Effects of 1,25-dihydroxyvitamin D3 and parathyroid hormone on bovine monocyte-mediated bone degradation and mitogen-induced lymphocyte DNA synthesis

Frank Gerard Edward Hustmyer

Iowa State University

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Effects of 1,25-dihydroxyvitamin D₃ and parathyroid hormone on bovine monocyte-mediated bone degradation and mitogen-induced lymphocyte DNA synthesis

by

Frank Gerard Edward Hustmyer

A Dissertation Submitted to the Graduate Faculty in Partial Fulfillment of the Requirements for the Degree of

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Iowa State University
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DEDICATION

I dedicate my efforts put forth in earning my Ph.D. to my heroes, my best buddies, my parents - Frank E. Hustmyer, Jr. and Rosemary H. Hustmyer. I thank Mom and Dad for the gift of life and the enriched world of my youth - for the joy of learning and love of knowledge that leads to the joy of recognition in future days - for blowing on the coal of my heart, lighting my spirit, and allowing their spirit to burn within me. One of the greatest joys of my life has been to have and love friends such as my Mom and Dad.

I also dedicate this work to the rest of my family, my siblings, who have cared for me throughout my years - Christine, our artist and beautiful butterfly; Kurt (K.J.) - "the Hospitality Suite Kid;" John - the Bonnie Prince who will be King someday; Roseanne - Zanne Bananne, "The Little One;" Pete, Petross, Twiggy - Arnie, an "A-1 good man with nice hair." Thanks for helping me through these times - we'll make it guys!

It is said that the true memorial or measure of someone is in the hearts of those who loved him. My Mom and Dad's legacy burns with white heat in the hearts of their children. It is that warmth that allowed me to accomplish what I have. I am eternally grateful!
GENERAL INTRODUCTION

Osteoclasts are the principal bone resorbing cells of the body. The bone calcium-mobilizing hormones, parathyroid hormone (PTH) and 1,25-dihydroxyvitamin D₃ (calcitriol), increase osteoclast numbers and their bone-resorbing activity (1-4). Monocytes also have been proposed to be mediators in the bone degradation process. Monocytes are found in areas of bone-resorbing osteoclasts and chronic inflammation (5-8), degrade devitalized bone (9), and are chemotactic to areas of resorbing bone (10). A particularly significant study reported that administration of a large dose of calcitriol to a child with osteopetrosis, a disease characterized by bone resorption dysfunction, resulted in an increase of osteoclast resorption and also an increase in monocyte-mediated bone degradation (11), thus suggesting calcitriol stimulation of both of these two cell types during bone resorption. Heersche (12) also proposed that the immediate bone resorption mechanism involves both osteoclast bone resorption and a "scavenger-helper" role of monocytes to remove and phagocytize bone debris. It is in the context of the monocyte as a scavenger cell distinct from the osteoclast bone resorption mechanism that I evaluated monocyte-mediated degradation of devitalized bone during both PTH and calcitriol administration. Additionally, I evaluated production of hydrogen peroxide by monocytes during PTH administration as a possible measure of an inflammatory-type mechanism for bone degradation activity by monocytes.
Calcitriol, in addition to its role in calcium homeostasis, is also an immunoregulatory steroid hormone. Recent studies have shown that calcitriol induces monocytic differentiation in murine and human myeloid leukemia cells (13-16) and that the thymus, monocytes, and activated T and B lymphocytes express the calcitriol receptor (17-22). The immunoregulatory role of calcitriol is demonstrated further by its inhibition of both mitogen-stimulated peripheral blood mononuclear cells (PBM) (23, 24) and murine medullary thymocyte proliferation (19,25) and its suppression of interleukin (IL-2) production (23, 24).

In contrast to the antiproliferative effects of calcitriol, Lacey et al. (26) reported that calcitriol stimulated the proliferation of a nontransformed T-helper cell line in the absence or at low concentrations of interleukin 1 (IL-1). At IL-1 concentrations necessary for optimal response, however, addition of calcitriol resulted in inhibition of proliferation. I sought, therefore, to characterize the in vitro response of bovine PBM to several mitogens 1) in the presence of calcitriol and 2) after in vivo administration of calcitriol.

Dissertation Format

This dissertation is presented in the alternate thesis format, which includes two manuscripts. The paper in Section I is coauthored by B. J. Nonnecke, D. C. Beitz, R. L. Horst, and T. A. Reinhardt, and has been published in Biochemical and Biophysical Research Communications (Vol. 152: 545-551). The paper in Section II is coauthored by J.
P. Goff, B. J. Nonnecke, D. C. Beitz, R. L. Horst, and T. A. Reinhardt
and is to be submitted to the Journal of Bone and Mineral Research.
Section III includes research not included in manuscripts for publica-
tion in Sections I and II. A brief review of the literature precedes
the first manuscript and is concerned principally with firstly the
scientific basis of monocyte-mediated bone degradation as related to
the bone calcium-mobilizing hormones PTH and calcitriol, and secondly
the immunoregulatory role of calcitriol. At the end of each section, a
separate Literature Cited section has been included. A general Summary
and Discussion section follows the final manuscript.

The doctoral candidate, Frank Gerard Edward Hustmyer, was the
principal investigator in each of these studies.
LITERATURE REVIEW

Monocyte-Mediated Bone Degradation

Origin of the osteoclast from blood-borne hematopoietic cells

The predominant and widely accepted theory on the origin of the osteoclast states that osteoclasts are of hematopoietic origin from phenotypically similar circulating cells of the monocyte–macrophage lineage. The blood-borne origin of osteoclasts initially was suggested by Jordan (27) in 1925 and Hancox (28) in 1949 in which they proposed the osteoclast precursor possibly to be a mononuclear wandering cell—either a histiocyte or macrophage. Jee and Nolan (29) supported this early hypothesis by demonstrating that phagocytilzed charcoal ingested by phagocytes later appeared in osteoclasts. Fischman and Hay, utilizing regenerating newt limbs, also suggested a mononuclear leukocyte origin for the osteoclast (30). Convincing evidence for the hematopoietic origin of osteoclasts, however, was provided by a series of experiments in which osteopetrotic animals were cured by hematopoietic infusion, parabiosis, and chimeric bone grafts.

Walker demonstrated that the defective osteoclast bone resorption that is characteristic of microphthalmic osteopetrotic mice could be cured by parabiotic union with normal siblings (31), thus suggesting a vascular origin of the cells responsible for rectifying the defect. Götlin and Ericsson employed parabiotic inbred rats to show the hematopoietic origin of osteoclasts. One animal was irradiated and the other was not. Tritiated thymidine-labeled cells from the non-
irradiated animal were identified later at sites of induced fracture in newly formed osteoclasts in the irradiated animal, thus again suggesting a blood-borne origin for the osteoclast (32).

Transplantation studies in irradiated osteopetrotic mice also have been conducted. Irradiated osteopetrotic mouse mutants infused with normal bone marrow or spleen cell suspensions were cured of the osteopetrotic malady (33, 34), and conversely, irradiated normal recipient mice injected with cell suspensions from osteopetrotic animals developed the disorder (35). Additionally, Ash et al. (36) found that irradiated osteopetrotic microphthalmic (mi/mi) mice infused with bone marrow cells with giant lysosomes from beige mice (bg/bg) resulted in formation of osteoclasts with giant lysosomes and normal bone resorption. Spleen cells from beige mice (bg/bg) that had been transplanted into irradiated microphthalmic mice also resulted in formation of osteoclasts with giant lysosomes, donor origin, and restored normal osteoclast function (37).

Further evidence for the hematopoietic origin of osteoclasts was demonstrated with chick-quail chimeras. Jotereau and LeDouarin (38) used the quail nuclei heterochromatin markings to distinguish nuclei in the newly formed osteoclast from chick nuclei that do not have heterochromatin markings following cross-transplantation of fetal limb buds. Chimeric osteoclast formation indicated the hematopoietic origin of formed osteoclasts. The heterochromatin markings of quail nuclei also were employed in chick-quail chimera experiments in which transplanted limb buds were fractured to initiate bone remodeling.
The resulting callus formation demonstrated that osteoclasts were of host origin, indicating a vascular path for nuclei in newly formed osteoclast (39).

**Origin of osteoclast from cells of monocyte-macrophage lineage**

The proposed blood-borne, extraskeletal origin of the osteoclast suggested that mononuclear leukocytes may be likely candidates for osteoclast precursor cells. The primary focus of investigators has been the study of cells of the mononuclear phagocyte system. Jee and Nolan (29) and Fischman and Hay (30) suggested a monocyte-macrophage origin for the osteoclast, but their studies could not unequivocally exclude histiocyte or lymphocyte origin. Several researchers then isolated "pure" populations of monocyte-macrophages, labeled these populations with an appropriate tag, reintroduced labeled cells into animals, and determined whether the marker appeared in developing osteoclasts. Stanka and Bargsten (40), Tinker et al. (41), and Göthlin and Ericsson (32) prepared labeled (Barr bodies, $^3$H-thymidine, and Thorotrast, respectively) peripheral blood mononuclear cells at several degrees of purity, reintroduced labeled cells into animals, and evaluated the newly formed osteoclasts for appropriate label content. All studies reported small percentages of labeled osteoclasts, but because of discrepancies in purity of originally isolated monocyte-macrophage populations and low amount of label in osteoclast, no definitive conclusion could be made.

The *in vitro* fusion of monocyte-macrophage cells with osteoclast-like cells has been suggested as evidence that circulating monocytes
are osteoclast precursor cells. Macrophages in non-skeletal tissues fuse into multinucleated giant cells (42). Zambonin-Zallone et al., in a series of photomicrographs and autoradiographic techniques, demonstrated fusion of monocytes in vitro with preformed osteoclasts (43, 44). One of the difficulties in interpreting the aforementioned studies has been based on the impure nature of either the isolated mononuclear phagocyte population or the osteoclast bone cultures. Ko and Bernard (45) and Burger et al. (46) sought to overcome these difficulties by using osteoclast-free fetal mouse bone explants cultured with either 1) nonadherent bone marrow-derived mononuclear cells (BMDM) - a highly purified immature mononuclear phagocytes (MNP) population, 2) adherent BMDM - a population of more mature MNP, fibroblasts, and adipocytes, 3) blood monocytes, or 4) resident and exudate peritoneal macrophages. Osteoclast formation was observed only in cultures of nonadherent BMDM, and not with adherent BMDM, monocytes, or peritoneal macrophages. Ibbotson et al. also demonstrated that osteoclast-like cells formed from nonadherent bone marrow monocytic progenitor cells that had been enriched by discontinuous Percoll gradient centrifugation (47). Burger et al. (48) later demonstrated that [³H]-thymidine-labeled nonadherent BMDM, when cultured with osteoclast-free bone explants, resulted in the labeling of 88% of nuclei in newly-formed osteoclasts. Exudate peritoneal macrophages, however, were inconsistent in ability to form osteoclasts, requiring longer culture periods for formation. Observations cited in this paragraph suggest that 1) the osteoclast precursor cell
is an immature monocyte, possibly a monoblast or promonocyte, and 2) that neither the mature tissue macrophage nor the mature blood monocyte will differentiate into osteoclasts.

The hypothesis that osteoclast development cannot occur via the circulating monocytes is strengthened further by several additional studies. In an in vivo study, no correlation was evident between the kinetics of \(^3\text{H}\)-thymidine labeling of monocytes and the subsequent detection of labeled DNA of nuclei in osteoclasts (49). Furthermore, defective osteoclastic bone resorption evident in osteopetrotic or radiation-damaged animals is not corrected by macrophage infusions (50, 51). Finally, no evidence has been presented yet of in vitro differentiation of peripheral blood monocyte into bona fide osteoclasts. These observations strongly suggest that the osteoclast progenitor cell is an immature BMDM and that a more differentiated and mature monocyte-macrophage is incapable of nascent osteoclast formation.

The role of monocyte-mediated bone degradation

Although monocytes and macrophages do not seem to have the capacity for nascent osteoclast formation, these cells secrete products, such as lysosomal enzymes and collagenase (52), that are believed essential for bone degradation and also degrade devitalized bone. Mundy et al. first reported that blood monocytes degrade devitalized \(^{45}\text{Ca}\)-labeled bone particles. In vitro addition of the bone calcium mobilizing hormones, PTH and calcitriol, did not increase monocyte-mediated bone degradation (9). Kahn et al. and Rifkin et al.
also demonstrated that contact of monocytes with devitalized bone particles was required for degradation to occur (53, 54). Resorbing bone is also chemotactic for monocytes (10). The degree of bone resorption in organ culture induced by PTH, calcitriol, or prostaglandin E₂, a potent stimulator of osteoclast activity (55), correlated with chemotactic activity (10). The bone matrix constituents Type I collagen and α₂HS glycoprotein, and osteocalcin, a major noncollagenous protein of bone, all exhibit a dose-dependent chemotactic response to monocytes (56-58). This response suggests that these substances released during the bone resorption process may provide a mechanism for recruitment of monocytes to areas of bone resorption. Moreover, the local production of factors, such as interleukin 1 (IL-1) (59) and prostaglandins (55), stimulate osteoclast bone resorption, suggesting a monocytic control mechanism for resorption in the microenvironment of the bone-resorbing osteoclast.

Additionally, monocytes may serve an important role in the osteolytic process evident at sites of inflammation. Macrophages are constituents of inflammatory infiltrates in diseases associated with osteolysis, such as periodontitis and rheumatoid arthritis, and are associated with osteoclasts during bone resorption (5-8). Induced inflammation-mediated osteopenia has been demonstrated in the rat (60), and endotoxin treatment of isolated monocytes resulted in enhanced in vitro monocyte-mediated bone degradation (61). In summary, studies on monocyte-mediated bone degradation demonstrate that monocytes 1) degrade devitalized bone in a contact-mediated mechanism,
2) are possibly recruited to regions of resorbing bone by bone matrix constituents, and 3) are found in regions of bone resorption in both chronic inflammatory disorders and normal bone resorbing osteoclasts. Heersche (12) has proposed a two-cell model for osteoclastic bone resorption that is particularly relevant to this summary. The monocyte as proposed by Heersche serves in a "janitorial-scavenger" role, assisting the osteoclast in removal of debris produced during the resorption process (12). It is in this context that the monocyte shall be examined in this project, i.e., a distinct monocyte-mediated bone degradation process accompanying but separate from osteoclastic bone resorption.

Bone remodeling sequence - relation to mononuclear phagocytes

The proposed role of the monocyte as a separate element in the bone resorption process requires an assessment of the possible contribution of this cell type throughout the bone remodeling sequence. Frost (62) first hypothesized that bone remodeling involved a sequence of cellular events occurring at different remodeling sites along the bone surface. The first phase, activation, involved the activation of osteoclast precursors and subsequent osteoclast formation. Formed osteoclasts then resorbed, during the resorption phase, a portion of bone followed by osteoblast differentiation and formation (F phase) of new bone at the resorption sites (63). A detailed analysis of this hypothesis was conducted later by Tran Van et al. (64, 65) based on the induction of a reproducible synchronized remodeling sequence during tooth egression in rats. These authors categorized bone
remodeling into five phases: 1) activation, 2) resorption, 3) reversal, 4) formation, and 5) resting.

The bone remodeling sequence seems to proceed in the following manner. During the resting phase, the osteoid tissue is covered by a uniform layer of active osteoblasts; osteoclasts and mononuclear phagocytes usually are absent. The activation phase of bone remodeling is characterized by recruitment, recognition, and attachment of osteoclast precursors to specific areas of bone surface (63). Two days after induction by extraction of maxillary molars to allow mandibular molars to egress, morphology of osteoblast changes to a more flattened, elongated shape with reduced cytoplasmic extensions. Cells along bone surface are now a heterogeneous mixture of types, with cells of mononuclear phagocyte (MNP) characteristics (fluoride-inhibitable nonspecific esterase-positive cells) in close apposition to bone surface and osteoblasts. The MNP contain numerous coated pits and pseudopodia in the membrane facing the bone surface. Internalization of some bone material seems evident in mononuclear phagocytes, and the plasma membrane becomes ruffled.

When the activation phase ends, the resorption phase begins with the fusion of osteoclast precursor cells to form nascent osteoclasts. Bone matrix solubilization becomes prominent with mature osteoclasts evident over most of bone surfaces. Uncharacterized mononuclear cells of undetermined cell type surround the osteoclast.

As the resorption phase ends, osteoclasts detach from bone surfaces, and the reversal phase begins when large, highly phagocytic
mononuclear cells characteristic of mononuclear phagocytes occupy the Howship's lacunae created by the osteoclast. At the end of the reversal phase, cells with morphological characteristics of preosteoblasts and osteoblasts appear in the area previously occupied by the phagocytic MNP-like cells and deposit new bone matrix along the calcified cement line signaling the beginning of the formation phase. Bone matrix formation by osteoblasts continues to replace bone in areas resorbed by osteoclasts during the resorption phase (64, 65). These studies by Tran Van et al. (64, 65) indicate that cells of the mononuclear phagocyte family may play a role in both the activation phase during recruitment of osteoclast precursors and/or the reversal phase during the increased phagocytic activity of mononuclear cells in Howship's lacunae.

Monocyte-mediated bone degradation in parturient paresis

In vitro monocyte-mediated bone degradation may be a useful tool to study the apparent bone calcium mobilizing defect evident in parturient paresis. Parturient paresis (milk fever) is a hypocalcemic disorder in dairy cattle evident at the onset of lactation in older, high milk-producing cows. The disease is characterized by a coma induced by the rapid decline in plasma calcium and phosphorus concentrations brought about by the high demand for calcium at onset of milk production. Immediate treatment is the infusion of calcium gluconate, which sufficiently corrects the malady (66). The etiology of parturient paresis is unknown. Sufficient concentrations of PTH and calcitriol in blood plasma, compared with nonparetic cows, are present at
the onset of clinical signs of milk fever. Increases of plasma PTH and calcitriol concentrations are not delayed in response to hypocalcemia compared to nonparetic controls (67, 68). Calcitonin concentrations are lower in paretic cows than in normal cows, and thus does not seem to be a factor in induction of milk fever (69, 70). Evidence that sufficient calcitropic hormones are indeed present during onset of parturient paresis has lead to the current theory that end organ tissues, bone and/or intestine, are resistant to PTH and/or calcitriol signals for increased calcium mobilization from bone to blood (71, 72).

The theory of end organ resistance as the etiology for parturient paresis and evidence that increased bone resorption does not increase until 2 to 3 days postpartum (66) suggest that the bone mobilizing mechanism is quiescent at peak calcium demand. This prompted the development of strategies for prevention of milk fever to be targeted at priming the calcium homeostatic mechanisms during the prepartal period. Goings et al. (73) demonstrated that parturient paresis could be prevented by feeding cows a low calcium diet during the 2-week prepartal period. The low calcium dietary regiment stimulated PTH secretion, which probably was followed by PTH-stimulated renal production of calcitriol and subsequently increased intestinal absorption of calcium (73). Goff et al. (74) later reported that prepartal intravenous infusions of PTH prevented parturient paresis, thus again supporting the efficacy of priming the calcium homeostatic mechanisms
to alleviate the apparent end organ resistance to PTH at the onset of parturient paresis.

It is in the context of parturient paresis as a disease characterized by end organ resistance to PTH and calcitriol that we sought to characterize the *in vitro* monocyte-mediated bone degradation activity of monocytes isolated from paretic and nonparetic cows. A similar study was conducted by Key *et al.* (11) and Glorieux *et al.* (75) with patients afflicted with osteopetrosis, which is an end organ-resistant disease characterized by deficiencies in bone calcium mobilization and resultant sclerotic, dense bone (76). Key *et al.* (11) found that administration of large doses of calcitriol to an osteopetrotic child resulted in increased osteoclastic bone resorption and also an increase in monocyte-mediated bone degradation. Glorieux *et al.* (75) also demonstrated induction of bone resorption by PTH in congenital malignant osteopetrosis. These results, coupled with the proposal of Heersche (12) that monocytic bone degradation may be a mechanism distinct from osteoclastic bone resorption, prompted me to determine whether monocyte-mediated bone degradation is altered during the hypocalcemia associated with parturient paresis.

**Bone Calcium Mobilizing Hormones - Effects on Cells of Mononuclear Phagocyte Systems**

To understand the role of monocytes in the bone resorption process, a brief review of the bone calcium mobilizing hormones, PTH
and calcitriol, must be examined. The effects of PTH and calcitriol on cells of the mononuclear phagocyte system also will be addressed.

**Vitamin D metabolism and mechanisms of action of metabolites**

Vitamin D₃ is derived from either dietary intake or via photosynthetic (ultraviolet irradiation) conversion of 7-dehydrocholesterol to pre-vitamin D₃, which, in turn, rapidly isomerizes to vitamin D₃ and enters the circulation from the skin (77). Dietary vitamin D₃ is absorbed by the small intestine, enters the lymphatic circulation, and then enters the bloodstream (78). In the liver, vitamin D₃ is hydroxylated by the microsomal enzyme vitamin D₃-25-hydroxylase to form 25-hydroxyvitamin D₃ (25-OHD₃) (79). 25-OHD₃ is the major circulating form of vitamin D and is present in concentrations of 10 to 40 ng/ml \((10^{-8} \text{ M})\) in man (80) and is associated primarily with vitamin D-binding protein (81). Kidney uptake of 25-OHD₃ and further hydroxylation by the kidney mitochondrial enzyme, 25-hydroxyvitamin D₃-1α-hydroxylase (1α-OHase), results in the formation of 1,25-(OH)₂D₃ (calcitriol) (82). Production of calcitriol is elevated under conditions of calcium shortage and decreases under conditions of calcium excess. The concentration range of calcitriol in plasma is from 25 to 65 pg/ml \((10^{-11} \text{ M})\). During calcium excess, 25-OHD₃ is converted predominantly to 24,25-(OH)₂D₃ (83), a metabolite whose function has yet to be established convincingly. Calcitriol is the most active form of vitamin D and is the principal mediator of biological effects associated with vitamin D (84-87).
The mechanism of action of calcitriol is virtually identical to that of other steroid hormones (Fig. 1). Calcitriol circulates bound to plasma protein, primarily vitamin D-binding protein. Hormone circulating in the free state enters the cell because of its lipophilic nature and binds to its intracellular receptor. Formation of hormone-receptor complex results in an increased affinity for nuclear components relative to that of unoccupied receptor (88, 89). Binding of the hormone-receptor complex to specific DNA domains causes repression or induction of mRNA transcription, specific alterations in protein synthesis, and subsequent cell function modulation. Specific examples of 1,25-(OH)₂D₃-mediated changes in the synthesis of several cell proteins are shown in Fig. 1.

**Effects of calcitriol on cells of the mononuclear phagocyte system**

**Vitamin D deficiencies** Urist and McLean (90) first suggested a role for vitamin D in hematopoietic cell differentiation by demonstrating a dramatic increase in number of bone marrow mast cells in rats fed a vitamin D-deficient, low calcium diet. Further evidence was provided by reports that children with vitamin D-deficient rickets often have increased severity and rates of infections (91), and anemia
Figure 1. Schematic model of calcitriol-receptor interactions and the induction of cellular responses. DBP = vitamin D binding protein; S = steroid; R = receptor; CaBP = calcium binding protein.
and extramedullary hematopoiesis (92). Isolated monocytes and macrophages from ricketic children and mice also have decreased phagocytic ability (93, 94). These defects in bone marrow cellularity and phagocytosis are improved by vitamin D administration (92, 94, 95). Random migration of neutrophils is decreased (96), and capacity of neutrophils to phagocytize Escherichia coli also is impaired (41) in patients with vitamin D-deficient rickets. An observation related to isolated macrophages in vitamin D deficiency was demonstrated by Kahn et al. (97): in vitro macrophage-mediated bone degradation dysfunction was observed in isolated macrophages from vitamin D-deficient animals, but normalized macrophage-mediated degradation activity was evident following administration of calcitriol to mice before cell isolation (97).

**Effect of calcitriol on transformed cell lines - myeloid leukemia cells**

Cell growth is decreased and the rate of differentiation to monocyte-macrophages is increased in human and murine myeloid leukemia cells in the presence of calcitriol as demonstrated in M1-mouse myeloid leukemia cells (13), HL-60-human myeloid leukemia cells (promyelocytic) (14-16), U937 cells-human histiocytic lymphoma cells (monoblast/histiocytes) (98-100), P388D1 cells-mouse macrophage-like lymphoma cells (101), and THP-1 cells-human monoblasts (102). The characteristics associated with monocyte-macrophage differentiation evaluated in these studies included morphologic changes, increased adherence, suppressed proliferation, induced phagocytic activity, C₃ rosette formation, chemical reduction of nitroblue tetrazolium,
increased α-naphthyl acetate esterase activity, expression of specific membrane antigens characteristic of cells of monocyte-macrophage lineage, and finally the expression of membrane surface antigens, including Fc and complement (C3) receptors (13-16, 98-102). Additionally, the acquisition of phenotypic characteristics of monocytes by HL-60 cells stimulated by calcitriol is preceded by a marked decrease in expression of c-myc oncogene (103).

The in vitro effects of calcitriol on myeloid leukemia cell growth and differentiation led Honma et al. (104) to study the effects of in vivo administration of calcitriol on the leukemogenicity of syngeneic SL mice and athymic nude mice inoculated with M1 leukemia cells. Mice treated with either calcitriol or 1α-hydroxyvitamin D₃ (1α-OHD₃) survived an average of 50% longer than did nontreated controls. These results indicate that calcitriol is effective in both inducing differentiation of M1 cells in vitro and in prolonging survival time of M1 cell-inoculated mice.

Evidence strongly suggests that calcitriol induction of differentiation in the transformed cell lines is receptor-mediated. Tanaka et al. (105) demonstrated that HL-60 cells express the calcitriol receptor protein, and Olsson et al. (99) reported that the U-937 cell line also expresses a similar high affinity receptor for calcitriol. Variant clones of HL-60, resistant to calcitriol-induced differentiation, express lesser amounts of calcitriol receptor protein (106). Mangelsdorf et al. (107) later reported that HL-60 cells responsive to calcitriol have approximately 4,000 high affinity calcitriol receptors
per cell (107), but a HL-60 variant line resistant to the growth and differentiation effects of calcitriol expressed less than 400 calcitriol receptor molecules per cell. These studies also demonstrated that the ability of several vitamin D metabolites to promote differentiation correlates with their affinity for the calcitriol receptor, thus indicating that calcitriol-induced differentiation of HL-60 to monocytes/macrophages is a receptor-mediated series of events.

**Effect of calcitriol on normal cells of the mononuclear phagocyte system**

The early studies in vitamin D-deficient animals that demonstrated a role for calcitriol in microbicidal host defense strongly suggested a direct effect of calcitriol on macrophage-associated effector functions. This is the case, for calcitriol activates the cytotoxicity of alveolar macrophages (108) and enhances secretion of hydrogen peroxide by human monocytes and by U937 monoblast cells (109, 110). The secretion of oxygen reduction products, such as hydrogen peroxide (H₂O₂) and superoxide anion (O₂⁻) by mononuclear phagocytes, is increased during phagocytosis and is essential for tumoricidal and microbicidal activity (111). Rook et al. (112) showed that calcitriol induced an anti-tuberculosis activity in monocytes by controlling the proliferation of intracellular *Mycobacterium tuberculosis*. Calcitriol also induces fusion of alveolar macrophages (108, 113) and HL-60 promyelocytic cells (16), and also enhances secretion of interleukin-1 (IL-1) by monocytes (114). IL-1 is an established modulator of T and B lymphocyte proliferation and also induces collagenase and proteoglycanase activity in chondrocytes.
(115), which, in turn, may facilitate IL-1 stimulation of in vitro bone resorption (59).

Other studies on the effects of calcitriol on monocytes have involved cellular adhesion and maintenance of adherence to substrate. Polla et al. (116) demonstrated that normal monocytes treated with calcitriol maintained adherence to tissue culture plastic as well as fibronectin-coated plastic. This study also reported calcitriol-mediated protection of monocytes from thermal injury by increasing synthesis of heat shock proteins, protecting normal protein synthesis, and increasing recovery rate of normal protein synthesis after thermal stress (116). Franceschi et al. (117) later reported that calcitriol increases cell-associated fibronectin and concentrations of fibronectin in growth media of MG-63 human osteosarcoma cells, TE-85 human osteosarcomas, SW-480 human colon carcinomas, and HL-60 myeloid leukemia cells. Accompanying the increased fibronectin synthesis by the MG-63 cells was an increase in cell adhesion (117).

Fibronectin is a constituent surface glycoprotein of many normal cells that is present in lesser amounts or absent in malignantly transformed cells (118, 119). Accompanying the loss of FN in malignant cells is lesser cell adhesion, rounded morphology, and loss of skeletal organization (120). The previous report with calcitriol-treated HL-60 promyelocytic leukemia cells also showed increased adherence to plastic (16) accompanying the differentiation of cells along the monocyte-macrophage lineage. This report, coupled with evidence of calcitriol-stimulated fibronectin synthesis and increased
adherence demonstrated by Franceschi et al. (117), suggests that increased adherent capacity by cells also may be mediated by increased synthesis of fibronectin. Polla et al. (121) also demonstrated that calcitriol induces the binding of U937 cells to collagen, a component of the extracellular matrix, thus suggesting another mechanism for calcitriol-induced maintenance of adherence in cells of the mononuclear phagocyte system.

The calcitriol-induced differentiation of transformed cells along the monocyte/macrophage pathway also has been investigated for normal cells. In the presence of calcitriol, normal bone marrow cells demonstrated increased adherence and esterase activity and suppressed colony stimulating factor (CSF)-dependent formation of colony-forming units (15, 122). Clohisy et al. (123), however, reported that the inhibitory effect of calcitriol on colony formation was independent of CSF-1 and targeted through the adherent bone marrow-macrophage precursor. Accompanying differentiation was the up-regulation of the mannose receptor, a cell membrane receptor specifically associated with mononuclear phagocyte differentiation (124-126). Provvedini et al. also reported that calcitriol-treated monocytes developed in vitro morphologic changes and increases in lysosomal enzyme activities that are characteristic of macrophage differentiation (127). In contrast to the studies reporting calcitriol-induced inhibition of myeloid leukemia cell growth and normal bone marrow-derived macrophage differentiation, Munker et al. (102) reported that cells from the CSF-dependent human myeloblast line (KG-1) and from normal human
granulocyte-monocyte stem cells (GM-CFC) were stimulated in their clonal proliferation by calcitriol in the presence of suboptimal CSF concentrations.

The studies presented in this section demonstrate that calcitriol mediates profound effects on normal cells of the mononuclear phagocyte system which include inhibition of growth, differentiation, activation and production of $\text{H}_2\text{O}_2$, maintenance of adherence, and finally enhanced proliferation under certain suboptimal conditions.

**Parathyroid hormone metabolism and mechanisms of action**

Parathyroid hormone acts directly on bone and kidney and indirectly on intestine through the stimulation of $\alpha$-OHase and the resulting synthesis of calcitriol. A decrease in plasma calcium concentration below normal results in a rapid increase in PTH secretion causing 1) an increase in calcium resorption from bone, 2) reduced renal excretion of calcium, 3) increased renal excretion of phosphate, and 4) enhanced efficiency of intestinal calcium absorption because of increased formation of calcitriol (128).

Parathyroid hormone is a protein produced by the chief cells of the parathyroid gland. Biosynthesis and secretion of PTH involves an initially large precursor molecule, pre-pro-PTH (115 amino acids [a.a.]) (129-132), that undergoes proteolytic cleavages to yield first pro-PTH (90 a.a.) (133-134) and then the native hormone PTH (84 a.a.) (135-137). Pre-pro-PTH contains an additional 25 a.a. (-31 to -7) on the NH$_2$ terminus of pro-PTH molecule. This 25 a.a. sequence is believed to be the "signal-leader" sequence that permits binding to
the endoplasmic reticulum of polyribosomes synthesizing the secreted protein and directs and facilitates the transport of newly synthesized protein across the intracellular membrane. The "pre" segment is believed to be removed and degraded by proteolytic cleavage during this transit (138, 139). Pro-PTH (~6 to ~1, 1-84 a.a.) then is transferred to the Golgi apparatus and converted to PTH (1-84 a.a.) (140). Parathyroid hormone subsequently is stored and secreted via secretory granules. Heterogeneity in the circulating forms, i.e., 1-84, 1-34, 35-84, of PTH exists because of proteolysis that seems to occur at two physiological sites — within the parathyroid gland and in peripheral organs (128). Proteolysis in the peripheral organs occurs rapidly as shown by the rapid disappearance of intact PTH from the blood ($t_{1/2} = 1-10 \text{ min}$) coupled with simultaneous appearance of PTH fragments (141-143). Principal among the peripheral organs responsible for proteolysis is the liver, where Kupffer cells contain specific endopeptidases that generate both -NH$_2$ and -COOH terminal fragments cleaved to form the NH$_2$ 1-34 a.a. PTH (144, 145). It is this 1-34 NH$_2$ terminal fragment that is required for biological activity (146). The kidney seems responsible for removal of proteolytically-formed carboxyl fragments (147, 148). Positions 1 and 2 of the 1-34 NH$_2$ segment of PTH are essential for bioactivity (146), and the region 3 through 34 contains the structural determinants necessary for receptor binding (149-151).

The mechanism of action of PTH involves interaction with a hormone-specific plasma membrane receptor. Hormone-receptor binding
initiates a series of biochemical events that include: generation of cAMP via adenylate cyclase, intracellular entry of calcium, and phosphorylation of specific intracellular proteins by PTH-activated kinases. This cascade of intracellular mechanisms ultimately leads to PTH-mediated biological activity in respective target cells.

**Effects of parathyroid hormone on mononuclear phagocytes, lymphocytes, and osteoblasts** Parathyroid hormone increases the metabolic activity and number of osteoclast *in vivo* (3,4). Parathyroid hormone also increases activity of osteoclasts in cultures of fetal rat bone, resulting in an increase in overall size of cells and an increase in area of the ruffled border and clear zone (152-155). Feldman et al. (156) reported that hormonally induced changes in osteoclast activity were correlated with PTH-induced fusion of pre-existing postmitotic mononuclear osteoclast precursors to form osteoclasts. The characteristics and identity of the osteoclast precursor cells are unknown, although, as detailed earlier, evidence strongly suggests their origin is the less mature (monoblast, promonocyte) cells of the mononuclear phagocyte system.

Monocytes and macrophages, more mature cells of mononuclear phagocyte system, have been investigated for PTH-induced effects. The results of these studies are confusing and unclear. Yamamoto et al. (157) demonstrated that circulating bovine lymphocytes, but not monocytes, contain the receptor for PTH. Adenylate cyclase activity was increased in PTH-treated lymphocyte cultures, but not in monocyte cultures treated with PTH. Perry et al. (158) also showed that
circulating human lymphocytes contain the PTH receptor and that cAMP accumulation increases threefold after a 5-min exposure of lymphocytes to PTH. PTH-induced changes in monocyte-macrophages, however, have been reported. Craig et al. (159) demonstrated increased lysosomal enzyme synthesis by culture monocytes treated with PTH, and Minkin et al. (160) reported increased cAMP accumulation in elicited mononuclear phagocytes in the presence of PTH. Stock et al. (161, 162), however, reported that PTH inhibits monocyte production of cAMP and also demonstrated that PTH decreased H₂O₂ production and chemiluminescence in monocytes.

The contradictory reports of a direct effect of PTH on monocytes, cells with no detectable PTH receptor expression, has led to the suggestion that cells, such as osteoblasts and/or lymphocytes that express the PTH receptor (157, 158, 163-165), serve a role in the PTH-induced effects on monocytes. A similar hypothesis for an intermediate cell role, i.e., osteoblast, has been proposed for the osteoclast during the resorption process (166). Osteoclast expression of PTH receptors is controversial (167-170). Isolated osteoclasts do not resorb bone in vitro in the presence of PTH (171), but addition of osteoblasts in the presence of PTH (172) or conditioned medium from osteoblasts cultured with PTH or calcitriol does stimulate osteoclast bone resorption activity (173-175). These studies suggest that the osteoblast, as an intermediate cell, may mediate PTH-induced osteoclast bone resorption. Parathyroid hormone effects on monocytes and monocyte-mediated bone degradation may be mediated in a similar
fashion, although conditioned medium from osteoblast-like cells in the presence of PTH did not affect *in vitro* monocyte-mediated bone degradation (175).

Lymphocytes also may mediate a PTH-induced effect on monocytes. Whitfield *et al.* (176) first reported increased mitotic activity and increased cAMP accumulation in PTH-treated, thymic lymphocytes. Mitogen-stimulated lymphocytes elaborate a potent bone-resorbing factor(s) that has been termed osteoclast activating factor (OAF) (177). Products of activated lymphocytes also affect monocyte function. Nathan *et al.* (178) demonstrated an increase in adherence, phagocytosis, spreading, motility, and direct oxidation in macrophages treated with conditioned medium from antigen-sensitized lymphocyte cultures. Enhancement of adenylate cyclase activity in macrophages by activated lymphocytes also has been demonstrated by Remold-O'Donnell *et al.* (179). Additional evidence reported by Perry (180) that PTH stimulates lymphocyte production of factors that increase bone resorption suggests a possible physiological role for PTH-lymphocyte interaction in bone resorption. These studies by Nathan *et al.* (178) and Remold-O'Donnell *et al.* (179) suggest that PTH-activated lymphocytes also may influence monocyte-mediated bone degradation activity in a mechanism similar to that proposed for activation of osteoclast bone resorption.
Calcitriol Effects on Immunoregulation

Calcitriol receptors in cells of the immune system

The initial unexpected findings that cells of hematolymphopoietic tissues express the calcitriol receptor suggested a role for calcitriol in the immune system. Reinhardt et al. (17) first discovered calcitriol receptors in calf thymus glands and lymph nodes. Further studies by Provvedini et al. (18) and Ravid et al. (19) demonstrated calcitriol receptor expression in the large, mature, mitotically active, medullary thymocytes, but not in the small, immature cortical thymocytes. Activated lymphocytes present in thymus and tonsils of normal children also contain a calcitriol receptor-like protein (182). Resting peripheral T and B lymphocytes, however, do not possess the calcitriol receptor, but in vitro activation of lymphocytes with Epstein Barr virus or mitogenic lectins, such as phytohemagglutinin (PHA) or concanavalin A (ConA), or by alloantigens in the mixed lymphocyte reaction results in expression of calcitriol receptor (20–22, 181, 182). Moreover, lymphocytes in patients with rheumatoid arthritis are activated and express the calcitriol receptor (183), and numerous T and B lymphocyte cell lines also express the calcitriol receptor (181).

Provvedini et al. (21) and Bhalla et al. (20) demonstrated that, in contrast to resting T and B peripheral lymphocytes, monocytes constitutively express the calcitriol receptor. Myeloid leukemia cell lines and several monocytic precursor cell lines also express the calcitriol receptor. These cells in the presence of calcitriol
differentiate to a more mature monocyte-macrophage cell (13, 14, 98, 101, 102, 105, 107).

**Effects of calcitriol on thymocytes**

The studies demonstrating calcitriol receptor expression in thymocytes suggested that the mature medullary thymocyte subpopulations are targets for calcitriol actions (18, 19). Provvedini et al. showed that primary cultures of rat thymic lymphocytes in the presence of calcitriol were protected against the spontaneous lytic involution evident in control culture in the absence of calcitriol (184). Ravid et al. (19) and Koizumi et al. (25) also reported that murine medullary thymocyte proliferative responses to PHA and IL-2, or IL-1 and IL-2 alone, was inhibited, but that calcitriol had no effect on the cortical thymocyte subpopulations. The calcitriol-induced inhibitory effect could be abrogated by addition of high concentrations of IL-2, suggesting IL-2 suppression may be a factor responsible for calcitriol-mediated inhibition. These results suggested a possible role for calcitriol in intrathyrmic differentiation of T cells.

**Effects of calcitriol on activated lymphocytes**

Stimulation of lymphocytes by mitogenic lectins leads to secretion of lymphokines, lymphocyte proliferation and differentiation into effector cells. Mitogenic lectins or antigens in association with monocytes promote the lymphokine cascade that includes secretion of interleukin 1 (IL-1) from activated monocytes, expression of the interleukin 2 receptor (IL-2Rec), and induction of IL-2 production by
activated T lymphocytes. The subsequent IL-2 binding to the IL-2Rec results in lymphocyte proliferation by an autocrine mechanism (185).

The discovery that stimulated lymphocytes express the calcitriol receptor initiated a series of experiments to determine the effects of calcitriol on lymphocyte proliferation, lymphokine production, and differentiation. Tsoukas et al. (23) demonstrated that calcitriol inhibited the proliferation and the secretion of IL-2 of phytohemagglutinin (PHA)-activated T lymphocytes. Other vitamin D₃ metabolites were less effective than calcitriol in suppressing IL-2. Their capacity to suppress IL-2 correlated with their affinity for the calcitriol receptor, thus suggesting a receptor-mediated event for IL-2 suppression (23). Rigby et al. (24) confirmed these findings and also reported that the calcitriol-induced inhibition of lymphocyte proliferation could be attenuated but not abrogated by addition of exogenous IL-2. This attenuation indicated that calcitriol might be exerting its inhibitory effects at other points in lymphocyte mitogenesis distinct from the inhibition of IL-2 secretion. Rigby et al. (186) later reported that calcitriol also markedly inhibited production of γ-interferon (γ-IFN) in PHA-induced T lymphocytes. Bhalla et al. (187) discovered that calcitriol inhibited the proliferative response of immune spleen and thymus cells to antigen but not to the lectin ConA. This report also demonstrated that calcitriol inhibited IL-2 production of cloned Ia-restricted T cell hybridomas (calcitriol receptor positive) activated with cloned Ia-bearing (calcitriol receptor negative) stimulator cells. This study suggests that
calcitriol may be interfering with early events of antigen-induced T cell activation, possibly by interfering with T cell recognition of relevant antigen on stimulator cell surfaces (187). Relevant to this hypothesis, Matsui et al. (188) examined the effect of calcitriol on several cell surface antigens on PHA-activated human PBM in vitro. Expression of OKT3+ (peripheral T-cell), OKT4+ (helper/inducer T cell), and OKT9+ (transferrin receptor cell) markers were decreased significantly in the presence of calcitriol, but IL-2 receptor (Tac+) expression was unaffected by calcitriol. The T3 (OKT3+) molecule is present on all T lymphocytes and is believed to be involved in the antigen-recognition process (189-191). Thus, these studies demonstrating diminished expression of this molecule in the presence of calcitriol suggests that calcitriol-mediated inhibition of lymphocyte DNA synthesis may be the result to some extent of a direct effect on the T lymphocyte, specifically the antigen-recognition mechanism. Finally, as shown by Matsui et al. (188), the evaluation of the effects of calcitriol on proliferation of mitogen-induced T lymphocyte subpopulations has demonstrated that both T helper/inducer and T suppressor cell populations are sensitive to the growth inhibitory effects of calcitriol (192, 193).

Cell cycle analysis of calcitriol-induced inhibition of mitogen-driven T lymphocyte proliferation has characterized more thoroughly this inhibitory effect. Rigby et al. (194) reported in flow cytometry studies of PHA-induced human PBM that calcitriol blocks transition from the early $G_{1A}$, low RNA compartment, to late $G_{1B}$, high RNA
compartment, and also demonstrated that calcitriol does not affect IL-2 receptor expression. Additions of IL-2 or indomethacin, an inhibitor of prostaglandin production, attenuated the calcitriol-induced suppression of DNA synthesis, but addition of IL-1 had no effect on reversal of calcitriol-induced inhibition. These findings suggested that, in addition to earlier reports of calcitriol-induced suppression of IL-2 production, calcitriol induces inhibition of proliferation via a prostaglandin-dependent component, possibly from enhancement of monocyte production of prostaglandin \( E_2 \) in the presence of calcitriol (195). Manolagas et al. (196) also demonstrated that the antiproliferative effect of calcitriol on lymphocyte proliferation was not caused by interference with the calcium-dependent lymphocyte activation process nor by extracellular calcium, thus suggesting that alteration of calcium translocation flux across the cell membrane was not a factor in the inhibitory effects of the steroid hormone.

Effects of calcitriol on B lymphocyte and cytotoxic lymphocytes also have been examined. Lemire et al. (197) demonstrated calcitriol-induced suppression of PWM-induced proliferation and immunoglobulin production by human PBM. Iho et al. (198) also showed that activation of isolated human B lymphocyte cultures with either PWM or \textit{Staphylococcus aureus} Cowan 1 in the presence of calcitriol resulted in suppression of proliferation and immunoglobulin production. Similar results showing calcitriol-induced inhibition of immunoglobulin secretion have been reported for Epstein Barr virus-infected cells (199) and PWM-activated human PBM in serum-free culture conditions.
Komoriya et al. (201), by using a T cell-dependent antigen, sheep red blood cells (SRBC), and a T cell-independent antigen, trinitrophenyl-lipopolysaccharide, to stimulate immunoglobulin synthesis in immune splenocytes, demonstrated that calcitriol-mediated inhibition of antibody production was evident only in the T cell-dependent, antigen-driven system. These results suggest that inhibition of immunoglobulin synthesis by calcitriol may be mediated by the T lymphocyte and/or macrophage. Manolagas et al. (181), however, reported that Epstein-Barr virus-induced antibody production (T cell-independent response) was inhibited by calcitriol addition and that IL-2 additions did not reverse these inhibitory effects, thus suggesting once again that the effects of calcitriol on immune response may be mediated through mechanisms other than suppression of IL-2 secretion.

In contrast to the antiproliferative effects of calcitriol, Lacey et al. (26) reported calcitriol-induced stimulation of ConA-induced proliferation of a nontransformed T-helper cell line (D10.G4.1) in the absence of IL-1 or at low IL-1 concentrations. If, however, IL-1 concentrations necessary for optimal response are employed, the calcitriol effect was inhibitory. My colleagues and I have demonstrated in a previous study that calcitriol both inhibits and enhances ConA-induced DNA synthesis in normal bovine PBM (202). We reported that the contrasting effects of calcitriol on ConA-induced response were dependent on lymphocyte density, monocyte numbers, and basal rates of DNA synthesis. Calcitriol-mediated enhancement of ConA-
induced proliferation was evident typically when proliferation was suboptimal, whereas calcitriol-mediated inhibition was evident in cultures with high proliferation rates. A similar pattern of calcitriol-induced enhancement of cell growth has been shown in bone marrow cultures. Munker et al. (102) demonstrated that normal human granulocyte-monocyte bone marrow stem cells were stimulated in their clonal proliferation by calcitriol in the presence of suboptimal concentrations of CSF.

**Effects of calcitriol on immune response-related monocyte functions**

Monocyte Class II MHC antigen expression and its role in antigen presentation (203, 204) and monocyte IL-1 production and its subsequent induction of IL-2 production (205, 206) and IL-2 receptor expression (207) are pivotal events in the early activation steps of lymphocyte proliferation. Evidence suggests that calcitriol increases both Class II MHC antigen expression and IL-1 production. Bar-Shavit et al. (16) reported increased expression of HLA-DR antigens (human Class II MHC) in HL-60 promyelocytic cells in the presence of calcitriol, and Morel et al. (208) demonstrated increased Ia antigen (mouse Class II MHC) expression in γ-IFN-induced myelomonocytic (monocyte tumor cell line) cell line WEHI-3. Enhanced expression of Ia molecules by WEHI-3 lead to the increased capacity of these cells to stimulate antigen-specific, Ia-restricted T cell activation (208). The effect, however, of calcitriol on Class II MHC expression on normal monocytes is unknown. Calcitriol also may affect monocytes by increasing IL-1 production. Amento et al. (209) reported that IL-1
production by a lymphokine-induced immature monocytic cell line, U937, was augmented in the presence of calcitriol. Hodler et al. (210) also demonstrated that calcitriol increased IL-1 production by P388D cells, a murine macrophage cell line; Bhalla et al. (211) later showed that peripheral blood monocytes produced increased IL-1 following calcitriol treatment.

Several recent discoveries also have suggested that calcitriol may act as an autocrine or paracrine factor in the microenvironment of immune responses. Extra-renal production of calcitriol initially was reported by Adams et al. (212, 213), who demonstrated in vitro production of calcitriol from 25-OHD₃ in primary cultures of alveolar macrophages from patients with sarcoidosis associated with hypercalcemia. Lymph node homogenates from a patient with sarcoidosis metabolized 25-OHD₃ to calcitriol, but lymph nodes from six normal persons failed to produce calcitriol (214). γ-Interferon- or lipopolysaccharide (LPS)-stimulated normal alveolar macrophages also convert 25-OHD₃ to calcitriol (215, 216), and Reichel et al. (217) also demonstrated that γ-IFN-stimulated normal bone marrow macrophages synthesize calcitriol. These results suggest that calcitriol concentrations may be regulated in a paracrine fashion in the bone marrow and lungs via activated macrophage synthesis of calcitriol to 1) induce differentiation of cells of the mononuclear phagocyte system, 2) enhance antigen processing via upregulation of Class II MHC antigen expression and IL-1 production by macrophages, and/or 3) induce
negative feedback loop on T lymphocyte proliferation by suppressing IL-2 production.
SECTION I.

CALCITRIOL ENHANCEMENT OF CONCANAVALIN A-INDUCED
BOVINE LYMPHOCYTE PROLIFERATION: REQUIREMENT
OF MONOCYTES
1,25-Dihydroxyvitamin D₃ (calcitriol) regulates calcium homeostasis and mediates its biological effects by interaction with a specific intracellular receptor (1). Recent studies have shown that calcitriol induces monocytic differentiation in murine and human myeloid leukemia cells (2-5) and that the thymus, monocytes, and activated T and B lymphocytes express the calcitriol receptor (6-9). Data from these studies suggested an immunoregulatory role for calcitriol. Calcitriol also inhibits proliferation of mitogen-stimulated peripheral blood mononuclear cells (PBMC) (10, 11) and murine medullary thymocytes (12) and also suppresses interleukin-2 (IL-2) production (10, 11). In contrast to the well-documented antiproliferative effects of calcitriol, Lacey et al. (13) reported that calcitriol stimulated proliferation of a nontransformed T-helper cell line in the absence of interleukin 1 (IL-1) or at low IL-1 concentrations while inhibiting proliferation at IL-1 concentrations necessary for optimal response. The present study demonstrates the effect of calcitriol on bovine lymphocyte mitogenesis and, more importantly, that calcitriol has both proliferative and antiproliferative activities in ConA-mediated mitogenesis of nontransformed cells.
MATERIALS AND METHODS

Cell Isolation and Enrichment

Peripheral blood mononuclear cells (PBM), from 1- to 2-year-old Jersey heifers were isolated as previously described by using Percoll (Pharmacia, Piscataway, NJ) (14). PBM were recovered, washed twice in Hank's balance salt solution (HBSS) without Ca\textsuperscript{++} and Mg\textsuperscript{++}, once in phosphate buffered saline (PBS), pH 7.2, and resuspended in RPMI 1640 media containing 10 mM HEPES, pH 7.3 (Gibco, Grand Island, NJ), penicillin G (100 U/ml), and streptomycin (100 mg/ml).

Nonadherent PBM were obtained by incubating PBM in RPMI 1640 with 10% heat inactivated fetal bovine serum (FBS) in 75 cm\textsuperscript{2} flasks (1.33 x 10\textsuperscript{6} PBM/cm\textsuperscript{2}) for 90 min at 39°C in 5% CO\textsubscript{2} and 95% air. Nonadherent PBM were recovered by washing the flask twice with HBSS, centrifuging the pooled washes at 300 X g for 10 min, and resuspending in RPMI 1640. These cells represented ~60% of PBM plated.

Adherent PBM were prepared by plating 0.2 ml of increasing numbers of PBM (0.12, 0.25, 0.38, and 0.50 x 10\textsuperscript{6} cells/ml) into 96-well, flat-bottomed microtiter plates and incubating for 90 min at 39°C in an atmosphere of 5% CO\textsubscript{2} in air. Nonadherent cells were removed by vigorous agitation of the plates and washing with HBSS. Nonadherent cells were reintroduced (0.25 and 0.5 x 10\textsuperscript{6} cells/ml) to evaluate mitogenesis with differing ratios of monocytes. Adherent cells prepared by this method represented an average of 18% of original PBM plated and consisted of 84% monocytes as determined by alpha-naphthyl acetate esterase staining. Adherent cells (monocytes) prepared
by this procedure did not incorporate significant $^3$H-thymidine into DNA in response to ConA or calcitriol (data not shown).

**Culture and Proliferation Conditions**

Cells were cultured in 0.2 ml of RPMI 1640 with 6% heat-inactivated FBS in 96-well plates. ConA, phytohemagglutinin (PHA), or pokeweed mitogen (PWM) (Sigma, St. Louis, MO) concentration, cell numbers, and relative percentages of monocytes (adherent) and nonadherent PBM varied as indicated in the figures. All culture conditions were examined in the presence or absence of $1,25(OH)_2D_3$ (obtained from Dr. M. Uskokovic, Hoffmann-La Roche, Inc., Nutley, NJ) added at the initiation of culture. Cells were cultured for 72 hr at 39°C in 5% CO$_2$ and 95% air, pulsed 18 hr with 2 μCi of [methyl-$^3$H]-thymidine (5.0 Ci/mmol, Amersham, Arlington Heights, IL), harvested onto glass fiber filters with a PHD cell harvester (Cambridge Technology, Inc., Cambridge, MA), and counted by liquid scintillation spectrophotometry. All assays were done in triplicate. Results were expressed as percentage of $[^3$H]-thymidine incorporation in cultures without calcitriol. Significant differences between treatment means were determined by least significant difference.
RESULTS

Effect of Calcitriol on Bovine PBM Proliferation

Bovine PBM mitogenic responses to PHA and PWM were inhibited by addition of $1-8 \times 10^{-10}$ M calcitriol (Fig. 1). The effect of calcitriol on ConA-mediated proliferation, however, was dependent on the magnitude of the proliferative response. Lymphocytes from cows with high inherent proliferative response ($134,904 \pm 15,488$ cpm/well) were unaffected by calcitriol, whereas lymphocytes from cows with low initial proliferative response ($46,317 \pm 6,000$ cpm/well) were significantly stimulated by $2$ to $8 \times 10^{-10}$ M calcitriol (Fig. 1).

Effect of Adherent PBM (Monocyte) Addition on ConA-Induced Proliferation of Low- and High-Density Nonadherent PBM Cultures

The data of Fig. 1 suggest that calcitriol-mediated enhancement of ConA-induced PBM mitogenesis is dependent on initial magnitude of the proliferative response. To establish in vitro conditions in which the magnitude of ConA-induced mitogenesis is either low or high, low- and high-density nonadherent PBM cultures supplemented with increasing numbers of monocytes (adherent cells) were examined for their proliferative responses to ConA (Figs. 2a, b). High-density nonadherent PBM without monocyte supplementation were more responsive to ConA than were unsupplemented low-density nonadherent PBM cultures. Increasing monocyte numbers in both low- and high-density nonadherent PBM cultures increased their proliferative response to ConA.
Figure 1. Effects of calcitriol on mitogenesis of bovine PBM. PBM (0.5 x 10^6/ml) in 0.2 ml of medium were supplemented with either PHA (25 µg/ml, △), PWM (2.0 µg/ml, ○), or ConA (8.0 µg/ml, low-response cows, ■, and high-response cows, ○). Responses are expressed as percent (X ± SEM) of individual controls in the absence of 1,25-(OH)₂D₃. Mean ± SEM [³H]-thymidine incorporation (cpm) of PBM controls were: PHA (101,054 ± 18,867, n = 5), PWM (86,411 ± 18,648, n = 4), ConA high response (134,904 ± 15,480, n = 4), and ConA low response (46,317 ± 6,000, n = 4).
Figure 2. ConA-induced mitogenesis of low-density (0.25 x 10⁶/ml), Fig. 2a) and high-density (0.5 x 10⁶/ml, Fig. 2b) non-adherent PBM with increasing monocyte additions. Responses expressed as CPM (X ± SEM, n = 6).
Proliferation in cultures of low-density nonadherent PBM was more dependent on monocyte supplementation than were cultures of high-density nonadherent PBM cultures. Proliferative responses of low-density cultures were less than those of high-density cultures regardless of the number of monocytes added.

Effect of Calcitriol on ConA-Induced Proliferation of Low- and High-Density Nonadherent PBM Cultures Supplemented with Monocytes

The ConA-induced proliferative response in cultures of low-density nonadherent PBM was inhibited significantly, p < 0.05, by 0.48 to 0.96 nM calcitriol. Monocyte additions to 7 and 13% of the total cells abrogated the inhibitory effects of calcitriol. Monocyte supplementation to 18 and 23% in the presence of calcitriol enhanced the proliferative responses by 80% (p < 0.01) as compared with responses of cultures without calcitriol (Fig. 3a).

Proliferative responses of high-density PBM cultures to ConA were inhibited by calcitriol additions at all monocyte supplementation concentrations (Fig. 3b). The magnitude of inhibition by 0.48 and 0.96 nM calcitriol, however, was attenuated significantly, p < 0.05, with increasing monocyte addition. Addition of calcitriol resulted in up to a 50% inhibition of the ConA-induced proliferation in cultures of high-density nonadherent PBM (without monocyte addition). Monocyte supplementation to 13% of total cells in high-density, nonadherent PBM cultures were, however, inhibited less than 15% with calcitriol addition (Fig. 3b).
Figure 3. Effect of calcitriol and monocytes on ConA-induced lymphocyte proliferation. Responses are expressed as a percent of the mean $[^{3}H]$-thymidine incorporation of controls not treated with calcitriol ($n = 6$). (a) Low-density nonadherent PBM supplemented with 0% (○), 7% (●), 13% (▲), 18% (▼), and 23% (■) monocytes (percent of total cells). ConA was present at 1 μg/ml. (b) High-density nonadherent PBM cells supplemented with 0% (○), 7% (●), 10% (▲), and 13% (■) monocytes. ConA was present at 8 μg/ml.
DISCUSSION

This study demonstrates that calcitriol inhibits PHA- and PWM-stimulated bovine PBM proliferation as previously reported for human lymphocytes (10, 11). We have shown that lymphocytes from cows that had a high initial proliferative response to ConA were unaffected by calcitriol. We also have shown that lymphocytes from cows that had a low initial proliferative response to ConA had significantly greater proliferation rates in the presence of calcitriol.

The role of initial proliferation rate on the calcitriol-induced effect was examined by supplementation of low- and high-density nonadherent PBM with different proportions of monocytes. The effect of calcitriol addition to low proliferation rate cultures (low cell density), in the absence of monocyte supplementation, was inhibition of proliferation. Monocyte supplementation to 18 and 23% of plated cells resulted in calcitriol-mediated enhancement of proliferation (Fig. 3a). The mechanism of calcitriol enhancement of ConA-induced proliferation is unknown. It may result from the ability of calcitriol to activate monocytes (15, 16) and increase Ia expression (Class II MHC antigen) in immature monocytic cell lines (4, 17). Calcitriol also increases IL-1 secretion in a macrophage cell line (18) and augments IL-1 secretion in a monoblast cell line induced with conditioned medium from lectin-stimulated T lymphocyte cultures (19).
These effects of calcitriol on monocytes may mediate the enhancement of suboptimal ConA-induced responses.

The addition of calcitriol to high proliferation rate cultures (high cell density) also inhibited proliferation in the absence of monocytes. This inhibition was attenuated by monocyte additions (Fig. 3b). Previous work has shown that addition of exogenous IL-2 (11), but not IL-1, attenuates the inhibitory effects of calcitriol (20) on proliferation. These data suggest that the monocyte-dependent attenuation of the inhibitory effect by calcitriol in high proliferation rate cultures, seen in our experiments, may involve reversal of calcitriol-mediated IL-2 suppression.

This study demonstrates that calcitriol possesses both proliferative and inhibitory effects on bovine ConA-induced proliferation, which are dependent on nonadherent PBM density, monocyte numbers, and basal rates of proliferation. These data suggest that monocytes mediate both calcitriol-induced enhancement of ConA-induced proliferation in low-density cultures and attenuation of inhibition in high-density cultures.
LITERATURE CITED


SECTION II.

PARATHYROID HORMONE ADMINISTRATION STIMULATES MONOCYTE-MEDIATED BONE DEGRADATION, AND CALCITRIOL ADMINISTRATION ENHANCES DNA SYNTHESIS IN CONCANAVALIN A-INDUCED BOVINE LYMPHOCYTES
INTRODUCTION

Monocytes are located in regions of osteoclasts, which are the principal bone-resorbing cell of the body, and in regions of chronic inflammation, such as rheumatoid arthritis and periodontal disease (1-4). Recent evidence suggests a role for monocytes in the bone resorption process. Monocytes secrete products believed essential for bone resorption, such as lysosomal enzymes and collagenase (6), and monocytes degrade devitalized bone via a contact-mediated process (7, 8). Additional studies suggest that monocytes may be recruited via chemotaxis to areas of resorbing bone (9, 10). Moreover, monocytes produce factors, such as interleukin 1 (IL-1) (11, 12), and certain prostaglandins (13) stimulate osteoclast bone resorption in vitro. Monocytes also may serve an important role in the osteolytic process at sites of chronic inflammation. Inflammation-mediated osteopenia has been demonstrated in the rat (14). In addition, endotoxin treatment of isolated monocytes has been shown to enhance in vitro monocyte-mediated bone degradation (15). These findings suggest that monocytes may play an important role in the bone resorption process.

The bone calcium mobilizing hormones, parathyroid hormone (PTH) and 1,25-dihydroxyvitamin D₃ (calcitriol), both mediate changes in monocyte function. Numerous studies have reported PTH-induced changes in monocyte functions. Craig et al. (16) demonstrated increased synthesis of lysosomal enzymes by cultured monocytes treated with PTH, and Minkin et al. (17) reported increased cAMP concentrations in
elicited mononuclear phagocytes treated with PTH. Stock et al., however, reported that PTH inhibits production of cAMP and decreases H$_2$O$_2$ production and chemiluminescence in monocytes (18, 19). Despite these reports demonstrating effects of PTH on monocyte function, expression of PTH receptors on monocytes has not been demonstrated (20, 21).

The effect of calcitriol on monocyte characteristics and functions are well established. Monocytes constitutively express the calcitriol receptor, and calcitriol has been shown to be a potent mediator of differentiation of mononuclear phagocytes. Calcitriol induces monocytic differentiation of myeloid leukemia cells (22, 23), normal bone marrow cells (24), and peripheral blood monocytes (25). Bar-Shavit et al. (26) also reported a direct dependency on calcitriol for macrophage phagocytic function. This steroid hormone also activates alveolar macrophages (27) and stimulates enhanced oxidative metabolism in monocytes (28, 29). Finally, high-dose administration of calcitriol to a patient with osteopetrosis, a disease characterized by osteoclast dysfunction, stimulated osteoclast bone resorption and increased in vitro monocyte-mediated bone degradation (30). These reports suggest that PTH and calcitriol affect functional characteristics of monocytes and may also affect bone degradation activity.

In addition to the roles of calcitriol as a regulator of calcium homeostasis and as an inducer of mononuclear phagocyte differentiation, recent studies have expanded the actions of calcitriol to include immunoregulation. The discoveries of expression of calcitriol
receptor in thymus, monocytes, and activated T and B lymphocytes (31-34) first suggested an immunoregulatory role. Further studies of immune response in lectin-induced peripheral blood mononuclear cells (PBM) demonstrated that calcitriol inhibited DNA synthesis and suppressed interleukin-2 (IL-2) production (35-37). In contrast to these antiproliferative effects of calcitriol, Lacey et al. (38) reported calcitriol-induced stimulation of lectin-induced proliferation of nontransformed T-helper cell line (D10.G4.1) in the absence of, or at low interleukin-1 (IL-1) concentrations. However, calcitriol inhibited proliferative responses of D10 cells when IL-1 concentrations were optimal. We have demonstrated that calcitriol both inhibits and enhances concanavalin A (ConA)-induced DNA synthesis by normal bovine lymphocytes (39). These contrasting effects of calcitriol on ConA-induced response were dependent on lymphocyte density, monocyte numbers, and basal rates of DNA synthesis. Calcitriol enhanced ConA-induced proliferation when conditions were suboptimal for proliferation, whereas calcitriol inhibited proliferation when culture conditions were optimal (39). It is evident that calcitriol can exert both inhibitory and stimulatory effects on cells of the immune system, and that these effects are dependent on culture conditions.

The studies cited in this report prompted the present investigation of the effect of in vivo administration of PTH and calcitriol on in vitro bovine monocyte-mediated bone degradation, $\text{H}_2\text{O}_2$ production by monocytes, and lectin-specific lymphocyte proliferation. Studies to date examining the immunoregulatory role of calcitriol and PTH have
concentrated on *in vitro* addition of these hormones. Thus, little is known of the effect of increased *in vivo* calcitriol or PTH concentrations on subsequent *in vitro* responses of monocytes or lymphocytes.
MATERIALS AND METHODS

Isolation of PBM

Peripheral blood mononuclear cells from Jersey cattle were isolated by gradient centrifugation by using 1.084 (g/ml) Percoll (Pharmacia, Piscataway, NJ) as previously described (40). PBMs were recovered, washed twice in Hank's balance salt solution (HBSS) without Ca<sup>++</sup> and Mg<sup>++</sup>, washed once in phosphate-buffered saline (PBS), and resuspended in 1.086 g/ml Percoll for further enrichment of monocytes with discontinuous gradient centrifugation. PBMs for lymphocyte proliferation studies were resuspended in RPMI 1640 containing 10 mM HEPES (Gibco, Grand Island, NJ), penicillin G (100 U/ml), and streptomycin (100 μg/ml).

Monocyte Isolation

The two procedures employed for monocyte isolation were: 1) adherence of PBMs to plastic and 2) Percoll discontinuous gradient separation of PBMs followed by adherence to plastic. Monocytes were isolated by adherence to plastic by incubating PBM in RPMI 1640 with 10% heat-inactivated fetal bovine serum (FBS) in 12-well (16 mm) plates (1.33 x 10<sup>6</sup> PBMs/cm<sup>2</sup>) for 60 min at 39°C in 5% CO<sub>2</sub>/95% air. Nonadherent cells were removed by vigorous agitation of plates followed by washing individual wells with HBSS. Adherent cells consisted of 84% monocytes as determined by alpha-naphtyl acetate esterase staining.
Total number of adherent cells was determined in a cell counter after removal of adherent cell population with trypsin.

Monocytes also were isolated by Percoll discontinuous gradient centrifugation: A 3.75-ml layer of HBSS and 7.5-ml layers of 1.068, 1.070, 1.072, 1.074, and 1.076 g/ml HBSS-diluted Percoll were underlayered with 185 x 10^6 PBM resuspended in 11.25 ml of 1.086 g/ml Percoll. The gradient was centrifuged at 320 X g for 30 min. Cells of the 1.068-1.070 g/ml interface band, which contained 60% nonspecific esterase-positive cells (monocytes), were harvested, washed twice in HBSS, resuspended at 2.5 x 10^6 cells/ml in RPMI 1640 with 10% HBS, plated in 16-mm diameter wells at 1.0 ml/well, and incubated for 90 min at 39°C in 5% CO₂/95% air. Nonadherent cells were removed by vigorous agitation of the plate followed by washing individual wells with HBSS to remove remaining nonadherent cells. A volume of 0.9 ml of RPMI 1640 with 10% FBS was added to each well containing adherent cells. Adherent cells were 60% esterase-positive.

**Culture Conditions for Proliferation of Mitogen-Induced Lymphocytes**

PBM (0.5 x 10^6/ml) were cultured in 0.2 ml of RPMI 1640 with 6% heat-inactivated FBS in 96-well plates. Concanavalin-A (ConA) (8.0 μg/ml), phytohemagglutinin (PHA) (12.5 μg/ml), or pokeweed mitogen (PWM) (2.5 μg/ml; Sigma, St. Louis, MO) was added to individual wells. Cells were cultured for 72 hr at 39°C in 5% CO₂/95% air, pulsed 18 hr with 2 μCi of [methyl-3H] thymidine (6.7 Ci/m mole, Amersham, Arlington
Heights, IL), harvested onto glass fiber filters with a cell harvester (Model PHD, Cambridge, MA), and counted by liquid scintillation spectrophotometry. All assays were done in triplicate. Results were expressed as percentage of pretreatment [3H]-thymidine incorporation (cpm) for each animal. Relative changes in [3H]-thymidine incorporation of control animals in the calcitriol administration experiment were used to correct for assay fluctuations on a per day basis in the [3H]-thymidine incorporation of calcitriol-treated animals.

Assay of Monocyte-Mediated Bone Degradation

Monocyte-mediated bone degradation was based on a modification of the method of Teitelbaum et al. (41). 45Ca-labeled bone was prepared in 100-g rats by seven subcutaneous injections of 150 uCi 45CaCl₂ (DuPont, NEN Research Products) on alternate days for 14 days. Rats were sacrificed five days after the last 45Ca injection. Long bones were removed, dissected free of periosteum, washed thoroughly in 0.9% NaCl, and air-dried for 7 days at 45°C. Fragments then were ground in a grinding mill (Brinkman/Retsch, Model ZM-1); the ground material was collected, dried in a desiccator for 3 days, and passed through a series of metal-sizing screens. Particles less than 38 µm in diameter were collected, sterilized by ultraviolet irradiation, resuspended in RPMI 1640 with 10% FBS at 4 mg/ml, and added in 0.1 ml volumes to individual monocyte culture at a final concentration of 400 µg bone fragments/well). Wells containing bone without monocytes served as controls. Wells containing monocytes without bone were used to
determine total monocytes per well. Monocytes were removed with trypsin following initial adherence and counted in a NOVA cell counter. Cultures were incubated at 39°C in 5% CO₂/95% air for 96 hr. Following incubation period, upper half (0.5 ml) of each well was removed carefully, and ⁴⁵Ca content determined by liquid scintillation spectro-photometry. Monocyte bone degradation activity was calculated by the following formula:

\[
\frac{45\text{Ca in cultures with monocytes + bone particles}}{45\text{Ca in cultures with bone particles}} \times 100 = \text{Net %} \quad \frac{45\text{Ca release/well}}{\text{per 2.0 x 10}^5 \text{ cells}}
\]

Monocyte numbers per well were not determined for PTH experiments and are reported as net % ⁴⁵Ca release/well. Triplicate wells of isolated monocytes from all animals were employed.

**Hydrogen Peroxide Production by Monocytes During PTH Injections**

Production of superoxide anion, O₂⁻, and resulting H₂O₂ was determined fluorimetrically by using p-hydroxyphenylacetate (PHPA; Sigma, St. Louis, MO) described by Hyslop and Sklar (42). PHPA is oxidized to the stable fluorescent product 2,2'-dihydroxybiphenyl-5,5'-diacetate (PHPA)_₂ by enzymatic reduction of H₂O₂ by horseradish peroxidase (Type VI, Sigma). Monocytes isolated by adherence and removed from plastic with trypsin (95% viable by Trypan blue exclusion)
were suspended in 0.15 M PBS, pH 7.2 at 25 x 10^7 cells/ml. An assay mixture (7.5 μl) consisting of PHPA (10 mg/ml): horseradish peroxidase (8 mg/ml): superoxide dismutase (8 mg/ml) in a ratio of 25:10:10 was added to 2.5 ml Earle's balanced salt solution (BSS), pH 7.3, in 3-ml cuvettes. Monocytes (2.5 x 10^6; 0.1 ml of stock cell solution) were added to each cuvette containing the assay mixture. Phorbol myristate acetate (PMA) was added at a volume of 250 μl to cuvettes for a final PMA concentration of 1.0 μg/ml. Nonstimulated monocytes containing no PMA received 250 μl of PBS. Fluorescence was measured every 15 min for a total period of 30 min in a spectrofluorometer (Model SLM 8000, SLM Instruments, Urbana, IL) with excitation and emission maxima of 323 and 400 nm, respectively. Net H_2O_2 released was evaluated by subtracting non-PMA-stimulated fluorescence values from PMA-stimulated fluorescence values; H_2O_2 concentration was determined from standard curve.

In vivo PTH Treatment of Jersey Calves

Six Jersey bull calves 4 to 5 months of age (70-115 kg) received intramuscular injections (60 IU PTH/kg body weight) of crude synthetic N-terminal 1-34 fragment bovine PTH (767 IU/mg) (lot #009002, Peninsula Laboratories, Inc., San Carlos, CA) in .01 M acetic acid diluted in sterile 0.9% NaCl solution with 0.2% bovine serum albumin (BSA). Injections were given at 12-hr intervals for a total of nine injections in bone degradation studies to six injections in H_2O_2 experiment. Plasma and PBM samples for assays of bone degradation and DNA
synthesis (pre-treatment, treatment, and post-treatment) were obtained. All samples during treatment phase were taken immediately prior to injections. Monocytes for assays of bone degradation and PBM for assay of DNA synthesis in mitogen-induced lymphocytes were obtained prior to morning injection (a.m.) of PTH. Plasma samples were isolated by centrifugation and stored at -30°C for later evaluation.

**In vivo Calcitriol Treatment of Jersey Cows**

ALZET osmotic pumps, Model 2 ML Series (Alza Development Corp., Palo Alto, CA), were inserted in subcutaneous region of the paralumbar fossa under local anesthesia in six Jersey cows weighing between 350 and 430 kg. Pumps contained 350 µg 1,25-(OH)2D3 in 95% propylene glycol:5% ethanol. Pump rate was 11.5 ± 0.3 µl/hr. Sham surgery was performed on three Holstein steers used as controls. Plasma and PBM samples for assays of monocyte bone degradation and DNA synthesis in mitogen-induced lymphocytes (pre-treatment, treatment, and post-treatment) were obtained. Plasma samples were isolated by centrifugation and stored at -30°C for later evaluation.

**Plasma Assays**

Plasma Ca concentrations were determined by atomic absorption (Perkin Elmer 5000) (43), and plasma calcitriol concentration was determined by calf thymus receptor assay (44).
Statistical Analysis

[^3H]-Thymidine incorporation was expressed as a percentage of the mean pretreatment[^3H]-thymidine incorporation (cpm) value for each animal; each animal served as its own control.

Relative changes in[^3H]-thymidine incorporation of control animals in calcitriol administration experiment were used to correct for assay fluctuations on a per-day basis in calcitriol-treated animals.

Significant differences of treatment means compared with pretreatment mean in all experiments were determined by least significant difference.
RESULTS

Plasma Calcitriol and Calcium Concentrations of PTH- and Calcitriol-Treated Animals

Calcitriol and calcium concentrations in blood plasma from calves administered PTH and from cows implanted with osmotic pump containing calcitriol are shown in Fig. 1 and 2. Injections of PTH caused a rapid, sustained hypercalcemia (day 1 to 5) (Fig. 2). Plasma calcitriol increased to 150 pg/ml after 24 hr of treatment. In response to hypercalcemia, calcitriol concentrations returned to pretreatment concentrations 36 hr after initiation of PTH treatment and then declined below pretreatment concentrations for the duration of the treatment period in a manner previously described (45).

Administration of calcitriol via osmotic pumps resulted in a sustained increase of plasma calcitriol concentration to 100-170 pg/ml (Fig. 1). Plasma calcium concentrations increased and remained at 11.5 to 12.5 mg/100 ml for days 2-8 (Fig. 2).

Bone-Degrading Activity of Monocytes Isolated from PTH- and Calcitriol-Treated Animals

In vitro degradation of devitalized bone by monocytes was enhanced significantly by in vivo administration of PTH (Fig. 3). Bone degradation activity increased significantly to 2.5X that of the pretreatment value on day 3 of treatment. Monocyte bone degradation activity
Figure 1. Plasma calcitriol concentrations in bull calves during PTH injection experiment (●, n = 6), and in cows during osmotic pump delivery of calcitriol experiment (○, n = 6). Plasma calcitriol concentrations were determined by method of Reinhardt et al. (46). Time of treatment initiation is designated as day 0. PTH injection schedule and osmotic pump implant duration are indicated. Data are means + S.E.M.
Figure 2. Plasma calcium concentrations in bull calves during PTH injection experiment (●, n = 6), and in cows during osmotic pump delivery of calcitriol experiment (○, n = 6). Time of treatment initiation is designated as day 0. PTH injection schedule and osmotic pump implant duration are indicated. Plasma calcium concentrations were determined by atomic absorption. Data are mean ± S.E.M.
Figure 3. Effects of PTH and calcitriol on bone-degrading activity of isolated bovine monocytes. Freshly prepared monocytes were isolated by Percoll discontinuous gradient centrifugation and adherence to plastic in PTH-treated animals ($\square$, n = 6) and by only adherence to plastic in calcitriol-treated animals ($\blacksquare$, n = 6). Devitalized $^{45}$Ca-labeled rat bone was added at initiation of culture (400 µg/well). Cultures were incubated for 4 days at 39°C in 5% CO$_2$/95% air. Upon completion of culture, 0.5 ml of medium from each control and treated wells was removed and counted. Bone-degrading activity in PTH experiment was expressed as net percentage increase in $^{45}$Ca release in wells containing monocytes and devitalized bone relative to wells containing devitalized bone alone. Bone-degrading activity in osmotic pump delivery of calcitriol experiment was calculated as net percentage $^{45}$Ca release per $2.0 \times 10^5$ cells/well. Data are mean $\pm$ S.E.M. Significance of difference from each pretreatment control culture designated as ** $P < 0.10$ and, **; $P < 0.05$. 
returned to pretreatment levels during post-treatment period. This peak in bone degradation activity occurred when plasma calcitriol concentrations were significantly below pretreatment calcitriol concentrations (Fig. 2). This strongly suggested that PTH treatment and not calcitriol caused the stimulation of monocyte-mediated bone degradation.

In vitro degradation of bone by monocytes was not affected by a sustained elevation of plasma calcitriol. Monocyte-mediated bone degradation activity also did not change in sham-operated controls (data not shown). These experiments strongly suggest that PTH is a potent mediator of monocyte-mediated bone degradation activity and that calcitriol, under the conditions examined, is without effect on subsequent in vitro monocyte-mediated bone degradation.

Phase Contrast Microscopy of Monocytes Isolated from PTH-Treated Calves During Bone Degradation Assay

Because of the enhancement of bone degrading activity of isolated monocytes from PTH-treated animals, we sought to determine if any changes occurred in monocyte localization in bone degradation assay at the time of peak bone degradation activity. Figure 4 shows the monocytes isolated from a calf prior to the start of PTH treatment. Photographs of monocytes in culture with devitalized bone were taken at 24-hr intervals during a 72-hr incubation period. Initially, monocytes and bone particles are distributed uniformly (Fig. 4A). Small aggregates of bone particles and cells were evident after 48 hr
Figure 4. Phase contrast microscopy of monocytes in bone degradation assay isolated from a bull calf before PTH administration (control cultures). Monocyte isolation and bone degradation assay were identical to that described in Fig. 1. "b" represents bone particles; "ag", bone particle aggregates; "arrows", localization of monocytes. Photographs represent different incubation times for same isolated monocytes from calf prior to PTH treatment. Incubation times were: A. 0 hr (initiation of culture), B. 24 hr, C. 48 hr, and D. 72 hr. Magnification was 200X.
in culture (Fig. 4C); monocytes are localized in areas of these bone aggregates after 72 hr in culture (Fig. 4D).

Figure 5 shows the appearance of monocytes that were isolated from the same calf used to obtain the photographs in Fig. 4 after 6 injections of PTH (day 3 of PTH treatment) in culture with devitalized bone. Monocytes and bone particles were distributed uniformly at initiation of culture (Fig. 5A) as shown for pre-PTH monocyte cultures (Fig. 4A). At 24 hr after initiation of culture, however, (Fig. 5B), large numbers of monocytes rapidly localized about bone aggregates and individual bone particles (Fig. 5B). Figure 5C shows the more rapid aggregation of bone particles associated with greater numbers of monocytes. Methylene blue staining of monocytes showed monocytes localized on top of bone particles (photographs not shown). Monocyte localization increased with increasing time of incubation as shown in 72-hr culture photograph (Fig. 5D). Monocytes surrounding these monocyte-bone aggregate islands appear larger and more elongated compared with pre-treatment cultures.

Hydrogen Peroxide Production by Monocytes Isolated from PTH-Treated Calves

To delineate further a possible mechanism for the PTH-induced increase in monocyte-mediated bone degradation, \( H_2O_2 \) production by monocytes from PTH-treated calves was measured (Fig. 6). As stated in the legend, \( H_2O_2 \) production of pretreatment samples from control and PTH-treated differed prior to treatment. Therefore, \( H_2O_2 \) production
Figure 5. Phase contrast microscopy of monocytes in bone degradation assay isolated from bull calf after 3 days of PTH treatment (6 injections). Monocyte isolation and bone degradation assay were identical to that described in Fig. 1. "b" represents bone particles; "ag", bone particle aggregation; "arrows" indicate localization of monocytes. Photographs represent different incubation times for isolated monocyte cultures from PTH-treated calf of Fig. 4. Incubation times were: A. 0 hr (initiation of culture), B. 24 hr, C. 48 hr, and D. 72 hr. Magnification was 200X.
Figure 6. Effect of PTH (6 injections) administration to bull calves on monocyte production of hydrogen peroxide. Monocytes from 6 PTH-treated calves and 4 carrier-treated calves were isolated by adherence as described in Methods section and were evaluated fluorimetrically for superoxide anion production by the method of Hyslop and Sklar (42). Phorbol myristate acetate (PMA)-stimulated O_2 production by monocytes was determined after subtraction of O_2 resting (non-PMA) monocytes. Results were reported as net % H_2O_2 released/30 min x 2.5 x 10^6 cells relative to pre-treatment values. Pre-treatment H_2O_2 concentration values were: PTH-treated animals (○) = 58 ± 10 pmol H_2O_2/30 min x 2.5 x 10^6 cells, n = 6; control animals (●) = 100 ± 21 pmol H_2O_2/30 min x 2.5 x 10^6 cells, n = 4.
by monocytes relative to pretreatment values for each calf are presented. As shown in Fig. 6, PTH treatment resulted in enhanced $H_2O_2$ production by day 3 of treatment to 4.5 X the pre-treatment values. Injection of carrier alone to control calves was without significant effect. These data suggest that PTH treatment enhances $H_2O_2$ production on the same day of peak monocyte-mediated bone degradation activity (Fig. 3). This enhancement suggests that PTH may be stimulating an inflammatory-type mechanism that mediates the enhanced bone degradation activity by isolated monocytes.

Mitogen-Induced DNA Synthesis in PBM Isolated from PTH- and Calcitriol-Treated Cattle

To determine the effect of plasma calcitriol on in vitro DNA synthesis in mitogen-induced PBM, plasma calcitriol concentrations were increased for 5 to 6 days by osmotic pump administration of calcitriol and for 1 to 2 days by PTH injections.

Parathyroid hormone administration resulted in a significant inhibition of ($P < 0.05$) in vitro DNA synthesis of PWM-stimulated lymphocytes (Fig. 7C). On day 1 of PTH treatment, DNA synthesis was 55% of pre-injection values (Fig. 7C). Inhibition occurred on the day of peak calcitriol concentrations (see Fig. 1). Lymphocyte proliferative response to PWM returned to pretreatment values on days 3, 4, and 5 of treatment, which is when plasma calcitriol concentrations were markedly lower (Fig. 1). PTH injections and subsequent increase in
Figure 7. Effect of PTH administration to bull calves on in vitro DNA synthesis in mitogen-induced PBM. PBM (0.5 x 10^6/ml) that were isolated by Percoll density centrifugation from PTH-treated bull calves were stimulated with PHA (12.5 µg/ml), (O, n = 5), ConA (7.8 µg/ml) (Δ, n = 5), or PWM (2.5 µg/ml) (⊗, n = 4), added at initiation of culture. PBM were incubated for 72 hr at 39°C in 5% CO₂/95% air, pulsed for 18 hr with 2 µCi [methyl-^3H]-thymidine, harvested onto glass fiber filters, and assessed for DNA synthesis by liquid scintillation spectrophotometry. Results are expressed as percentage of pretreatment [^3H]-thymidine incorporation, allowing each calf to act as its own control. Mean ± S.E.M. of [^3H]-thymidine incorporation (CPM) of pretreatment controls were: ConA (453,193 ± 45,456), PHA (436,037 ± 57,031), and PWM (269,633 ± 19,013).
plasma calcitriol had no effect on DNA synthesis in ConA- or PHA-stimulated lymphocytes (Fig. 7A and 7B).

Treatment of cows with calcitriol also caused a significant inhibition ($P < 0.05$) of DNA synthesis in PWM-stimulated lymphocytes (Fig. 8C). Inhibition of proliferative response to 60% of that for pretreatment value was evident on day 3 (Fig. 8C). This inhibition coincided with an increase in plasma calcitriol concentrations to 160 pg/ml (Fig. 1). ConA- and PHA-induced PBM mitogenic responses, however, were enhanced greater than 70% of pretreatment by day 2 of calcitriol treatment (Figs. 8A and 8B). Mitogenic response to PHA was unaffected by sustained plasma calcitriol increase on treatment days 2-3 but increased to values twice that of pretreatment values during post-treatment interval (Fig. 8B). ConA-induced proliferative response, however, remained markedly enhanced throughout the treatment period and coincided with increases in plasma calcitriol (Fig. 1 and Fig. 8A). ConA-induced proliferative response returned to pretreatment values within 3 days after pump removal (Fig. 8A) and was associated with a return of plasma calcitriol concentrations to pretreatment levels (Fig. 1).
Figure 8. Effect of calcitriol administration to cows on in vitro DNA synthesis in mitogen-induced PBM. Osmotic pumps calcitriol were implanted surgically in the subcutaneous region of paralumbar fossa in 6 Jersey cows. PBM were isolated and cultured as described in Methods section and legend of Fig. 4. Results are expressed as percentage of pretreatment $[^3]$H-thymidine incorporation relative to control (non-implant) cows. Mean ± S.E.M. of $[^3]$H-thymidine incorporation (CPM) of pretreatment controls were: ConA (246,531 ± 32,274), PHA (223,428 ± 30,467), and PWM (40,797 ± 6,804).
DISCUSSION

The study of monocyte function in this report demonstrates that PTH administration in vivo stimulates both in vitro monocyte-mediated bone degradation and \( \text{H}_2\text{O}_2 \) production. These results suggest a possible inflammatory-type mechanism for the PTH-induced increase in bone degradation activity of monocytes.

The cellular events directing the PTH-induced enhancement of monocyte-mediated bone degradation is unknown. Previous studies have demonstrated that in vitro PTH treatment increases cAMP production in mononuclear phagocytes and decreases chemiluminescence and cAMP production while increasing lysosomal enzyme production in monocytes (16-19). These studies suggested that PTH treatment can mediate a direct effect on cells of monocyte-macrophage lineage. These studies would suggest that PTH administration directly stimulates monocyte-mediated bone degradation via a cAMP-mediated process or by an increase in lysosomal enzymes, or collagenase. This proposal seems unlikely because previous studies have been unable to demonstrate PTH receptor expression in the monocyte (20, 21). A more plausible explanation for our results is that a PTH responsive accessory cell mediated the observed PTH effect.

An intermediate role for the osteoblast in stimulating osteoclast bone resorption has been proposed by Rodan et al. (46). This hypothesis was formulated because of the inability to demonstrate PTH receptor on osteoclasts (47, 48) and the lack of evidence for any direct
effect of PTH on isolated osteoclasts (49, 50). These data and data demonstrating that PTH stimulates osteoblasts to release factors that increase osteoclast bone resorption (51) points to the osteoblast as a mediator of PTH effects on osteoblasts. Therefore, a PTH responsive cell such as the lymphocyte (20, 21) may play an intermediate role in stimulation of monocyte-mediated bone degradation we observed.

Studies have suggested a role for the thymus gland and lymphoid cells in the bone resorption mechanism. Milhaud et al. (52) reported that thymus atrophy and associated bone resorption dysfunction in the op/op (osteopetrotic) rat could be cured by transplantation of normal thymus gland into affected animals. Marks et al. (53) also demonstrated that bone resorption was restored in ia/ia (inciser absent-osteopetrotic) rats by infusion of mononuclear cell isolates from thymus (99% lymphocytes, 1% monocytes) of normal littersmates. Furthermore, bovine and human lymphocytes express the PTH receptor and demonstrate increased cAMP accumulation after PTH treatment (20, 21). PTH stimulation of lymphocyte synthesis of factors that increase bone resorption (54) are further evidence that a physiological role for PTH-lymphocyte interaction in bone resorption may exist. Although these reports suggest a role of lymphocytes in osteoclast bone resorption, the role of lymphocytes in monocyte-mediated bone degradation has yet to be examined.

The increase in \( H_2O_2 \) production by isolated monocytes from PTH-treated animals suggests that PTH activates circulating monocytes in a manner similar to that evident in an inflammatory response. Monocytes
are found in regions of chronic inflammation and associated osteolysis evident in rheumatoid arthritis and periodontitis (1-5) and H_2O_2 is formed and released during the respiratory burst of phagocytes during the inflammatory response. Inflammation-associated secretory products of macrophages include lysozyme and collagenase (55), and macrophages activated with endotoxin, a modulator of inflammatory response, secrete increased amounts of collagenase (56, 57). Endotoxin also increases in vitro chicken monocyte- and murine macrophage-mediated bone degradation (15), but another study reported inhibition of bone degradation activity in endotoxin-activated murine macrophages (58). Associated with PTH activation of monocyte inflammatory functions in this study, i.e., H_2O_2 production, may be an increase in collagenase production by monocytes thus possibly increasing the bone-degrading activity of isolated monocytes from PTH-treated calves.

Heersche (59) and Mundy (60) have proposed an accessory-"helper-scanvenger" role for monocytes in the removal of debris from areas of osteoclast bone resorption. Baron et al. (61) also demonstrated that initiation of bone remodeling, as studied in tooth egression in the rat, occurs during the activation phase of bone remodeling and involves the recruitment of cells with mononuclear phagocyte characteristics to areas of bone. Additionally, Chambers and Fuller (62) demonstrated that digestion of unmineralized organic material by osteoblasts exposes bone mineral surfaces to osteoclasts. Monocytes, recruited to areas of bone resorption during the recruitment/activation phase of bone remodeling, also may remove osteoid matrix by secretion of
collagenase and thus facilitate osteoclast contact and resorption of mineral surfaces. Our data suggest that the circulating monocyte is responsive to PTH, either directly or indirectly via an intermediate cell such as the lymphocyte. PTH-activated monocytes may be recruited to areas of bone resorption as shown in our in vitro model, indicating that isolated monocytes from PTH-treated animals rapidly accumulated around bone aggregates. Once recruited, monocytes may aid osteoclast bone resorption by removal of the osteoid matrix and/or removal of bone debris resulting from osteoclast activity.

In addition to the investigation of monocyte-mediated bone degradation, I examined the in vitro proliferative response of lymphocytes to mitogens following calcitriol administration. Periods of increased plasma calcitriol were of 1- to 2-day duration in the PTH experiment and of 7-day duration when osmotic pumps continuously infused calcitriol. The present study suggests that, as shown in previous in vitro studies in humans and cattle (37, 39), that calcitriol inhibits the PWM-stimulated bovine lymphocyte proliferative response of bovine lymphocytes during the period of increased plasma calcitriol concentration. Our results demonstrating enhanced PHA- and ConA-induced responses of isolated lymphocytes during calcitriol treatment contrast with studies reporting in vitro inhibition of PHA- and ConA-stimulated lymphocyte proliferation in the presence of calcitriol (35, 36, 39). Rigby et al. (36), however, reported that calcitriol must be present during the first 12-18 hr of PHA-induced mitogenesis; otherwise, a significant loss in the inhibitory effect of
calcitriol occurs. Calcitriol is not present during mitogenesis in the present study, and the findings demonstrate that calcitriol administration to cattle prior to lymphocyte isolation results in the enhancement of DNA synthesis in PHA- and ConA-induced lymphocyte.

The mechanism of enhanced ConA- and PHA-induced proliferation resulting from calcitriol treatment is unclear. Earlier studies have shown calcitriol-induced enhancement of lymphocyte proliferation. Tsoukas et al. (35) reported a significant increase in PHA-induced PBM proliferative response at $1 \times 10^{-12}$ M calcitriol. This observation was evident in a 2-day incubation study at low proliferation rates, but the authors were unable to reproduce this phenomenon. Lacey et al. (38) also have demonstrated calcitriol-induced augmentation of proliferation of ConA-induced nontransformed T helper cells. Studies conducted in our laboratory demonstrated that low proliferation rate cultures of ConA-stimulated bovine lymphocytes supplemented with increasing monocyte additions showed enhanced proliferative response in the presence of calcitriol (39). We suggested that the monocyte may be the mediator of the calcitriol-induced enhancement either by increasing Ia expression and/or increasing IL-1 synthesis, which are two pivotal events in the early activation steps of lymphocyte proliferation. Monocytes constitutively express the calcitriol receptor, whereas T and B lymphocytes do not (32, 33). This differential expression suggests that the monocyte and not T and B lymphocytes is responsive to calcitriol in this study, and thus the monocyte is the
likely mediator of the calcitriol-induced enhancement of proliferation in the present study.

The manner by which the monocyte may mediate calcitriol-induced enhancement of ConA-stimulated proliferative response, as stated earlier, is unclear. Several studies have shown that ConA-stimulated lymphocyte proliferation requires an Ia-bearing accessory cell (63-65), and this requirement may be a mechanism of the enhancement of ConA-stimulated proliferative response in the present study. Calcitriol increases expression of HLA-DR (human Class II) antigens in HL-60 leukemia cells (23), and also increases Ia antigen expression in IFN-α-induced monocyte tumor cell line WEHI-3 (66). Circulating monocytes, which all express Class II antigen to varying degrees, may possess higher Class II expression on a per-cell basis as a result of in vivo calcitriol treatment. This isolated monocyte population then would be more effective at antigen presentation than isolated pre-treatment monocytes in ConA-stimulated PBM culture conditions resulting in augmentation of proliferation. Additionally, calcitriol treatment may cause an increase in IL-1 synthesis by monocytes. Calcitriol-treated monocytes, a macrophage cell line, and lymphokine-induced U937 cells (immature monocyte cell line) demonstrate increased production of IL-1 (67-69). Lacey et al. (70) also reported that a normal nontransformed IL-1 dependent T helper cell line (variant of D10 G4.1), when exposed to IL-1 and IL-2, showed a marked synergistic effect on growth promotion. In the present study, if monocytes isolated from calcitriol-treated animals do indeed have enhanced IL-1
production capacity, then the increased IL-1 production may act with IL-2 that is produced by T cells during in vitro ConA-induced response to cause a synergistic enhancement of lymphocyte proliferation.

The calcitriol-induced enhancement of ConA-induced lymphocyte proliferation also may result from the induction of growth factor receptors. Lacey et al. (38) reported that calcitriol increased IL-2 receptor expression in D10 G4.1-T helper cells. Recently, a study by Lichtman et al. (71) demonstrated that the D10 G4.1 cell line, classified as a type 2 helper (TH2) cell (72), responds in an autocrine growth-type mechanism by secreting B-cell stimulatory factor 1 (BSF-1, IL-4) and also proliferating in response to BSF-1. Lichtman et al. (71) also observed that D10 cells respond to IL-1 and BSF-1 together but not to BSF-1 alone and that D10 cells also respond to IL-2 alone. In the present study, calcitriol administration may increase IL-1 secretion in circulating monocytes as suggested earlier, and this increase in IL-1 secretion then may potentiate the responsiveness of TH2 subpopulations to BSF-1 or IL-2 by upregulating the BSF-1 or IL-2 receptor, thus resulting in augmentation of ConA-induced proliferation.

Furthermore, the enhanced ConA-induced lymphocyte proliferative response may result from shifts in circulating T lymphocyte subpopulations during calcitriol administration. The OKT4/OKT8 (TH-helper-inducer/Ts-suppressor T cell) ratio is reported to be significantly greater in osteopetrotic patients compared with that of nonosteopetrotic patients (73). Administration of 1α-hydroxyvitamin D3,
however, resulted in a significant decrease in the $T_H/T_S$ ratio suggesting either a decrease in $T_H$ cells or an increase in $T_S$ cells (73). Hersey et al. (74) also reported that solarium exposure to normal volunteers increased in $T_S$ suppressor/cytotoxic T cells and a significant decreased $T_H/T_S$ ratio. Exposure to sunlight increased vitamin D synthesis in the skin, but unfortunately Hersey et al. (74) did not evaluate calcitriol plasma concentrations in these volunteers. In our present experiment, calcitriol administration may cause a shift in the $T_H/T_S$ ratio and thus alter the ConA-stimulated DNA synthesis of lymphocytes isolated from calcitriol-treated animals relative to pre-calcitriol-treated lymphocyte response.

The inhibition of in vitro PWM-induced lymphocyte proliferation during calcitriol treatment and during PTH-stimulated increases in plasma calcitriol also suggests an inhibitory role of calcitriol. These results correspond to in vitro studies of calcitriol-induced inhibition of PWM-stimulated human and bovine lymphocytes (37, 39). This inhibition of PWM-induced proliferation suggests, as proposed earlier for calcitriol-induced enhancement of ConA-stimulated lymphocytes, that the monocyte, which expresses the calcitriol receptor during calcitriol administration, may mediate the apparent calcitriol-induced inhibition of lymphocyte proliferation. The lack of sustained inhibition of PWM-induced lymphocyte proliferation during the 6-day period of elevated plasma calcitriol is, however, puzzling and warrants caution in attributing any inhibitory role for calcitriol in
experiments of in vivo calcitriol administration and the subsequent PWM-induced lymphocyte proliferation.

This study investigated the effect of PTH and calcitriol administration in the bovine on the subsequent in vitro DNA synthesis in mitogen-induced lymphocyte. PTH administration did not change either the ConA- or PHA-induced lymphocyte proliferation but did inhibit PWM-induced proliferative response on the day of peak plasma calcitriol concentration. Calcitriol administration, however, either did not affect or enhanced the PHA-induced proliferative response. ConA-induced proliferative response was enhanced markedly throughout calcitriol administration. The lymphocyte proliferative response to PWM also was unaffected by calcitriol administration, except for a one-day period of inhibition. The principal finding of this study suggest that calcitriol administration to animals does not inhibit the subsequent in vitro DNA synthesis by lymphocyte. On the contrary, DNA synthesis in ConA-induced lymphocyte is augmented significantly following calcitriol administration. I propose as for my earlier finding of in vitro calcitriol-induced enhancement of ConA-induced proliferative response under low proliferative rate cultures conditions (39) that the monocyte is the mediator of the enhanced proliferation. Calcitriol may act to increase monocyte Ia expression and/or IL-1 synthesis, which are two pivotal events in the early activation steps of lymphocyte DNA synthesis. This potentiation of the early activation steps may result in the enhancement of stimulation of
subset populations of T cells, i.e., Type 2 helper cells, possibly by upregulation of IL-2 and/or BSF-1 receptor expression.
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SECTION III.

MILK FEVER
INTRODUCTION

My discovery that PTH administration to bull calves resulted in an increase in monocyte-mediated bone degradation prompted me to evaluate the bone degradation activity of monocytes isolated from cows with parturient paresis (milk fever). Milk fever is a disease characterized by end-organ resistance to the calcium mobilizing hormones, PTH and calcitriol, seen at the onset of lactation (1). Preventative measures include a prepartal low-calcium diet (2) and prepartal exogenous PTH administration (3). Both of these strategies are designed to prime bone calcium mobilization for calcium demand at lactation. The basis for evaluating monocyte-mediated bone degradation is a study by Key et al. (4) in an osteopetrotic child. Osteopetrosis is also an end-organ-resistant disorder characterized by dysfunction of bone resorption mechanism. Key et al. (4) administered high-dose calcitriol to this osteopetrotic child and found that both osteoclast bone resorption and monocyte-mediated bone degradation were increased significantly as a result of calcitriol treatment. We, therefore, sought to determine if monocyte-mediated bone degradation was affected during clinical milk fever relative to pre- and post-milk fever bone degradation activity.
MATERIALS AND METHODS

Parturient Paresis (Milk Fever) Experiment

Periparturient Jersey cows with histories of parturient paresis were fed a high calcium diet that predisposes the cow to milk fever beginning at 6 weeks prepam. The diet for the first 4 weeks of the 6-week prepartal period supplied approximately 110 g Ca and 33 g P daily. At approximately 2 weeks prepam and for the first 2 weeks of lactation, cows were fed a diet that supplied approximately 150 g Ca and 78 g P daily. Plasma and PBM samples for monocyte isolation and bone degradation assay were taken at regular intervals, but more frequently at days around parturition. At onset of clinical signs of milk fever, calcium borogluconate was administered immediately after plasma and PBM samples were taken. The prepartal and postpartal periods were divided into different day intervals as indicated in the figures.

Monocyte Bone Degradation Assay

Monocytes were isolated and evaluated for bone degradation activity as detailed in the Materials and Methods of Section II.

Plasma Evaluation During Periparturient Period

Calcium, PTH, and calcitriol concentrations in plasma were determined by methods detailed in the Materials and Methods of Section II.
RESULTS

Monocyte-Mediated Bone-Degrading Activity and Calcitriol, PTH, and Calcium Concentrations in Plasma of Parturient Paretic (Milk Fever) Cows

Plasma calcium and calcitriol concentrations (Fig. 1B) and plasma PTH concentrations (Fig. 1A) from three paretic cows are shown in Fig. 1. Plasma calcium concentrations begin declining on days -2 to -1 and are decreased significantly (P < 0.01) at onset of milk fever and on days 1 and 2 post-milk fever (P < 0.01) (Fig. 1A). PTH stimulation of renal 1α-(OH)ase activity and low plasma calcium concentrations (5) result in an increase in plasma calcitriol concentrations at onset of milk fever and on days 1 to 2 and 3 to 4 (P < 0.01) post-milk fever. Horst et al. (6) previously have shown that PTH and calcitriol concentrations are not different in paretic and nonparetic cows and suggested that a primary hormone resistance to these calcitropic hormones may be a cause for onset of milk fever. We have measured the bone-degrading activity of monocytes from milk fever cows at the onset of milk fever and compared this activity to bone degradation activity of pre- and postparietic cows. As shown in Fig. 1A, monocyte bone degradation activity decreased on days -4 to -3 relative to days -9 to -5 degradation activity, and then increased significantly on days -2 to -1 to 2.5 x the activity of days -4 to -3. This increase in bone degradation activity of monocytes was coincident with an increase in plasma PTH concentration (Fig. 1A). At the onset of milk
Figure 1. Plasma calcium, calcitriol, and PTH concentrations and the bone-degrading activity of monocytes from Jersey cows before, during, and after onset of milk fever. Determination of PTH, calcitriol, and calcium concentrations in plasma are described in Materials and Methods of Section II. Experimental protocol for monocyte isolation and bone degradation assay is the same as for PTH administered to calves, as detailed in Materials and Methods of Section II. Days were combined into group intervals as indicated on the abscissa of each figure. Figure 1A. Plasma PTH (pg/ml) and bone degradation activity of monocytes (net % Ca release) of milk fever cows for 20-day periparturient period. Figure 1B. Plasma calcium (mg/100 ml) and calcitriol (pg/ml) concentrations of milk fever animals for 20-day periparturient period.
fever and on days 1 to 2 post-milk fever, however, a marked significant decline in bone degradation activity occurred relative to peak activity on days -2 to -1. Monocyte bone-degrading activity during onset of milk fever was unresponsive to the significant increases in plasma PTH \((P < 0.01)\). The bone-degrading activity did, however, also increase on days 3 to 4 post-milk fever.
DISCUSSION

The present study demonstrates that monocyte-mediated bone degradation increases prepartally with increasing plasma PTH concentrations in paretic cows. Our earlier study in Section II demonstrated that exogenous PTH resulted in a significant increase in monocyte-mediated bone degradation. Bone degradation activity of monocytes, however, was not increased on the days of peak plasma PTH concentrations that occurred at onset of milk fever. These results suggest that at onset of milk fever, a time that calcium homeostatic mechanisms are resistant, monocyte-bone degradation activity is also unresponsive to the bone calcium mobilizing hormone PTH. This resistance to PTH action suggests that the hypothesized "scavenger-helper" role of monocytes in bone resorption proposed by Heersche (7) may be resistant to PTH stimulation at the onset of milk fever, and thus possibly contributes to the resulting hypocalcemia and paresis. The role of the monocyte in bone resorption must be established firmly with further study, though, before this hypothesis is accepted.


SECTION IV.

PROLIFERATION OF LYMPHOCYTES AND BONE-DEGRADING ACTIVITY
OF MONOCYTES FROM COWS WITH EDTA-INDUCED HYPOCALCEMIA
INTRODUCTION

We evaluated the effect of hypocalcemia induced by EDTA infusion on proliferation in mitogen-induced lymphocytes and on monocyte-mediated bone degradation. EDTA-induced hypocalcemia was used as a model of milk fever to evaluate mitogen-induced lymphocyte response and monocyte bone degradation activity. Several previous studies have used EDTA as a chelating agent of calcium to experimentally induce hypocalcemia in cattle (1-3).
MATERIALS AND METHODS

Ethylene Diaminetetraacetate (EDTA) Infusion

Procedure for EDTA infusion was a modification of methodology of Ramberg et al. (3). Six Jersey heifers weighing between 250 and 320 kg were maintained in individual stanchions and fed a diet that met NRC requirements for mineral content and caloric intake (4). Teflon catheter was placed in one external jugular vein under local lidocaine anesthesia. Cows were infused intravenously and constantly for 36 hr with disodium EDTA (5 g/hr) sufficient to chelate 541 mEq Ca/hr. An additional 12-hr infusion of 0.9% NaCl solution completed the infusion protocol. Plasma samples and PBM samples for monocyte isolation and bone degradation assay, and for DNA synthesis in mitogen-induced lymphocytes were obtained at regular intervals in pre-treatment, treatment, and post-treatment phases by jugular venipuncture on the side opposite the catheter infusion.

Assay of Monocyte Bone Degradation

Monocytes were isolated and evaluated for bone degradation activity as detailed in Materials and Methods of Section II.

Assay of DNA Synthesis in Mitogen-Induced PBM

PBM were isolated and cultured for evaluation of mitogen-induced DNA synthesis as described in Materials and Methods of Section II.
RESULTS

Monocyte-mediated bone degradation did change during EDTA infusion relative to pre-EDTA values (data not shown).

PBM DNA synthesis, in response to PHA, ConA, or PWM, were also unchanged relative to pre-EDTA values (data not shown).

Plasma calcitriol concentrations are shown in Table 1. Calcitriol concentrations during the two pre-treatment days did not exceed 13 pg/ml. Infusion of EDTA caused an increase in calcitriol concentration to 28.6 ± 4.0 pg/ml. Cessation of infusion protocol caused calcitriol concentrations to return to pre-EDTA infusion concentrations.
Table 1. Plasma calcitriol concentrations during 36-hr EDTA infusions in 5 Jersey heifers

<table>
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<tr>
<th>Treatment day</th>
<th>Plasma calcitriol (pg/ml)</th>
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<tr>
<td>Pre-1</td>
<td>12.8 ± 3.0</td>
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<tr>
<td>Pre-2</td>
<td>6.9 ± 1.0</td>
</tr>
<tr>
<td>1</td>
<td>28.6 ± 4.0</td>
</tr>
<tr>
<td>2</td>
<td>17.7 ± 4.0</td>
</tr>
<tr>
<td>3</td>
<td>N.D.*</td>
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<td>4</td>
<td>6.2 ± 0.4</td>
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*Not determined.
DISCUSSION

No change in DNA synthesis proliferation of PBM or activity of monocyte-mediated bone degradation was observed during EDTA infusion relative to pre-EDTA values. Plasma calcium was not evaluated, but the observation of increased plasma calcitriol concentrations suggests that hypocalcemia was induced (5), but not to the degree evident in milk fever animals (see Fig. 1B of Milk Fever study). This experiment, therefore, probably did not induce sufficient hypocalcemia to induce plasma PTH and calcitriol concentration increases sufficient to affect proliferation of mitogen-induced PBM and/or rate of monocyte-mediated bone degradation as demonstrated in PTH and calcitriol administration studies (see Section II).
LITERATURE CITED


SECTION V.

EFFECT OF 24-FLUORO-1,25-DIHYDROXYVITAMIN D₃

ON PROLIFERATION IN MITOGEN-INDUCED PBM
INTRODUCTION

24-Fluoro-1,25-dihydroxyvitamin D₃ [24F-1,25-(OH)₂D₃] is a fluorinated analogue of 1,25-(OH)₂D₃ that is similar to 1,25-(OH)₂D₃ in its hypercalcemic potency (1). This study was designed to evaluate the effect of 24F-1,25-(OH)₂D₃ administration on *in vitro* proliferation of mitogen-induced PBM.
MATERIALS AND METHODS

24-F-1,25-Dihydroxyvitamin D₃ Implant

Three Jersey heifers weighing between 250 and 320 kg were given subcutaneous implants of 24-F-1,25-(OH)₂D₃ pellets (0.5 mg/pellet) (Innovative Research of America, Gaithersburg, MD) in the paralumbar fossa under local anesthesia with lidocaine. Three control heifers were given implants of pellets containing only carrier (cholesterol:methyl cellulose:a-lactose). Plasma and PBM pretreatment, treatment, and post-treatment samples were obtained.

Assay of DNA Synthesis in Mitogen-Induced PBM

PBM were isolated and cultured for assay of mitogen-induced DNA synthesis as described in Materials and Methods of Section II.

Plasma 24-F-1,25-(OH)₂D₃ and Calcium Concentrations

Plasma calcium and 24-F-1,25-(OH)₂D₃ concentrations were evaluated as described in Materials and Methods of Section II. The method of Reinhardt et al. (2) was modified for assay of both 1,25-(OH)₂D₃ and 24-F-1,25-(OH)₂D₃ in plasma. 24-F-1,25-Dihydroxyvitamin D₃ and 1,25-(OH)₂D₃ comigrated on C18 Sep-Pak silica columns. Separation was achieved by subjecting the dihydroxyvitamin D metabolite fraction from the initial C18 Sep-Pak column to high-performance liquid chromatography (HPLC) on a Zorbax Sil column (0.45 x 25 cm, Dupont) developed in 3:97 (v:v) isopropanol:methylene chloride. Once separated, concentrations of both sterols were determined by using a radioreceptor
assay (2). Recovery estimates for 1,25-(OH)_{2}D_{3} were used to estimate recovery of 24-F-1,25-(OH)_{2}D_{3}.
RESULTS

Plasma 24-F-1,25-(OH)$_2$D$_3$ concentrations are shown in Table 1. 24-F-1,25-(OH)$_2$D$_3$ concentrations peaked at 450-500 pg/ml on days 1-2 and then remained at about 150 pg/ml for the next 11 days.

Results of PHA-, ConA-, and PWM-induced lymphocytes proliferation from treated and control cows are shown in Fig. 1. No changes in proliferation in PHA- or ConA-induced PBM was evident in 24-F-1,25-(OH)$_2$D$_3$-treated cows. Proliferation of PWM-induced PBM seemed inhibited in the 24-F-1,25-(OH)$_2$D$_3$ treatment group, but large standard errors caused a lack of significant treatment effects (P > 0.05).
Table 1. Plasma calcium and 24-F-1,25-(OH)_2D_3 concentrations during pretreatment period and during implant of pellet containing 24-F-1,25-(OH)_2D_3

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<tr>
<th>Treatment day</th>
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<th>Plasma 24-F-1,25-(OH)_2D_3 (pg/ml)</th>
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<td>Pre-2</td>
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Figure 1. Effect of 24-F-1,25-(OH)₂D₃ administration on in vitro proliferation in mitogen-induced PBM. Pellets containing 24-F-1,25-(OH)₂D₃ (0.5 mg/pellet) (Innovative Research of America, Gaithersburg, MD) were implanted in the subcutaneous region of the paralumbar fossa under local anesthesia in 3 Jersey cows. Pellets containing carrier alone were implanted in 3 control cows. PBM were isolated and cultured as described in Methods section of Section II. Results are expressed as mean ± S.E.M. [³H]-thymidine incorporation (cpm). All assays were done in triplicate.
DISCUSSION

The 24-F-1,25-(OH)$_2$D$_3$ pellet implant did not change rate of proliferation in mitogen-induced PBM. The experiment may have been hampered by the low number of cows per group (n = 3) and the wide range of plasma 24-F-1,25-(OH)$_2$D$_3$ concentrations during the experiment. It is interesting to note, though, that proliferation in PHA- and ConA-induced PBM was not affected by increased plasma 24-F-1,25-(OH)$_2$D$_3$. This is in marked contrast to 1,25-(OH)$_2$D$_3$-induced inhibition of \textit{in vitro} DNA synthesis in PHA- and ConA-induced lymphocytes demonstrated in earlier studies (3, 4).
LITERATURE CITED


SUMMARY AND DISCUSSION

Calcitriol is an immunoregulatory steroid hormone that suppresses IL-2 production and inhibits proliferation in mitogen-induced lymphocytes. In Section I of this dissertation, the effects of calcitriol on in vitro proliferation in bovine PBM was evaluated. Calcitriol inhibited DNA synthesis in PHA- and PWM-induced PBM, but the effect on ConA-stimulated PBM was dependent on the initial rates of DNA synthesis. PBM from cows with low proliferative response to ConA were stimulated significantly by calcitriol, whereas PBM from cows with high proliferative responses were unaffected by calcitriol addition. Cells with low initial proliferative response to ConA (low-density nonadherent cells) with increasing monocyte additions resulted in significant enhancement of DNA synthesis in the presence of calcitriol. Cells with high initial proliferative response to ConA (high-density nonadherent cells) were inhibited in the presence of calcitriol at all monocyte additions, but successive increases in relative monocyte additions significantly attenuated the calcitriol-induced inhibition of DNA synthesis. These studies suggest that the calcitriol-induced enhancement of ConA-stimulated lymphocytes is mediated on the monocyte, possibly by increased Class II MHC molecule expression and/or increased IL-1 synthesis by the monocyte. In Section II, the effects of in vivo administration of calcitriol on subsequent in vitro proliferation in mitogen-induced PBM were examined. In vivo administration of calcitriol resulted in enhancement of in
vitro proliferation in PHA- and ConA-induced PBM and inhibition of proliferation in PWM-induced PBM relative to pre-calcitriol values. These studies of in vivo administration of calcitriol also suggest that the monocyte mediates the enhanced ConA-induced DNA synthesis because the monocyte, and not the T and B lymphocyte, constitutively expresses the calcitriol receptor during the calcitriol treatment.

This research project also examined the effects of the bone calcium mobilizing hormones, PTH and calcitriol, on monocyte-mediated bone degradation. Monocytes degrade devitalized bone and are found in areas of osteoclast bone resorption and in areas of osteolysis associated with chronic inflammation, such as periodontitis and rheumatoid arthritis. The monocyte has, therefore, been proposed as playing a role in the bone resorption process distinct from the osteoblast.

In Section II of this dissertation, I have demonstrated that PTH administration significantly increased monocyte-mediated bone degradation, whereas calcitriol administration had no effect. PTH administration also resulted in enhanced monocyte production of H₂O₂ on the same day as peak monocyte-mediated bone degradation. This relationship suggests that PTH induces an inflammatory-type mechanism during the increase in monocyte-mediated bone degradation.


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