts reversibly, while irreversible toxicity occurs at solution [Ga^{3+}] > 50 μM.

Key words: bone resorption, osteoclast, gallium, hypercalcemia, osteoporosis

The skeleton is both the principal supporting organ and the repository of calcium in the air-breathing vertebrates. Hence, bone is produced by osteoblasts and degraded by osteoclasts throughout life to maintain mechanical strength and to regulate serum calcium activity. However, when bone resorption exceeds formation over a prolonged period, osteoporosis and consequent mechanical failure may result. Moreover, rapid uncontrolled bone resorption causes life-threatening hypercalcemia (Muggia, 1990). Medical science has identified factors contributing to these problems, such as reduced estrogen levels in women and production of hypercalcemic factors, like the PTH-like peptide, by tumors (Strewler and Nissenson, 1990). However, general means to control excess bone resorption have proved elusive.

Gallium is a group IIIa (aluminum period) metal that has shown promise as a therapeutic agent for hypercalcemia of malignancy (Warrell et al., 1984). The detailed effects of Ga(III) on cellular physiology are unknown. Solutions at 0.01% w/v (100 μg/ml) have been shown to inhibit osteoclastic bone pitting ~50% during 6 h incubation in vitro (Hall and Chambers, 1990). In contrast, however, gallium nitrate inhibits tumor growth, bone resorption with Paget's disease, and tumor invasion of the skeleton at concentrations 30–50 times lower; effective steady state $Ga(NO_3)_3$ serum levels are 1.9–3 μg/ml (7–12 μM Ga^{3+}) at the clinical doses used (Foster et al., 1986; Matrovic et al., 1990). Clinical studies have also demonstrated the effectiveness of $Ga(NO_3)_3$ in reducing hypercalcemia of

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malignancy in similar dose ranges (Warrell et al., 1984). Gallium nitrate doses higher than ~50 μg/g body weight are fatal (at substantially lower measured serum concentrations) (Foster et al., 1986).

We previously determined that another class of anti-osteoclastic drugs, bisphosphonates, targets to osteoclasts at low micromolar levels via hydroxyapatite binding (Carano et al., 1990). We hypothesized that Ga^{3+}, which accumulates in the skeleton but is chemically dissimilar to bisphosphonates (Bockman et al., 1990), also targets to osteoclasts via local concentration on bone. However, since these chemicals are otherwise disparate, they may affect osteoclast metabolism differently, raising the possibility of synergistic or alternative means for controlling bone degradation and minimizing toxicity.

We sought to resolve the paradoxical finding of osteoclast resistance to Ga(III) at clinically useful doses via studies of the kinetics, distribution, and cellular effects of this drug over several days in vitro. In the present study, we use $^{67}Ga^{3+}$ to measure the concentration and time dependence of gallium binding to bone, and we study the effects of bone bound and soluble gallium (III) on osteoclast activity and viability as a function of time in culture.

METHODS

Cell Cultures

Osteoclasts were isolated from medullary bone of calcium-starved laying hens, *Gallus domesticus*. Briefly, the birds are placed on a diet without calcium carbonate and dicalcium phosphate (Purina 5070-9); since the birds move grams/day of Ca to make their $CaCO_3$ eggshells, large numbers of osteoclasts are produced in the medullary bone to provide the Ca. These cells, as much as 50% of the cellular mass in the medullary bone, can then be easily isolated by washing the medullary bone through a 100 μm nylon filter; the large and dense osteoclasts are further purified and collected by sedimenting the cell population through 70% serum, which raises the osteoclast content to ~80%. To provide osteoclasts at still higher purity, ~95–98%, they are allowed to attach to 25–50 μm bone fragments, and the bone with essentially only osteoclasts attached separated by sedimentation after 18–36 h of incubation in MEM at 37°C in 5% CO_2. Cells were then plated in 2 cm^2 wells in Eagle's minimal essential medium, α-modification, containing 10% fetal calf serum, 100 μg/ml streptomycin, 100 units/ml penicillin, and 5 μg/ml cytosine-β-D-arabinofuranoside (α10-MEM) as described (Blair, et al., 1986). Cultures were maintained at 37°C in humidified air with 5% CO_2.

Resorption Assays

Bone-affinity purified osteoclast cultures with 10^4 cells in 1 ml of medium in 2 cm^2 wells of 24-well tissue culture plates were used. To determine bone resorption, 100 μg of 25–50 μm fragments of devitalized rat bone metabolically labeled with L-[5-^{3}H]-proline was added (Teitelbaum, Stewart, and Kahn, 1979). Bone resorption, relative to time in culture, is thus indicated directly by ^3H recovered in supernatants of 10^4 osteoclasts. Activity of cell-free controls was subtracted, and substrate specific activity was then used to convert counts to μg bone resorbed. Substrate specific activity was determined by total hydrolysis (6 N HCl, 24 h at 110°C) and scintillation counting. Unless specified, bone particles were suspended in PBS and added to the osteoclast cultures without any treatment. All experiments included no-cell controls with identically treated substrates.

Pre-binding was generally performed in PBS with gentle agitation for 48 h at 37°C. Bone (as 25–50 μm fragments) was suspended at 100 μg per ml PBS, with the indicated concentrations of gallium (as $Ga(NO_3)_3$), followed by washing and resuspension in tissue culture medium (α10-MEM). When expressed as bound gallium/μg bone, binding is calculated assuming, for convenience, complete binding under these conditions, except at 0.1 mM Ga^{3+}, where binding is taken from Figure 2C. These differences are much less than the variation of cellular measurments using the bone-bound Ga, and therefore regarded as insignificant for our present purposes: cf Figure 2C, 3, and 4. Pre-binding using tissue-culture medium and PBS gave similar results in preliminary osteoclast inhibition studies. However, steady-state binding was slower in α10-MEM and varied from 20% to 70% efficiency, with standard deviations ~3 times those for PBS measurements in ^{67}Ga binding studies (described below). Thus, accuracy of gallium exposure was improved with PBS as the pre-binding medium. Bone powder for resorption assays was ^3H labeled and pre-bound with unlabeled Ga, whereas bone to be used in measuring Ga distribution was unlabeled (but labelled gallium was of course used for these experiments).

Quantifying Gallium Distribution and Bone Association Constants Using ^{67}Ga

One hundred micrograms of 25–50 μm fragments of devitalized rat bone in 500 μl of PBS, or in α10-MEM (α-MEM with 10% FCS, pH 7.40, described above) with and without 10^4 bone attached osteoclasts, were incubated with indicated concentrations of gallium (as $Ga(NO_3)_3$ with 10^{-12} M ^{67}Ga as a tracer). Incubation was at 37°C in sealed tubes (to prevent alkalinization due to loss of CO_2) and assay tubes were centrifuged (10,000*g*, 1 min) to separate bone and supernatant at indicated times. Bone (pellet) and medium ^{67}Ga were then determined by γ-counting. As this isotope has an ~3 day half-life, all samples were counted at the same time (±1 h) to control for decay.

With these data, surface association constants were determined by applying Langmuir's adsorption equation: $\theta_1 = \alpha\mu/(\nu_1 + \alpha\mu)$ (Langmuir, 1917). For our purposes, θ_1 (binding saturation) is Ga_{bone}/Ga_{max}; μ (concentration) is Ga_{free}; and ν_1 (off rate at saturation; the dissociation constant) is the inverse of $K_{association}$. Gallium activities (α) at the concentrations used, all $<10^{-4}$ M, are assumed to approach unity, making this term unnecessary. Hence, multiplying the right side of Langmuir's adsorption equation by ν_1^{-1}/ν_1^{-1}, making the indicated substitutions, and multiplying through by Ga_{max}, we obtain:

$$Ga_{bone} = \frac{Ga_{max} \cdot Ga_{free} \cdot K_{association}}{(Ga_{free} \cdot K_{association} + 1).}$$

$K_{association}$ values are calculated from a least squares fit of our data (n = 9 for PBS and n = 12 in α10-MEM) to this modified Langmuir adsorption equation (Fig. 3B). Ga_{max} values were calculated for each concentration tested using the $K_{association}$ values from the least squares fit, and are reported as mean ± SD. Ga_{max} and association constants are given as moles Ga/g bone and M^{-1}, respectively; g bone represents the surface of one gram of bone.

Cell Quantification and Protein Synthesis

Viability of cells was measured in parallel with resorption by counting cells excluding trypan blue. Protein synthesis by cultured cells was assessed using [^{3}H]-leucine incorporation (Blair et al., 1989): Cells, incubated in media including 1 μCi/ml of ^{3}H-leucine for 6 h, were washed twice with PBS, and isotope incorporated into macromolecules was fixed (30 min, 10% trichloroacetic acid and 30 min, 5% trichloroacetic acid). Lipids were removed with ethanol/ether (3:1, v/v) to reduce background, and protein solubilized by digestion in 0.1 M NaOH for scintillation counting.

Statistics

Error ranges are standard deviations, except in Fig. 3B, where errors indicate residuals after least squares fitting. Quadruplicate measurements were performed unless noted. Where ^{67}Ga bone-binding was less than no-bone control due to statistical variation, bound Ga is reported as zero and free Ga as 1.0. Where a difference is concluded, comparisons reject the null hypothesis, at 0.05, by Student's t-test.

RESULTS

Gallium Inhibition of Bone Resorption by Osteoclasts

Addition of gallium to osteoclasts cultured on 20–50 μm bone particles produced no inhibition until $Ga^{3+}_{(Total)}$ was 1 mM (Fig. 1). Furthermore, after 48 h at 1 mM Ga^{3+} the osteoclasts had detached from their substrate, did not exclude trypan blue, and were dead, signifying that the mechanism of this inhibition was cell death. This was inconsistent with clinical studies and studies in vivo, which indicated that much lower concentrations of gallium should effectively inhibit bone resorption and that inhibition at low [Ga^{3+}] does not reduce osteoclast number (Foster et al., 1986; Matrovic et al., 1990; Warrell et al., 1984). Previously we reported that binding to bone is critical to the effectiveness of bisphosphonates on osteoclasts (Carano et al., 1990).

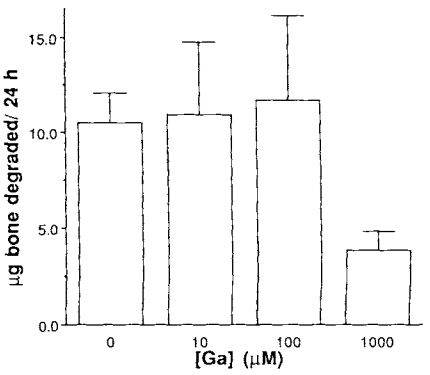

Fig. 1. Effect of gallium on bone resorption by 10^4 osteoclasts in vitro. Bone resorption was measured at 0–48 h of incubation using 100 μg of 25–50 μm fragments of ^{3}H-proline labeled rat bone (Methods). Gallium, as $Ga(NO_3)_3$, was added at time zero.

We reasoned that if gallium was accumulated by bone this would effectively increase its local concentration and inhibit osteoclastic bone resorption without requiring toxic levels of Ga^{3+} in solution. Such a bone matrix accumulation could be quite slow, as the in vivo studies were performed over days to weeks. It was also unknown whether or not osteoclasts would affect Ga^{3+} binding at their matrix attachment, an important corollary measurement if the bone-concentration hypothesis were confirmed. Thus we studied distribution of gallium in media, bone, and cells incubated several days of in vitro.

^{67}Gallium(III) Interaction With Bone Particles

We found that ^{67}Ga(III) binding to bone fragments is avid but slow, with 20–64 h required to reach steady state at initial total ^{67}Ga(III) concentrations of 100 to 0.001 μM (Fig. 2). Significant uptake at 1 nm shows the avidity of bone for the metal. However, at the highest initial concentration, 100 μM, gallium binding by bone reduced medium [Ga] only ~10%, suggesting that binding saturation is significant at micromolar concentrations (see below). Binding to bone particles was qualitatively similar in medium with fetal calf serum added (Fig. 2A) in saline (Figure 2C), and to bone particles with attached osteoclasts (Fig. 2B). In the absence of bone, osteoclasts did not alter ^{67}Ga(III) distribution (not illustrated).

Quantitative analysis, on the other hand, reveals significant concentration and medium dependent differences in gallium-bone binding (Fig. 3). Bone binding, in PBS and α10-MEM with and without cells present, is compared as a function of time, at 1 and 100 μM initial added gallium, in Figure 3A. Uptake was faster in PBS than in serum containing medium. Gallium binding was reduced ~40% in 10% fetal calf serum as compared with saline solution; a further ~50% reduction in ^{67}Ga(III) binding was consistently observed when 10^4 osteoclasts were added to the bone particles 24 h before the addition of gallium. Note that 24-h incubation with osteoclasts has the effect of allowing cells to bind to the bone, covering much of its surface and mea-

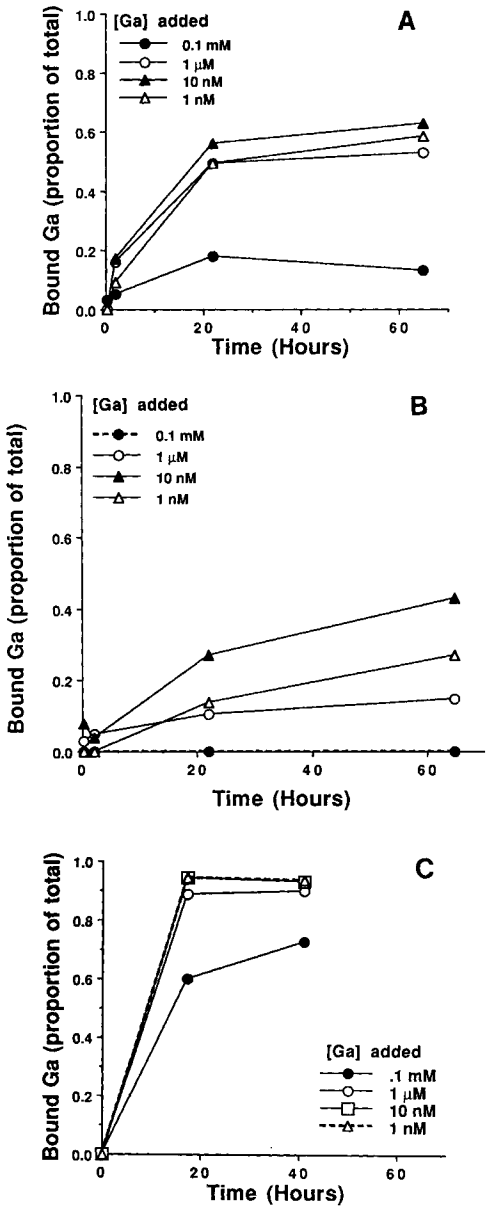

Figure 2.

Fig. 2. ^{67}Ga bound to bone or osteoclast-coated bone from tissue culture medium, or to bone from PBS, as a function of [Ga], 10^{-9}—10^{-4} M, and time. pH of all media was 7.40, and all incubations were at 37.0°C. Standard deviations, not shown for clarity, average 5% of each value (direct comparison of binding in different media is shown, with standard deviations, in Fig. 3A). **A:** Bound fraction of gallium as a function of time, 0–64 h (free fraction is 1 minus the bound fraction). Tissue culture medium with 10% serum (α10-MEM), 500 μl, pH 7.40, was incubated with the indicated concentration of gallium (III) and 10^{-12} M ^{67}Ga in sealed tubes at 37°C for the indicated times with 100 μg of unlabeled 25–50 μm rat bone. The fraction of bound Ga was then determined by γ-counting after separation of bone from medium by centrifugation. N = 3. **B:** Bound fraction of Ga as a function of time in α10-MEM, as in (A) above, but using bone aliquots pre-incubated 48 h with 10^4 osteoclasts. Note that the fraction bound (difference from time zero) is ~50% of that resulting when this value was determined in the absence of cells. N = 3. **C:** Bound fraction of Ga as a function of time, as in (A) above, but with the experiment performed in room air in PBS. Note that the fraction bound is higher than in the tissue culture medium. N = 2.

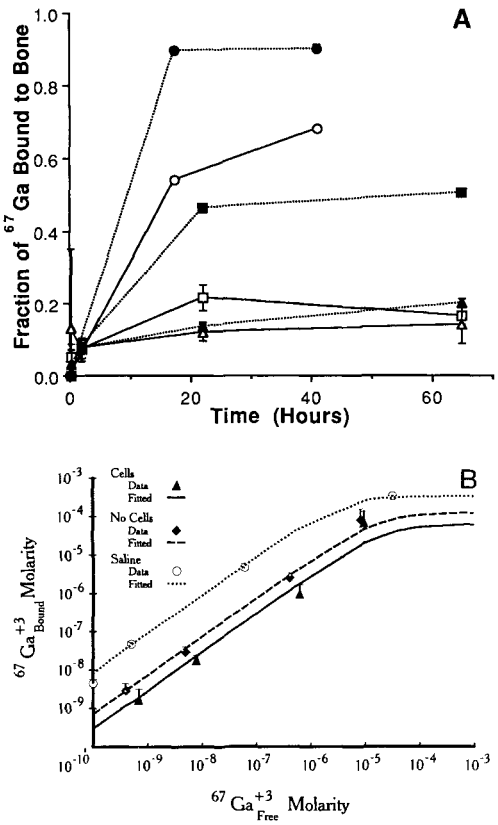

Fig. 3. Comparison of Ga binding time course, and relative fractions bound at steady state, in PBS and serum-containing tissue culture medium (α10-MEM) with and without osteoclasts. **A:** Comparison of fraction of 100 μM (open symbols and solid lines) and 1 μM Ga (closed symbols and dotted lines) bound to bone as a function of time. Gallium binding to osteoclast-coated (triangles) or unmodified bone (squares) was studied in α10-MEM, and Ga binding to unmodified bone (circles) was also studied in PBS to show by comparison the effect of the serum containing medium. N = 3, mean ± SD, except for PBS (N = 2). Where error is not shown, SD is less than symbol size. **B:** Relative binding, at steady state, to bone at 10^{-9} to 10^{-4} M initial [Ga]. Binding was assayed and fitted to Langmuir's adsorption equation as described in Methods. Free gallium (i.e., that remaining in solution at steady state, rather than added Ga) is expressed as Log(M); Ga bound is expressed as Log(moles per g bone). The purpose of the log-log scale is to allow comparison over widely differing concentrations. Note that values at $\sim 10^{-4}$ M deviate significantly from linearity, an indication of saturation (except with cells on bone, where Ga_{max} may vary with Ga_{free}, see text).

surably degrading the matrix, while not reducing the amount of bone by more than ~10% (Blair et al., 1986). Thus, the differences in bound Ga^{3+} due to osteoclast attachment are far too large (~50%) to be attributed simply to reduction of available matrix by degradation. This conclusion is strongly confirmed by similar saturation binding of naked and cell bound bone at very high [Ga^{3+}] (Fig. 3B at 10^{-4} M [Ga], discussed below).

Steady-state differences of gallium binding to bone under the various conditions used are expressed as inverse log ratios of bound and free gallium in Figure 3B. Langmuir association constants and gallium adsorption maxima were calculated from these data (Methods and Fig. 3B). Bone-Ga(III) $K_{association}$ in PBS was 79 ± 3 × 10^3 M^{-1}, but decreased ~25% in the presence of serum (58 ± 10 × 10^3 M^{-1}) and decreased a further 40% when bone with cells attached was used (34 ± 4 × 10^3 M^{-1}). Binding capacity (Ga_{max}) was 8.0 ± 2.6 × 10^{-4} moles/g bone in PBS and decreased in the presence of α10-MEM and α10-MEM + cells to 1.09 ± 0.16 × 10^{-4} moles/g bone and 0.65 ± 0.2 × 10^{-4} moles/g bone, respectively (excluding the cell-bone measurement at 10^{-4} M added Ga, which gave 1.01 ± 0.1 × 10^{-4} moles/g bone, n = 3; see Discussion).

Additionally, under all conditions and concentrations tested, a minimum 10% of the ^{67}Ga is bound from 1 ml of medium onto 100 μg of bone particles (volume < 1 μl), indicating over 100-fold concentration of gallium in the bone fraction at any medium concentration compatible with life (see Discussion). Effective concentration (activity) at the actual bone surface is not directly comparable to solution concentration, but is undoubtedly much higher still: It is established that the interior of mineralized bone matrix is impervious to even such small cations as H^+ (Neuman and Neuman, 1958) and Ga binding to bone is consistent with essentially ideal Langmuir surface adsorption kinetics (Methods and Fig. 3B).

Effect of Bone Associated Ga^{3+}

These results also suggested that osteoclasts would be affected differently by bone pre-incubated with gallium than when gallium was added concurrently with osteoclast exposure to bone. Therefore, we repeated the initial study, but with bone pre-incubated 48 h at 37°C in PBS containing various concentrations of gallium. This substrate was washed prior to osteoclast addition to insure that the experiment would reflect only the effect of bone-bound Ga^{3+}. We found that, under these conditions, gallium was a potent inhibitor of osteoclastic activity. Figure 4 shows inhibition of osteoclastic bone degradation as a function of substrate preincubation [Ga] and time. Half-maximal inhibition was seen

at ~1 μM (yielding 10 pmoles Ga^{3+} per μg of bone, Fig. 3), more than 100 times less than the dose having an effect when added to supernatants (Fig. 1). In various experiments, half maximal inhibition at 48 h incubation was seen following a Ga^{3+}-bone preincubation at 1–3 μM.

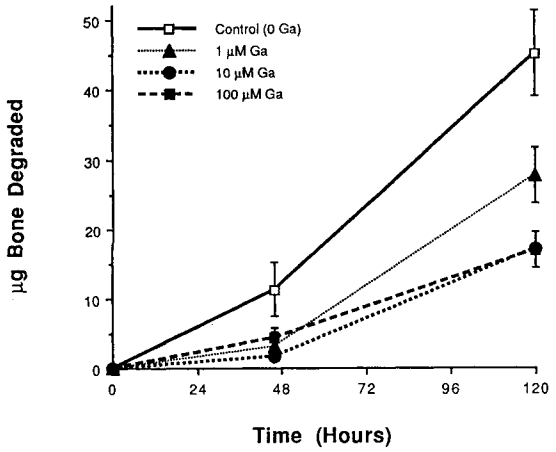

Fig. 4. Time course of bone degradation by 10^4 osteoclasts in α10-MEM, as in Figure 1, when bone had been pre-incubated 48 h at 37°C in PBS in indicated concentrations of gallium nitrate. At 46 and 120 h, supernatants were counted for ^3H activity to determine total bone degradation. Bone-bound gallium was 10 pmoles/μg bone at 1 μM Ga^{3+}, 100 pmoles/μg bone at 10 μM Ga^{3+}, and ~300 pmoles/μg bone at 100 μM Ga^{3+} (Fig. 2C).

Time Dependence of Gallium Inhibition of Bone Resorption

It remained unclear, after the distribution of gallium was studied, why, in the first experiment (Fig. 1), no inhibition was seen at ~10 μM, since by ~48 h even this osteoclast-coated bone should have ~30% as much Ga^{3+} bound as naked bone in PBS (Fig. 2B,C). We had noted, however, that the inhibition of bone resorption by bone associated gallium tended to be less pronounced after longer times in culture (120 h in Fig. 4, for example), suggesting that the effect of bound gallium might decrease with time in culture. Hence, in an attempt to further reconcile resistance to Ga^{3+} in solution and sensitivity to pre-bound Ga^{3+}, we measured the time course of effect of gallium bound to bone. We pretreated bone with 0 to 50 μM Ga(III) (i.e., the only gallium added in these experiments was bone-bound) and determined its osteoclast degradation rate during two time periods (0–72 and 72–135 h). As expected, at 72 h [Ga] over 1.5 mM significantly reduced bone resorption (Fig. 5A). However, at 72–136 h, only bone pretreated with >15 mM Ga^{3+} inhibited bone degradation (Fig. 5B).

Thus, substrate-bound gallium loses its effectiveness after ~48 h (Fig. 4) and is essentially neutralized by 72 h (Fig. 5) under the conditions

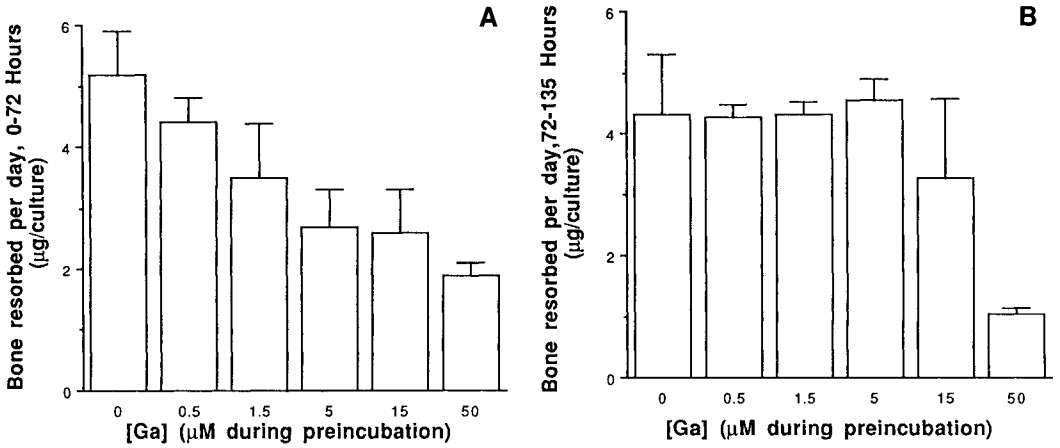

Fig. 5. Rate of bone resorption is inhibited by substrate preincubation in micromolar Ga^{3+} at 0–72 h, but not at 72–135 h of incubation. Bone-bound gallium at time zero, in picomoles Ga^{3+} per μg bone, is 10 times the solution Ga^{3+} concentration, in μM, in which the binding occurred, except at 50 μM Ga^{3+} where saturation would limit bound gallium to <300 pmoles/μg bone (Methods and Fig. 2C). For this experiment, N = 3. **A:** Dose response of bone resorption rate per day when measured from 0–72 h in α10-MEM (as in Fig. 1, but with preincubation of substrate in gallium at the indicated concentrations). Half-maximal inhibition is seen with bone preincubation between 1.5 and 5 μM Ga^{3+}. Note that inhibition is less complete than that measured at 46 h (Fig. 3). This finding is reproducible and indicates that some loss of effectiveness is already likely at 3 days. **B:** Bone resorption rates with substrate pre-incubated in 0–50 μM gallium, as in (A), but measured at 72–135 h of incubation. Only 50 μM gallium gives a significant difference at this time point.

studied (which included a very high osteoclast density and 10% serum, see Discussion). This, and the slow rate at which substrate binding occurs (Fig. 2), would account for the differences of Figure 1 and Figure 4. Put another way, a single dose of Ga appears to lose potency at roughly the same rate that binding occurs. This, incidentally, also suggests that periodic small doses of Ga^{3+} would be advisable for study of gallium effects where pre-binding would be impractical, such as in long-term in vitro incubation.

Effect of Ga(III) on Osteoclast Viability

Dissipation of the gallium effect with time showed that the drug, at least at the lower concentrations, bound to bone and inhibited but did not kill the cells. Indeed, no effect on trypan-blue exclusion was seen at [Ga^{3+}] below 100 μM. This was confirmed by measurment of ^{3}H-leucine uptake by osteoclasts as a function of [Ga^{3+}], which showed no significant effect of the metal on osteoclastic protein synthesis, even at concentrations dramatically depressing bone resorption (Fig. 6).

DISCUSSION

Gallium (III) has been evaluated as an anti-neoplastic drug (Foster et al., 1986). In this setting, however, anti-hypercalcemic properties and effects on bone destruction were prominent (Warrell et al., 1984; Matrovic et al., 1990). Consequently, gallium was proposed as an anti-osteoclastic agent, and indeed Hall and Chambers (1990) found that pit formation by grazing

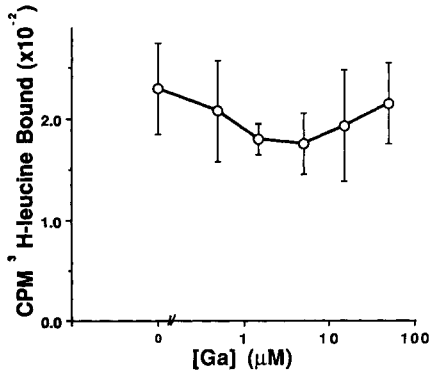

Fig. 6. Six hour ^{3}H-leucine uptake by osteoclasts in α10-MEM after 72 h incubation with bone pre-incubated in 0–50 μM Ga^{3+}. The differences are not significant. This experiment gives variable results at 0.1 mM Ga^{3+}, and does not work at 1 mM gallium, because the cells detach under these conditions (see text).

osteoclasts could be reduced ~50% by 100 μg/ml $Ga(NO_3)_3$ (391 μM Ga^{3+}). However, it is unlikely that this observation represents replication of the clinical activity of gallium in vitro, because the concentrations of $Ga(NO_3)_3$ required to partially inhibit bone resorption would be uniformly fatal in vivo. Indeed, our observations would suggest that, had Hall and Chambers's study been carried to longer time points, [Ga^{3+}] > 100 μM would probably have produced morphological differences between groups (Fig. 1).

Typical serum concentrations for in vivo trials have been 1.9–3 μg/ml (7–10 μM), or ~2% of those used in the cited in vitro study. Hall and Chambers found no morphologic effect of these high concentrations of gallium nitrate at 6 h of incubation. However, we found that concentrations above 50–100 μM Ga(III) are toxic in 48 h of tissue culture. Nevertheless, when we performed an inhibitory dose dependence study in 48-h osteoclast cultures, unreasonably high Ga^{3+} concentrations were, as reported by Hall and Chambers, required to reduce bone degradation (Fig. 1). Therefore, we investigated the possibility that the manner in which osteoclasts are exposed to the drug affects the sensitivity of the cells.

In vivo studies on $^{67}Ga^{3+}$ distribution report that it binds slowly to metaphyseal bone after 14 day exposure to micromolar levels (Bockman et al., 1990), and Hall and Chambers observed that bone exposed 18 h to Ga(III) duplicated the effects of direct application of the drug to tissue culture. Hence, we undertook a detailed quantitative examination of the binding of this metal to bone in vitro using ^{67}Ga(III). To determine whether or not Ga distribution, or time of Ga exposure, affects osteoclast sensitivity to the drug, we also studied the kinetics of Ga(III) action on osteoclastic bone resorption and protein synthesis.

We found that equilibration of Ga(III) distribution in the presence of bone is slow, requiring ~48 h under tissue culture conditions (Fig. 2). Bone-Ga(III) $K_{association}$ in PBS was $79 \pm 3 \times 10^3$ M^{-1}, but $58 \pm 10 \times 10^3$ M^{-1} in the presence of serum, and $34 \pm 4 \times 10^3$ M^{-1} when bone with cells attached was used (Fig. 3A). The decreases in $K_{association}$ with serum containing medium, and further decrease of $K_{association}$ in serum containing medium + cells, indicate reduced affinity of the bone for gallium under these conditions.

Bone-gallium binding capacity (Ga_{max}) was $8.0 \pm 2.6 \times 10^{-4}$ moles/g bone in PBS, and de-

creased in the presence of α10-MEM to 1.09 ± 0.16 × 10^{-4} moles/g bone. Ga_{max} measurements at ~10^{-4} (~30–80% saturation) to ~10^{-10} M gave the same results, confirming the validity of the single-affinity adsorption model for Ga(III) on bone in both of these media. However, the differences of Ga_{max} and $K_{association}$ between serum containing medium and PBS indicate that the serum-containing medium competes with bone for Ga binding, or that medium elements compete for the bone-gallium adsorption sites.

The situation in the presence of cells is more complex. Under these conditions, Ga_{max} was 0.65 ± 0.2 × 10^{-4} moles/g bone, when calculated using results from 10^{-6}–10^{-10} M free Ga. The effect of the osteoclasts on Ga_{max} at low Ga_{free} suggests that attached cells may reduce the area of high affinity binding (by ~50%). At 10^{-4} M added gallium, Ga_{max} was 1.01 ± 0.1 × 10^{-4} moles/g bone, more closely resembling the result for the same medium but without cells, although it should be noted that this is the result of triplicate determination only. If confirmed, this result would suggest that osteoclasts affect binding (presumably at the cell attachment site) by reducing affinity, but leave capacity essentially unaffected. Presence of cells did reduce the binding constant, further suggesting that cell-associated bone may have a significantly lower affinity for gallium than naked bone. In the absence of bone, osteoclast cultures did not alter ^{67}Ga(III) distribution, indicating that the cells alone do not bind significant quantities of Ga (at any concentration tested). Thus, the osteoclast may reduce gallium binding by excluding the metal from its bone interface. However, the alternative that the physical environment at the cell's attachment, perhaps its acid pH (Blair et al., 1991), reduces $K_{association}$ would appear to be more likely.

At gallium solution concentrations over ~100 μM, we observed that toxicity and cell death was responsible for inhibition of bone resorption. Gallium concentrations of 100 μM are not effectively bound out of tissue culture media by bone (Fig. 2) and are thus exposed to the cells directly (the higher apparent gallium-binding capacity in PBS is not relevant in this regard). High concentrations of Ga^{3+} in serum may mediate the observed toxicity, at high doses, to other organ systems than bone (Foster et al., 1986).

Because it is easily quantifiable and more efficient than binding from tissue culture medium, we used pre-binding in PBS to determine the effects of bone-bound gallium on osteoclasts. Under these conditions, osteoclasts were exquisitely sensitive to gallium, with half maximal inhibition at 10 pmoles Ga/μg bone, produced by pre-binding 1 μM gallium to bone (Fig. 4). Above 50 pmoles Ga/μg bone, resorption at 48 h was effectively abolished.

We thus propose that the concentration of gallium by binding to the bone permits it to achieve a high local concentration at the site of attachment, which suppresses bone resorption without killing the osteoclast. We had previously proposed that similar bisphosphonate-bone binding is important in the function of the bisphosphonates as anti-osteoclastic drugs, although the specific level of bone binding was not quantified (Carano et al., 1990). Recent observations of Lakatos, et al. (1991), support this proposition. Inhibition of osteoclastic bone resorption by bone-bound gallium occurred at [Ga^{3+}] ~100 times lower than previously reported in vitro. Furthermore, total inhibition was achievable by this method, in contrast to the effect of Ga(III) in solution (Hall and Chambers, 1990). Another remarkable finding differentiating the effects of bone-bound and soluble Ga was that the inhibition by bound Ga is reversible with time, as the bound gallium is released into the medium and diluted or bound to proteins (Fig. 5). Inhibition by bone-bound gallium is not associated with reduced cellular protein synthesis, as indicated by ^{3}H-leucine incorporation into proteins (Fig. 6). These results indicated that the cells had not been killed, and, furthermore, that the biological life of bound gallium has a definite limit, ~48 h at the minimum inhibitory level of bone-bound gallium under the conditions tested in vitro (Figs. 4, 5). This could be an advantage in clinical application of Ga(III), as spontaneous decay of activity with time might prevent complication by secondary hyperparathyroidism and osteitis fibrosa if an excess of drug were to accumulate on bone.

It is important to note that cells in vivo are unlikely to encounter levels of bone-bound Ga^{3+} exceeding ~100 pM Ga^{3+}/μg bone, corresponding to PBS binding from 10 μM solution. This follows from the medium (serum) level required to achieve this level of bone-binding, ~20 μM Ga^{3+}. This level, which is ~twice that required to produce 100 pM Ga/μg bone in PBS (Fig. 2A,C), approximates the serum concentration after a fatal dose, and is hence an approximate physiologic maximum (assuming that bone-

binding from serum is similar to that from serum-containing tissue culture medium). Furthermore, since gallium effects on osteoclasts were reversible below ~100 pmoles/μg bone (Fig. 5), the calculated maximum physiologic bone [Ga] is below the osteoclast toxicity threshold. In other words, our results suggest that under conditions compatible with life, Ga(III) should not reduce the number of functional osteoclasts. Warrell et al. (1984) found no change in bone histology, including number of active osteoclasts, after gallium treatment, supporting this conclusion. In vivo studies, incidentally, used moderate, frequent doses of gallium (Matrovic et al., 1990; Warrell et al., 1984). Our results suggest that this is an ideal way of administering the drug, since the life of the drug is short, and low micromolar [Ga] for a period of days is required to achieve adequate bone binding (Fig. 2). Thus, this approach would likely be more effective while avoiding the toxicity of a large doses.

It is also important to consider that the culture model used differs in other important respects from the situation in the whole organism. Gallium has recently been shown to have significant effects on other bone cells, which are not considered here (Lakatos et al., 1991). Furthermore, the short life of bound gallium observed may be influenced by a culture situation where the osteoclasts cover ~50% of the bone surface. This differs substantially in vivo, except in disease states with greatly increased bone resorption. If, in our culture model, osteoclasts release the surface-bound gallium in attempting to resorb treated bone, Ga would be diluted in the medium and be subject to binding by serum or cellular proteins. It would therefore be less likely to re-bind to the bone surface. Released gallium in solution would not inhibit bone resorption unless its concentration exceeded ~50 μM, which would not be likely to occur, since prebinding of gallium employed < 20 μM $Ga(NO_3)_3$. Therefore, after a cycle of attempted bone resorption it is not surprising that we observe the Ga inhibition diminish. In vivo, where osteoclasts usually cover a smaller fraction of total bone surface, gallium inhibition of bone resorption could in theory be much more persistent, since the osteoclasts would clear a smaller fraction of the total surface per unit time. In the living organism, circulating Ga is cleared fairly rapidly, although significant retention of the metal (as tissue binding) occurs, suggesting that gallium is bound into stable complexes or chemically modified in the presence of cells and becomes inactive (Foster et al., 1986; Warrell et al., 1984). Thus, persistence of the anti-osteoclastic effect in vivo may vary with the rate of bone turnover or with binding to other tissues than bone and may be significantly longer than the ~2 days measured in vitro.

The mechanism of action and time course of Ga(III) effects contrast with bisphosphonate mediated osteoclast inhibition, although matrix binding is important in both classes of compounds (Carano et al., 1990). Bisphosphonates are anti-osteoclastic agents, several of which are metabolic inhibitors at micromolar levels, reducing protein synthesis. Additionally, the anti-osteoclastic effects of bisphosphonates tested in vitro did not dissipate over several days in tissue culture.

The degree of concentration at the site of action of the osteoclast suggests that gallium is a fairly specific inhibitor of this cell at solution activities in the high-nanomolar to low micromolar range. This may be useful both for further studies of osteoclastic activity and clinical applications. However, close attention must be paid to kinetics in reproducing the effect. Under the conditions described here, gallium inhibition is limited to ~48 h after osteoclasts contact Ga(III) carrying bone.

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