Defective Binding of Macrophages to Bone in Rodent Osteomalacia and Vitamin D Deficiency

IN VITRO EVIDENCE FOR A CELLULAR DEFECT AND ALTERED SACCHARIDES IN THE BONE MATRIX

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ABSTRACT In the osteomalacic as well as normal skeleton, few osteoclasts are associated with osteoid-covered bone surfaces. The reason for this particular cellular deficit is not clear, but may relate to the inability of osteoclasts and/or osteoclast precursors (monocyte-macrophages) to attach to immature, unmineralized bone matrix, a step apparently essential for normal resorptive activity and osteoclast differentiation. In this study, we have examined cell-bone binding using macrophages (MØ) and bone isolated from vitamin D-deficient rats and hypophosphatemic, osteomalacic mice and from their normal counterparts. The data show that MØ-bone attachment is greatly reduced ($P < 0.001$) in both vitamin D deficiency and hypophosphatemia, but that the mechanisms responsible for this reduction are apparently different in the two disorders. In hypophosphatemia, the reduction in binding appears solely attributable to the absence or inaccessibility of bone matrix oligosaccharides or glycoproteins essential to the attachment process. In vitamin D deficiency, on the other hand, not only is the bone matrix defective as a binding substrate, but the MØ, per se, is limited in its capacity to attach to normal, vitamin D-deficient, and hypophosphatemic bone.

INTRODUCTION

In the remodeling skeleton, osteoclasts are ordinarily observed only in proximity to mineralized bone surfaces and, as such, might be expected to exhibit changes in population size as a function of the efficiency and extent of the bone mineralization process. Nowhere is this relationship more apparent than in severe osteomalacia where, because of a reduced rate of mineralization, most bone surfaces are covered by uncalcified matrix (osteoid) and proportionally fewer than normal numbers of osteoclasts are observed (1, 2). The latter observation is in a sense surprising, since osteomalacia is frequently attended by increased circulating levels of parathyroid hormone, an agent which, in normal circumstances, is among the most potent stimulators of osteoclastogenesis (3).

There are two possible explanations for the paucity of osteoclasts seen in osteomalacia. The first proposes that there is a deficiency in the composition of osteoid that limits the attachment of osteoclasts and/or osteoclast precursors to the bone surface. Since such binding appears essential for efficient resorptive activity (4) and for the development of the osteoclast phenotype (5), its absence would sharply curtail both these functions, even in the presence of an osteoclast-promoting agent like parathyroid hormone. The second hypothesis suggests that the principal deficiency in osteomalacia is cellular in nature and, specifically, that osteoclasts and/or osteoclast precursors are defective in their ability to bind to bone and osteoid surfaces. Such cellular defects would have similar effects as a matrix deficiency (i.e., fewer osteoclasts) but would probably be achieved by different mechanisms.

It is now confirmed that osteoclasts are derived from cells belonging to the monocyte-macrophage family (6, 7). However, whether the immediate precursor is an immature cell (e.g., a promonocyte) or a differ-

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entiated one (e.g., a resident or exudate macrophage) has not been established.

We have previously shown that peripheral blood monocytes and elicited rodent peritoneal macrophages (MØ)[1] bind and subsequently resorb bone in vitro in a manner similar to the osteoclast in vivo (8, 9). Moreover, we have demonstrated that elicited MØ can be induced to form multinucleated giant cells and that these polykaryons, like osteoclasts, are more efficient in binding and resorbing bone than the mononuclear cells from which they are derived (10). Thus, the elicited MØ would seem to exhibit essential features of both an osteoclast and osteoclast precursor.

In a companion study, we showed that the attachment of elicited MØ to bone is a highly reproducible event that can be precisely quantitated, in vitro, using radiolabeling techniques (11). Moreover, we demonstrated that such binding is due largely to saccharides and lectins (carbohydrates "receptors") located on both the cell and bone surface. In the present investigation, we have used the methods developed for measuring MØ-bone attachment to test the "defective matrix" and "defective cell" hypotheses of osteoclast deficiency in osteomalacia. Our data show, in support of the first hypothesis, that bone derived from hypophosphatemic and vitamin D-deficient animals is bound by MØ with considerably less avidity than the same tissue from normal animals. Moreover, in keeping with the second postulate, our results also indicate that cells isolated from vitamin D-deficient, but not hypophosphatemic, animals attach to mineralized bone and osteoid as well as plastic less effectively than MØ isolated from healthy donors. On the basis of competitive-binding studies with carbohydrates and fetuin, the diminution in attachment associated with vitamin D-deficient and hypophosphatemic bone seems to be due to alterations or modifications in matrix oligosaccharides or glycoproteins. On the other hand, the reduction in attachment observed with vitamin D-deficient MØ appears to be part of a more generalized phenomenon in which the MØ ability to migrate and engage in phagocytosis are also impaired.

METHODS

Osteomalacia

Hypophosphatemia. Calvaria and MØ were obtained from 30-d-old *Hyp*/Y (C57BL/6J) male mice (circulating P 4–5 ng/dl) and compared with that of their normal sex-matched litter mates (circulating P 7–10 mg/dl) (12).

Vitamin D deficiency. Vitamin D-deficient bone particles and MØ were obtained from male Sprague-Dawley rats maintained on a vitamin D-deficient diet (test diet 170670, Teklad, Madison, WI) in the dark for 5 wk following weaning. At the time of killing, the circulating 25-hydroxyvitamin D levels were <3 ng/ml (13). Normally maintained littermates served as controls.

MØ isolation

Elicited peritoneal cells were recovered by lavage from the rodents 3 d after intraperitoneal injection of a 10% thioglycollate solution. The cells were suspended in α-MOPS (Eagle's minimal essential medium [MEM] buffered with 3 [*N*-morpholino] propane sulfonic acid to pH 7.4), and were plated into tissue culture dishes to initiate purification of the MØ population by differential adhesion (see below). The MØ monolayers were used directly in particle-binding assay, or after resuspension, in the calvarial-binding assay.

Bone-binding assays

Particle binding. Peritoneal cells were seeded into 96-well microtiter plates (KC Biological, Kansas City, MO) at 1×10^5 cells/well and after 1-h incubation rinsed to remove most of the nonadherent fraction. α-LH medium (α-MEM supplemented with 0.1% lactalbumin hydrolysate [Gibco Laboratories, Grand Island, New York] and bicarbonate buffer) was added to each well and, after an additional 20–23 h culture period, the plates were rinsed again leaving monolayers consisting exclusively (99%) of MØ as determined by nonspecific esterase activity (14). (Note: lactalbumin hydrolysate was used in these experiments to avoid contamination with vitamin D present in serum.) Bone particles were obtained from normal and vitamin D-deficient rats administered $^{45}CaCl$ for 2 wk before killing as described (9). Radiolabeled particles, 23–45 μm in diam suspended in α-MOPS were added to each culture at the specified concentrations (generally 0.2 mg in 0.2 ml) and the plates incubated at 37°C in air for up to 2 h. The nonadherent bone particles were removed by vigorously rinsing the plates in three successive changes of phosphate-buffered saline (PBS) and the residual (attached) bone was dissolved by adding concentrated trichloroacetic acid (TCA) to each well. The radioactive mixture was then diluted in scintillation fluid (Scintiverse, Fisher Scientific Co., Pittsburgh, PA) and counted. Cell-associated bone particle binding was determined by subtracting the disintegrations per minute obtained from identically treated cell-free wells from those recorded in the presence of MØ monolayers. The results are expressed as net micrograms of bone bound per well and represent the mean±SEM of six replicate cultures. In experiments comparing cells from normal and osteomalacic rodents, each group consists of three to four animals. The bone in each experiment was taken from one animal of each type.

Calvarial binding. Elicited cells in α-MOPS were dispensed into 60-mm culture dishes and incubated for 1 h to allow the adherent fraction to attach to the plastic surfaces. The nonadherent cells were then removed by rinsing and cultures continued for another 20–23 h in α-LH. The plates were rinsed again, and the adherent MØ removed from the substrate by a 20-min exposure to cold (4°C) Ca^{++}/Mg^{++}-free PBS and gentle scraping. The MØ were then exposed to $Na^{51}CrO_4$ (sp act 250–500 mCi/mg Cr, Amersham Corp.,

[1] *Abbreviations used in this paper:* α-LH medium, α-minimal essential medium supplemented with 0.1% lactalbumin hydrolysate; MØ, macrophage(s); α-MOPS, minimal essential medium buffered with 3(*N*-morpholino) propane sulfonic acid; $1,25(OH)_2D_3$, 1,25-dihydroxyvitamin D_3; TM, tunicamycin.

Arlington Heights, IL) (1 μCi/10^6 cells in α-MOPS) for 1 h at 37°C, washed three times in cold medium and suspended in α-MOPS to 1 × 10^6 cells/ml. 100-μl samples of cell suspension were then aliquoted on the stripped endocranial surface of calvaria, freshly dissected from 30-d-old mice, and the cells allowed to attach for periods up to 2 h. The nonadherent MØ were removed by sequential rinsing in PBS, and the calvaria with attached, radioactive cells subjected to gamma counting. The data are expressed as MØ bound per calvarium and represent the mean±SE of six replicate cultures.

RESULTS

Attachment of normal MØ to hypophosphatemic and vitamin D-deficient bone. Elicited MØ from normal animals bind to hypophosphatemic and vitamin D-deficient bone more slowly and with less apparent avidity than mineralized matrix obtained from untreated donors. For example, at each of several sampling intervals, significantly fewer normal cells attach to osteoid-covered *Hyp* calvaria (Fig. 1) than to min-

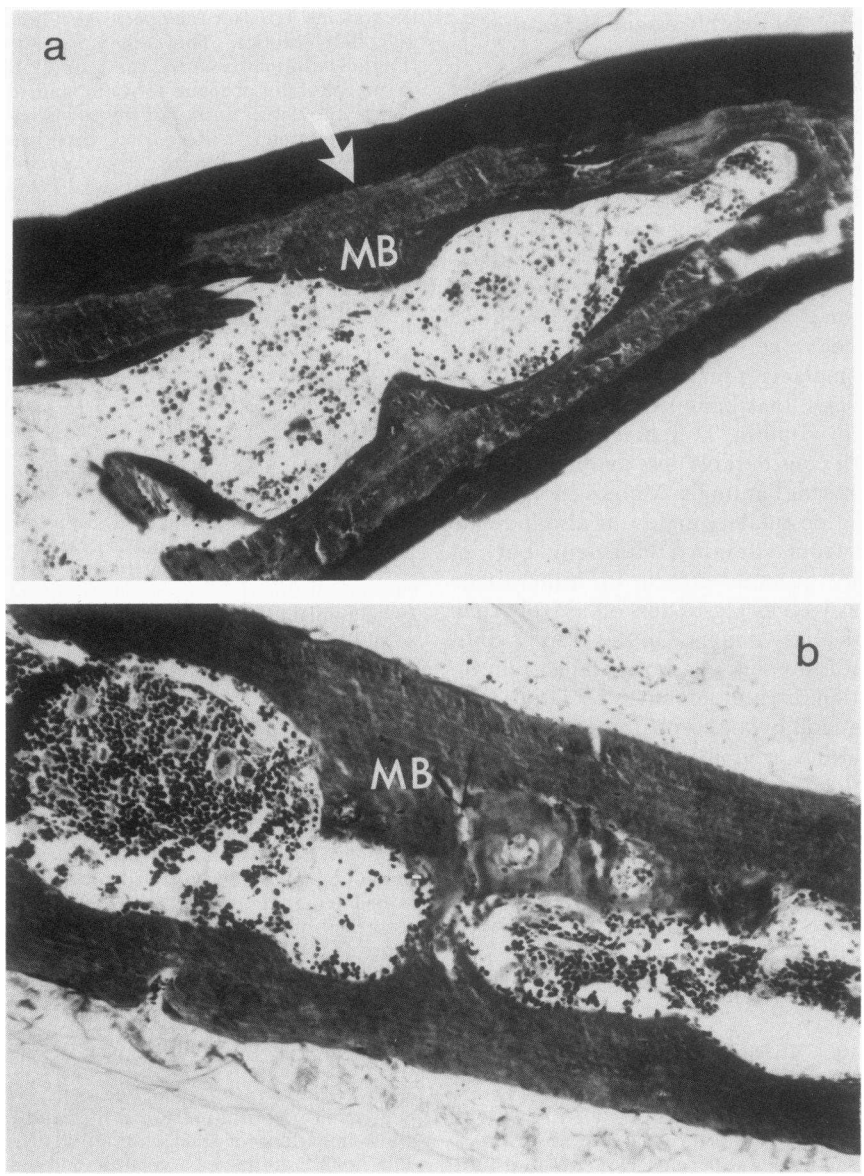

FIGURE 1 Calvaria from (*a*) phosphate-deficient *Hyp* mouse and (*b*) normal littermate. The endocranial surface of the affected mouse is covered by a thick osteoid seam (arrow). MB, mineralized bone, nondecalcified. Goldner stain, ×100.

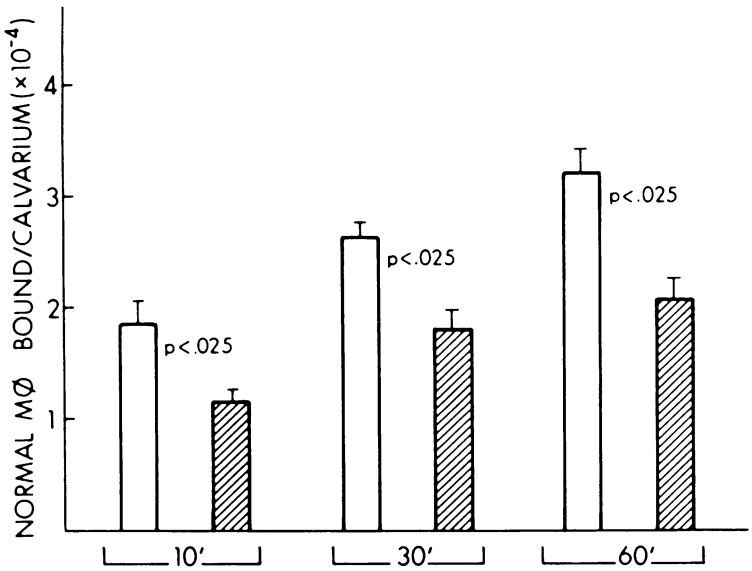

FIGURE 2 Binding of ^{51}Cr-labeled normal mouse MØ to calvaria from normal (□) and hypophosphate-mic (*Hyp* ■) mice. The numbers on the abscissa represent the incubation period, in minutes, at which binding was determined. Note that at all three sampling intervals, fewer MØ bind to *Hyp* calvaria than to normal controls. Each bar represents the mean±SE of six replicate determinations.

eralized calvaria isolated from littermate controls (Fig. 2). Similarly, normal MØ bind bone particles prepared from vitamin D-deficient animals less rapidly and to a lesser extent than mineralized particles (Fig. 3). Finally, at any given particle concentration, a smaller percentage of the vitamin D-deficient bone is attached by normal MØ than comparable particles derived from control animals (Fig. 4 *A*). (It should be noted that the sedimentation rate of the vitamin D-deficient and normal particles is the same, and therefore not a factor in differential binding [data not shown].)

Binding of vitamin D-deficient and hypophosphatemic MØ to bone. Elicited MØ isolated from vitamin D-deficient animals attach to tissue culture plastic and normal bone (particles and calvaria) less effectively than elicited cells recovered from control animals (Figs. 4 and 5). This difference is detectable within 5 min of culture (Fig. 5) and corresponds to a decrement of 20–35% relative to controls as measured in the calvarial assay (Fig. 5) and ~50% as determined with particle binding (Fig. 4). It is of interest that the abnormality in vitamin D-deficient cells is apparent on plastic as well as both types of bone, suggesting that the attachment defect is general rather than specific in nature.

In contrast to MØ isolated from vitamin D-deficient rats, elicited cells isolated from *Hyp* mice bind bone

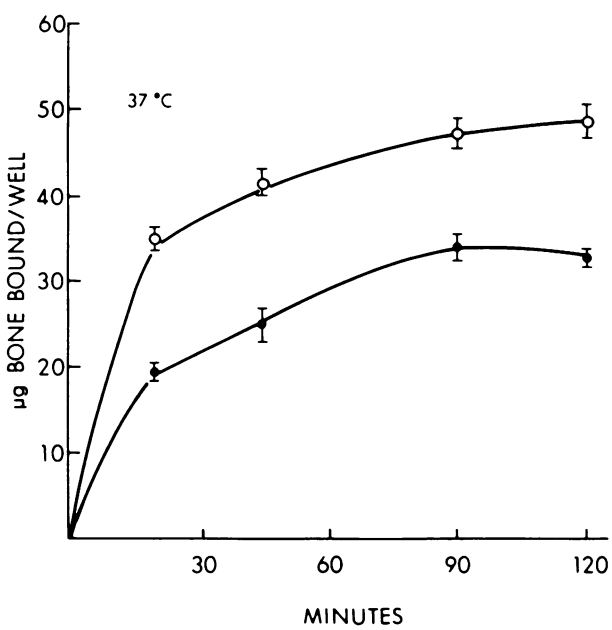

FIGURE 3 Binding of bone particles from normal (○) and vitamin D-deficient (●) rats by normal rat MØ. Note that at any sampling time up to 120 min MØ bind particles from vitamin D-deficient animals less well than particles from control animals. Each point represents the mean±SE of six replicate cultures.

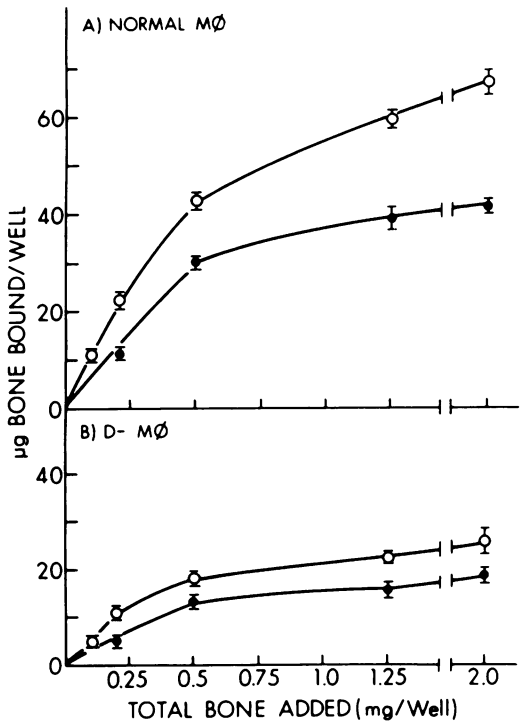

FIGURE 4 Binding of bone particles from normal (○) and vitamin D-deficient (●) rats by normal (*A*) and vitamin D-deficient (*B*) rat MØ. Note that both normal and vitamin D-deficient MØ bind vitamin D-deficient bone less effectively than normal bone. Moreover, MØ from normal rats bind both types of bone more efficiently than do cells from vitamin D-deficient rodents. Incubation period, 2 h. Each point represents the mean±SE of six replicate determinations.

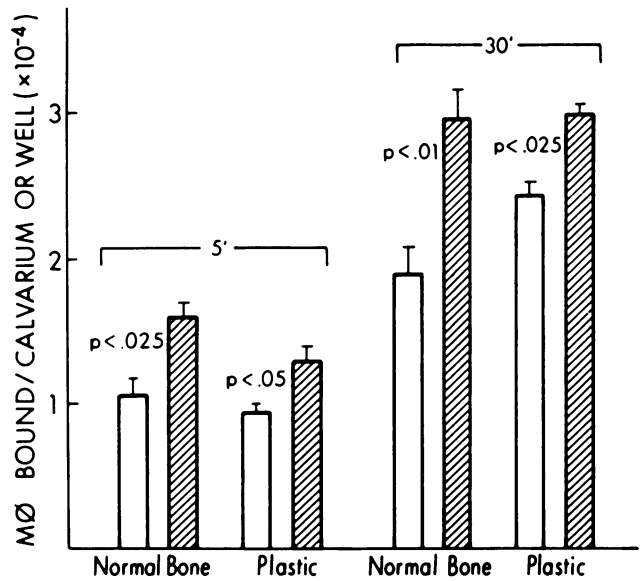

FIGURE 5 Binding of MØ from normal (■) and vitamin D-deficient (□) rats to bone (calvaria) and plastic. Note that cells from vitamin D-deficient animals bind significantly less well to either substrate than normal cells ($P < 0.01$ to < 0.001). Sampling intervals, 5 and 30 min. Each bar represents the mean±SE of six replicate cultures.

as effectively as MØ recovered from control animals (Fig. 6). This equivalency is demonstrable with particles from both normal and vitamin D-deficient skeletons and, in fact, the two cell populations display identical particle saturation kinetics with either substrate (Fig. 6).

Cell-bone binding in the presence of carbohydrate, fetuin, and following tunicamycin (TM) treatment. In a related study (11), we demonstrated that MØ attachment to normal bone is inhibited by the fetal glycoprotein, fetuin, some specific sugars, and TM treatment, suggesting that saccharide recognition plays an important role in MØ-bone binding. In contrast to normal bone, however, fetuin (Fig. 7) or its asialo-derivative (Sigma Chemical Co., St. Louis, MO) (data not shown) fail to reduce MØ attachment to vitamin D-deficient particles, regardless of whether cells were obtained from control (Fig. 7) or vitamin D-deficient rodents (data not shown). Moreover, simple carbohydrates (Sigma Chemical Co.), which consistently in-

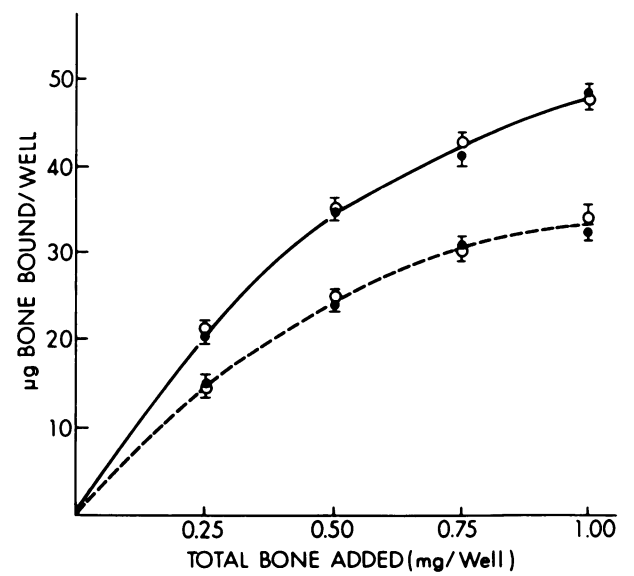

FIGURE 6 Binding of MØ from normal (●) and *Hyp* (○) mice to bone particles from normal (——) and vitamin D-deficient (- - -) rats. Note that, in contrast to cells from vitamin D-deficient animals (Fig. 5), MØ from *Hyp* mice are as effective in binding bone particles as MØ from littermate controls. Moreover, they show the same reduced affinity for particles from vitamin D-deficient animals. Assay period, 2 h. Each point represents the mean±SE from six replicate cultures.

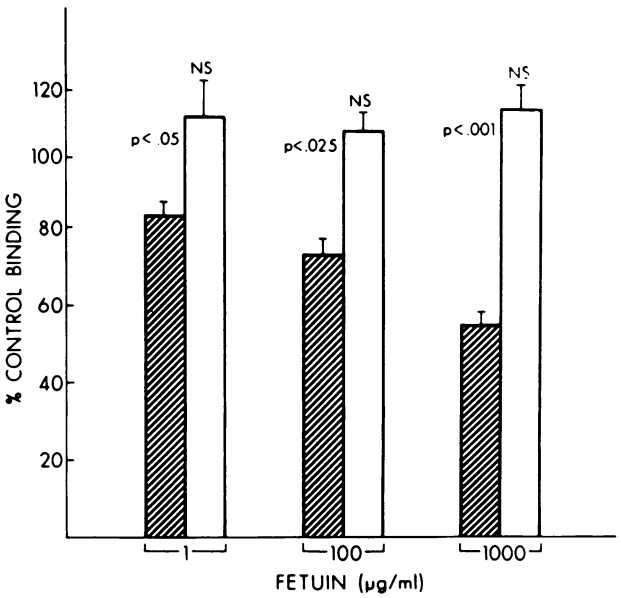

FIGURE 7 Effect of fetuin on the binding of bone particles from normal (■) or vitamin D-deficient (□) rats by normal rat MØ. The experiment was performed in glucose-free medium and data are expressed as percent control binding (i.e., binding in the absence of added fetuin). Attachment was determined after 2 h. Note that while fetuin inhibits MØ binding to normal bone in a dose-dependent manner, it has no such effect on attachment to vitamin D-deficient bone. Each bar represents the mean±SE from six replicate determinations.

hibit the attachment of MØ to normal bone, are uniformly less effective in suppressing the binding of these same cells to vitamin D-deficient particles (Fig. 8). In fact, in two instances (fucose and arabinose), carbohydrates that are moderately inhibitory against normal matrix fail to show any suppressive action against vitamin D-deficient bone.

As previously reported, preincubation of cells with 0.4 μg/ml TM (Sigma Chemical Co.) for 18 h, which does not affect cell survival or protein synthesis (11), suppresses the attachment of MØ to normal bone by ~20%. The inhibitory effect of the antibiotic is even more pronounced when vitamin D-deficient bone is used as a substrate. In the experiment shown in Fig. 9, the attachment of TM-treated, vitamin D-deficient MØ to vitamin D-deficient bone was reduced by ~60% relative to untreated vitamin D-deficient cells and by ~88% relative to normal cells binding to normal bone. The inhibitory effect of TM on vitamin D-deficient cells is not blocked by the protease inhibitor leupeptin (Sigma Chemical Co.) and is reversible with time (data not shown).

DISCUSSION

In the United States, the two most common causes of osteomalacia are renal insufficiency and X-linked hypophosphatemia. It is believed that osteomalacia associated with kidney disease reflects, at least in part,

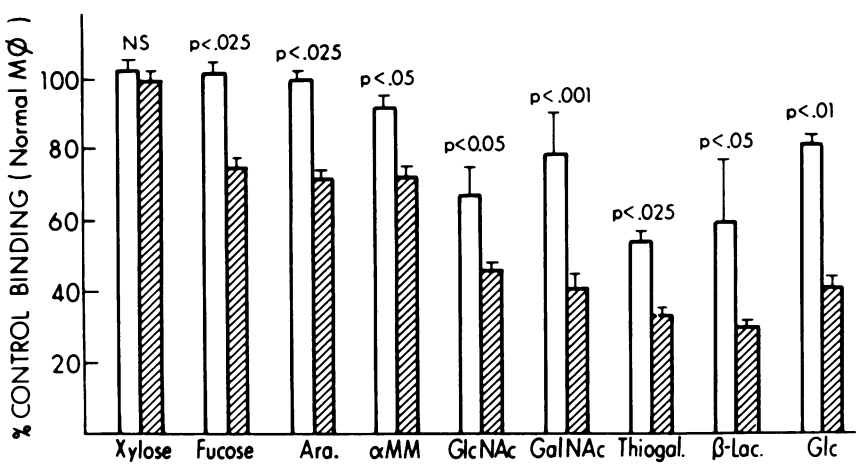

FIGURE 8 Effect of specific carbohydrates on the binding of bone particles from normal (■) or vitamin D-deficient (□) rats by normal rat MØ. In all cases, 0.1 M carbohydrate was added and attachment determined after 2 h. Data are expressed as in Fig. 7. Note that in each instance in which binding is suppressed, the inhibition with vitamin D-deficient bone is proportionally less than that with normal particles. In contrast to cell-bone attachment, binding to plastic is not affected by any carbohydrate tested (data not shown). Each bar represents the mean±SE from six replicate cultures. Ara, arabinose; αMM, α-methylmannoside; GlcNAc, *N*-acetylglucosamine; GalNAc, *N*-acetylgalactosamine; Thiogal, thiogalactoside; β-Lac, β-lactose; Glc, glucose.

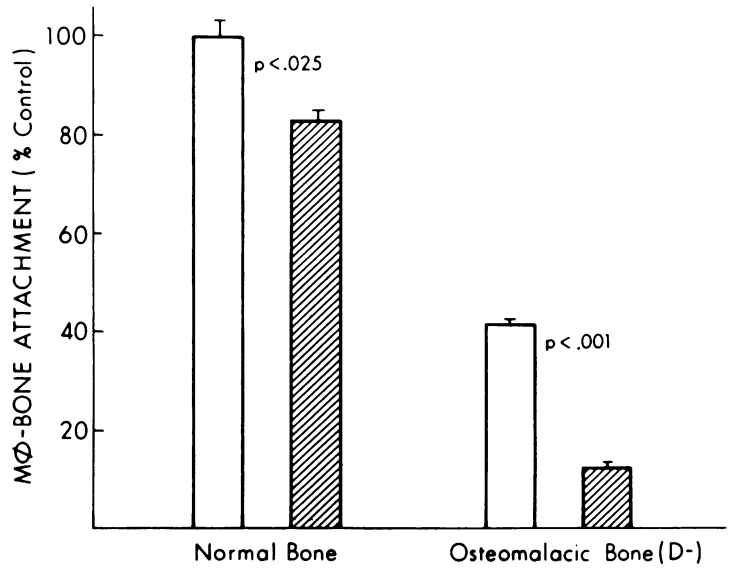

FIGURE 9 Binding of bone particles from normal or vitamin D-deficient rats by normal or TM-treated rat MØ. Normal rat MØ were incubated for 18 h in 0.4 μg/ml TM (■) before the addition of bone. Data are expressed as percent control (i.e., binding of normal cells to normal bone). Attachment was assayed after 2 h. Note that when TM-treated cells are incubated with vitamin D-deficient bone, ~90% of normal cell-bone attachment is lost. Each bar represents the mean±SE from six replicate cultures.

deficient production of 1,25-dihydroxyvitamin D_3 ($1,25[OH]_2D_3$) (15). Hypophosphatemia, on the other hand, elicits osteomalacia because the levels of circulating phosphorus are inadequate to support normal bone mineralization (16).

In the introduction we presented two hypotheses to account for the reduced number of osteoclasts associated with osteomalacia. In both, the essential thesis is that osteoclast precursor cells (MØ) bind poorly to osteoid and, with this diminished cell-substrate contact, MØ fail to differentiate into osteoclasts. We speculated that this binding deficiency might occur as a consequence of the altered characteristics of the bone matrix or because the cells of osteomalacic animals are defective in their ability to recognize and bind bone.

The data from the present study clearly demonstrate that bone derived from hypophosphatemic and vitamin D-deficient animals is modified such that it is less readily bound by MØ (osteoclast precursors) than normal bone. In both hypophosphatemia and vitamin D deficiency, osteoid accumulation represents the most apparent matrical abnormality. However, it is also known, at least in the case of vitamin D deficiency, that other matrical defects such as a predominance of poorly cross-linked collagen (17) and a diminution in glycosaminoglycan content (18) represent major changes in the bone matrix. In both forms of osteomalacia, however, bone is bound at a slower rate and to a lesser extent than normal by MØ from control animals. Furthermore, specific carbohydrates, fetuin, and asialofetuin are uniformly less effective in suppressing the attachment of cells to osteoid-covered, and vitamin D-deficient bone than to normal bone and, in some cases, such as fucose, arabinose, and fetuin, the compounds are totally noninhibitory. The latter findings are of particular interest since they indicate that, at least in part, changes in matrix sugars (oligosaccharides or glycoproteins) are responsible for diminished attachment.

The situation is more complicated with regard to the bone-binding behavior of cells derived from vitamin D-deficient and hypophosphatemic animals. Here, MØ isolated from *Hyp* mice are indistinguishable from controls, while those prepared from vitamin D-deficient rodents show marked deficits in their ability to bind to normal and abnormal bone as well as to plastic. In view of the distinctive differences in the binding activities of the two cell populations, the conclusion seems inescapable that vitamin D plays an essential role in regulating MØ function or phenotypic expression. Bar-Shavit et al. (19) recently reported that MØ and polymorphonuclear leukocytes from vitamin

D-deficient mice show diminished spontaneous migration and phagocytosis in vitro, and that these deficiencies are correctable with the addition of $1,25(OH)_2D_3$. Similarly, we demonstrated that MØ from vitamin D-depleted rats resorb bone poorer than control cells in vitro and that this deficiency could be overcome with the application of vitamin D (20). However, in this case, in contrast to Bar-Shavit et al. (19) studies the vitamin had to be introduced into the vitamin D-deficient animal to be effective. We have no firm explanation for this difference in result, but we speculate that the in vivo finding may relate to the purported ability of vitamin D to enhance the differentiation of monocytes and MØ from precursor cells. This view is consistent with the observation of Miyaura et al. (21) that $1,25(OH)_2D_3$ induces the differentiation of murine and human myeloid leukemia cells in culture, and our own findings that vitamin D promotes the transformation of the human myeloid line HL-60 into monocytes (22).

As noted above, osteomalacia and its associated relative osteoclastopenia are most commonly observed in patients with X-linked hypophosphatemia (23) and end stage renal disease (24). The diseases differ distinctly in the probable cause of osteoid accumulation (calcium vs. phosphate deficiency) and the circulating levels of vitamin D metabolites (normal in hypophosphatemia [25]; lacking $1,25[OH]_2D_3$ in end stage renal insufficiency [26]). Based upon the concept that osteoclast development is dependent upon the attachment of precursor cells (MØ) to bone, we have examined in the present study the binding of MØ to bone using materials isolated from hypophosphatemic, vitamin D-deficient, and normal animals. The data show that cell-bone attachment is decreased in the two disorders thereby accounting, in theory, for the osteoclastopenia, and that this decrement in binding results from alterations in the bone matrix and the MØ. The alterations in bone matrix are apparent in both vitamin D-deficient and hypophosphatemic animals, and probably involve changes in sugar residues in the matrix; the modification in MØ function is evident only in vitamin D-deficient animals and probably relates to the failure of MØ to fully mature in the absence of vitamin D.

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