An Evaluation of Cells Recruited to Ectopically Implanted Bone Matrix

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AN EVALUATION OF CELLS RECRUITED
TO ECTOPICALLY IMPLANTED BONE MATRIX

By
Janet Dvonch Kelly

A Dissertation Submitted to the Faculty of the Graduate School
of Loyola University of Chicago in Partial Fulfillment
of the Requirements for the Degree of
Doctor of Philosophy

APRIL 1990
DEDICATION

To Dan and Colleen,
with love

Forsan et haec olim meminisse juvabit
The Aeneid
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VITA

The author, Janet Dvonch Kelly is the daughter of Gertrude R. and the late William J. Dvonch, M.D. She was born on July 28, 1961 in Chicago, Illinois.

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The author is married to Daniel P. Kelly and they have one daughter, Colleen Anne.
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CHAPTER I

INTRODUCTION

Bone resorption is the process by which the organic and inorganic components of the skeleton are degraded and removed. Bone resorption, closely linked with bone formation, allows for growth, maintenance and repair of the skeleton. The principal effector cell of the osteolytic process is the osteoclast, however bone forming cells, monocytes, macrophages and their derivatives are capable of bone removal. The direct and/or indirect roles of these accessory cells in the physiological and pathological processes of osteolysis may be significant. Because of its destructive potential to the organism, bone resorption is highly regulated. If this process dysfunctions due to a cellular or regulatory defect, bone loss may result either throughout the skeleton as in osteoporosis or in a localized area as in rheumatoid arthritis. In either case the effects can be debilitating or life threatening. Although the effects of physiological and pathological bone loss are apparent, knowledge of the process, especially in the disease state, is limited and further investigation is necessary.

Implantation of mineralized bone matrix into subcutaneous sites results in a rapid, cell-mediated osteolytic response (Glowacki et al., 1981; Glowacki and Cox, 1986). Histological evaluation of the implant site revealed mononuclear and multinucleated giant cells (MNGCs)
contacting the bone particles. Although it has been suggested that the MNGCs surrounding the bone surface are indeed osteoclasts, indicating this system can be used to study physiological bone resorption; it has been shown that MNGCs derived from the mononuclear phagocyte system also have osteolytic capabilities and may be present at the site (Walters and Schneider, 1985). Multinucleated giant cells are formed by fusion of mature mononuclear phagocytes in response to a foreign body infiltrate or chronic inflammation (Adams, 1976). Sufficient evidence has not been presented to substantiate the use of this model system for physiological bone resorption. This model may be better suited for the study of pathological bone loss which is mediated by mononuclear and multinuclear phagocytic cells. In either case, this model system may be a valuable tool to study the osteolytic process.

The osteoclast is both unique morphologically and functionally. However, other than the morphological features of multiple nuclei and ruffled borders, which are highly reliable but not always apparent in a histological section, there are few means of easily and quickly distinguishing osteoclasts from other bone cells and macrophages. Recently, the presence of tartrate resistant acid phosphatase (TRAP) in osteoclasts and their precursors has been used for identification in both normal and experimental situations. TRAP can be easily demonstrated, however the specificity of the enzyme to osteoclasts and the reliability of using it in experimental situations have been questioned (Hattersley and Chambers, 1989). In the first study of this dissertation, a method using image analysis was devised for quantitating TRAP
activity and defining a level which was specific for the osteoclast. To define the level of TRAP expression, osteoclasts from rat proximal tibiae and cells generated at the subcutaneous site of implanted mineralized bone matrix were reacted for TRAP and compared. This level of TRAP expression could be used to characterize osteoclasts in both normal and experimental situations. Previously, only the presence of the enzyme or a subjective evaluation of TRAP expression had been used.

In the next study the subcutaneous bone implant was assessed as a model system for bone resorption. The influence of three preparations of bone matrix—mineralized, demineralized and a mineralized/demineralized composite—on the recruitment of multinucleated giant cells to the implant site was investigated. The cells surrounding each of the three types of bone matrix were characterized using level of TRAP expression and ultrastructure as criteria. TRAP expression and ultrastructure of the MNGCs were compared to TRAP expression and ultrastructure of normal osteoclasts. The final studies employed the use of hormones and a cytokine to modulate the cellular response to the three types of bone implants. The histochemical and morphological effects of these substances to modulate or to transform the MNGCs into osteoclasts at the site were determined.

In summary, the primary goal of this investigation is a better understanding of the process of bone resorption through characterization of the cells elicited by the implanted bone matrix. The osteolytic potential of cells, other than osteoclasts, that are commonly present and may be active in osteolytic disease states must be assessed to
determine their role in the process of pathological bone resorption. Clinically, bone implants and transplants are used extensively in corrective neurological and orthopedic surgeries and craniofacial reconstructive surgeries, mainly to promote localized bone formation. The extent of mineralization of a devitalized bone implant has a direct effect on its ability to form bone. The implant system described here may be used to evaluate the effectiveness and subsequent cell-mediated resorption of some clinical bone implants. It may also be used to assess the direct role of monocytes and/or macrophages in pathological bone destruction. Characterization of the cells elicited to implanted bone matrix will determine whether the in vivo model system reflects osteoclastic resorption or monocyte-macrophage bone destruction, which has been implicated in osteolytic disease states. Bone resorption is a cell-mediated process, coupled with bone formation, which is able to precisely mold and define the form of the growing and fully developed skeleton. Most of the details of this process are yet to be elucidated.
CHAPTER II

REVIEW OF RELATED LITERATURE

Osteoclast Morphology and Function

Osteoklast was the name given to the multinucleated cell found in the bone microenvironment. Based on circumstantial evidence, as early as 1852, Tomes and de Morgan proposed that bone resorption was a cell-mediated process, however, they were unable to fully characterize the cells, the factors responsible for their occurrence, their distribution or regulation. Subsequent observations described the multinucleated cell in areas of bone loss but did not propose a function for it. Although the multinucleated cell of bone had been described over twenty years previously, the suggestion that bone removal was the principal function of the osteoclast was not proposed until 1872, by Kolliker when he named the cell. Since the osteoclast's identification, the mechanism of its function, its regulation and origin have been the subjects of numerous investigations and controversies.

The osteoclast can be distinguished from other types of multinucleated giant cells by its location next to the bone surface and a combination of characteristic membrane specializations indicative of its function, bone removal. At the light microscopic level, the osteoclast is characteristically a large cell with multiple nuclei. Present in the area of bone removal, the osteoclast is found along the bone
surface in cavities or pits called Howship’s lacunae. The cytoplasm is variable and can range from basophilic to acidophilic (Gothlin and Ericsson, 1976). The definitive feature of an active osteoclast however, is the brush or striated border which is made up of fine cytoplasmic processes located along the bone-cell interface.

Ultrastructurally, the morphological features of the osteoclast have been described in detail. As was seen at the light microscopic level, the most conspicuous features are the large size and multiple nuclei arranged in a cluster. However, the feature that morphologically defines the osteoclast is the ruffled border. The ruffled border, which is equivalent to the brush border at the light microscopic level, is a specialized area of the cell membrane in contact with the bone surface. The ruffled appearance is due to an irregular complex system of cytoplasmic infoldings which exhibit a coated membrane structure (Kallio et al., 1971). Subjacent to the ruffled border are vacuoles and vesicles, which have been shown to contain bone salt crystals. The ruffled border area has been implicated as the area of bone resorption based on the coated cell membrane, which may facilitate formation of the cytoplasmic vesicles, and the presence of bone salt crystals within vacuoles in the area subjacent to the ruffled border. Baron et al. (1985) have shown that the extracellular space bound by the ruffled border and the bone surface is actively acidified by the secretion of lysosomal enzymes by the osteoclast. Also, the extracellular surface of the ruffled border membrane expresses a 100-kD protein similar or identical to those expressed in the limiting membrane of
secondary lysosomes and other acidified organelles. Antibodies to the 100-kD protein cross-react with a proton-pump ATPase from pig gastric mucosa and may perform a similar function of acidification via proton transfer across the membrane. Another feature of the osteoclast, which is not exclusive but necessary for bone resorption, is the clear zone. The clear zone, as the name implies, is an organelle free zone, which contains actin and surrounds the ruffled border in an active cell. It may also be present in inactive osteoclasts and active macrophages and monocytes cultured with devitalized bone particles (King and Holtrop, 1975; Teitelbaum and Kahn, 1980). The clear zone aids in the resorption process by anchoring the cell firmly to the bone substrate and isolating the active resorption site, the ruffled border area. The cell characteristically exhibits a high concentration of lysosomes and ribosomes. Mitochondria are also seen in large numbers, scattered throughout the cytoplasm except in the area of the ruffled border. An extensive Golgi apparatus is present around the clustered nuclei. Also, found in a clustered arrangement, are numerous centrioles, which form a centrosphere (Matthews et al., 1967). Prior to its identification in foreign body giant cells, the centrosphere was believed to be an exclusive feature of the osteoclast (Sapp, 1976). The centrosphere has provided a plausible explanation for the lack of mitotic figures and formation of the osteoclast by cell fusion. Because of their clustered arrangement at a distance from the nuclei, the centrioles would probably not be avaliable for nuclear/cell division.

Histochemically, the osteoclast can be characterized by the
presence of a specific hydrolytic enzyme, the tartrate resistant form of acid phosphatase (TRAP). Acid phosphatases are lysosomal enzymes that have been classically divided into two groups, based on their sensitivity to tartrate. The tartrate sensitive isoenzyme has been identified in all animal cells except the erythrocyte (Anderson and Toverund, 1986). The tartrate resistant form has been identified in human leukocytes and more recently in bone (Li et al., 1970; Minkin, 1982). TRAP has been proposed to be a marker for osteoclast differentiation and function (van de Wijngaert and Burger, 1986; Minkin, 1982). Scheven et al. (1985) have shown the immediate osteoclast precursor is a mononuclear, nonproliferative cell exhibiting TRAP activity. Minkin (1982) observed significant increases in TRAP activity in the media of newborn mouse calvaria cultures which had been stimulated to induce bone resorption, relative to control cultures.

Another biochemical marker proposed for characterization of the osteoclast is carbonic anhydrase. The function of this enzyme is to catalyze the reversible hydration of CO₂. It can facilitate gas transfer or produce H⁺ or HCO₃⁻ rich solutions. Gay and Mueller (1974) isolated carbonic anhydrase in chicken osteoclasts and suggested that its role in H⁺ secretion which could be directly linked to mineral resorption. Vaananen and Parvinen (1983) were able to identify the c-isoenzyme of carbonic anhydrase in rat calvarial osteoclasts. Carbonic anhydrase inhibition studies which showed successful inhibition of bone resorption suggest a role for the enzyme in osteoclast-mediated bone resorption. Administration of osteotropic hormones,
parathyroid hormone, calcitonin and 1,25 dihydroxyvitamin D₃, have been shown to influence either the location or the activity of carbonic anhydrase in osteoclasts in vitro (Cao and Gay, 1985; Hall and Kenny, 1985).

The function of the osteoclast as the effector of bone resorption was proposed over 100 years ago and was initially supported by histological evidence. The classic theory of the bone resorption process indicates that the osteoclast dissolves the bone mineral extracellularly by secretion of acid and chelating agents and then phagocytoses the resorption products, both inorganic mineral and organic matrix components (Sakamoto and Sakamoto, 1986). Although this theory has been accepted, there are areas of uncertainty and controversy; specifically, the collagenolytic potential of osteoclasts and the sequence of events in the process. Bone mineral has been identified within the vacuoles of osteoclasts substantiating osteoclastic uptake of the inorganic components. However, bone collagen has not been seen within the osteoclast and investigators have used this to support the possible role of an auxiliary cell responsible for the removal of the organic matrix components. Heersche (1978) proposed a combined effort by the osteoclast, responsible for the mineral dissolution, and a fibroblast or macrophage, responsible for organic matrix removal. Sakamoto and Sakamoto (1982) suggested that bone resorption occurs in two phases. The first phase involves enzymatic removal of matrix collagen by osteoblastic collagenase followed by a second phase of osteoclastic digestion of the bone crystal. However, Chambers et
al. (1984), using in vitro techniques, have shown the ability of the osteoclast to digest both components of the bone matrix.

**Hormonal Control of Bone Cell Function**

Osteoclastic formation and function is regulated directly and indirectly by a host of calcemic hormones, cytokines and growth factors. Calcemic hormones not only regulate the function of the cells responsible for bone remodelling but also play a role in calcium homeostasis. Parathyroid hormone (PTH) and 1,25 dihydroxyvitamin D$_3$ (1,25(OH)$_2$D$_3$) have stimulatory effects on bone resorption, however receptors for these hormones have not been demonstrated on the osteoclast (Rouleau et al., 1986; Silve et al., 1982). The effects of PTH and 1,25(OH)$_2$D$_3$ must therefore, be mediated by an indirect mechanism. When osteoclasts are mechanically removed from neonatal rat long bones and allowed to adhere to slices of cortical bone, excavation of the bone surface by the isolated osteoclasts begins within hours and can be quantified by scanning electron microscopic analysis of the bone surface. This technique forms the basis for a bioassay by which the effects of hormones on bone resorption can be assessed (Chambers et al., 1985). PTH and 1,25(OH)$_2$D$_3$ administration did not influence the resorption potential of the isolated osteoclasts (Chambers et al., 1985). However, if the osteoclasts are cultured with either osteoblastic cell lines, primary cultures of osteoblasts or the supernatant from PTH or 1,25(OH)$_2$D$_3$ stimulated osteoblastic cells, the increase in resorption is 2 to 4 times that seen without hormone (McSheehy and
In vitro investigations using isolated osteoblasts and osteoclasts indicated that the activation of osteoclasts by PTH was dependent on the presence of osteoblasts. Rodan and Martin (1981) suggested a role for the osteoblast in physiological bone resorption based on identification of receptors and its responsiveness to the bone resorbing hormones. Osteoblasts, which form a cellular barrier over the bone surface, have been shown to change their shape and uncover the bone surface in response to PTH and prostanoids. The uncovered bone surface is then exposed to the osteoclasts for degradation. Also, osteoblasts secrete collagenase and increase the rate of collagenase secretion when stimulated by PTH (Puzas and Brand, 1979; Sakamoto and Sakamoto, 1982). They are able to remove the unmineralized organic layer to expose the subjacent mineral (Chambers and Fuller, 1985). This is an important finding since it has been shown in vitro, that osteoclasts excavate the bone surface only if placed on a mineralized surface and were not able to excavate pits if placed on an unmineralized surface.

PTH facilitates bone resorption in vivo by increasing (1) the number of osteoclasts; (2) the number of nuclei per cell, denoting an increase in formation by increased fusion; (3) the proportion of osteoclasts exhibiting ruffled borders; and (4) the extent of the ruffled border. In vivo, increases in osteoclast number in the rat rib have been detected as early as 30 minutes after the administration of PTH (Burger et al., 1986). An increased number of nuclei per cell has been documented to occur as early as 1 hour after PTH administration in kit-
tens (Addison, 1979). Holtrop et al. (1979) demonstrated an increase in the average size of the ruffled borders, the clear zones and the cells after injection of 50 U of purified bovine PTH. The effects of PTH on osteoclast function include increased synthesis and secretion of acid phosphatase and β-glucuronidase and increased acidity in the extracellular space bound by the ruffled border and bone. (Vaes 1965, 1968; Anderson et al., 1985, 1986).

It remains questionable whether PTH has an effect at the level of osteoclast differentiation. Fuller and Chambers (1987) incubated rabbit bone marrow cells or cells from neonatal rabbit spleen for up to 4 weeks on plastic cover glasses and bone slices in the presence of PTH, 1,25(OH)$_2$D$_3$ and interleukin-1 (IL-1) or a combination of these hormones and the cytokine. Using an osteoclast specific antibody (Horton et al., 1985) and the ability to excavate bone slices as criteria for osteoclast identification, osteoclasts were seen only in cultures of bone marrow or spleen incubated with 1,25(OH)$_2$D$_3$, indicating PTH had no effect on differentiation in this culture system. Perris (1971) however, reported PTH administration in vivo stimulated mitosis in rat bone marrow, thymus and liver. This response to PTH was shown to be mediated by an increase in plasma calcium. In vitro, short term incubations with PTH stimulated mitogenesis in hemopoietic stem cells (Gallien-Lartigne and Carrez, 1974). Since osteoclasts are of hemopoietic origin (Marks, 1983; Chambers, 1985), the increase in osteoclast number seen with PTH administration may be a result of mitogenic stimulation of the bone marrow.
PTH has also been shown to reduce bone formation by directly affecting osteoblast activity. In vitro investigations revealed that the administration of the hormone reduced osteogenesis by inhibiting the synthesis and secretion of matrix proteins including collagen and osteocalcin, profoundly decreased citrate decarboxylation, and possibly inhibited alkaline phosphatase (Kream et al., 1980; Dietrich et al., 1976; Lian and Canalis, 1985; Beresford et al., 1984; Luben and Cohn, 1976; Luben et al., 1976). In vivo, PTH has been shown to cause both inhibition as an acute effect (Bingham et al., 1969) and stimulation as a long term effect (Tam et al., 1982). Intermittent exposure to low levels of hormone similar to the physiological release, produces increased number and activity of osteoblasts. Recently, Slovik (1986) reported increased bone mass in osteoporotic patients with a treatment regimen of intermittent low doses of PTH and 1,25(OH)₂D₃. Sustained exposure of PTH, both in vitro and in vivo, causes a catabolic response including reduced collagen synthesis and increased resorption by secretion of plasminogen activator and collagenase (Hamilton et al., 1985).

The effects of 1,25 dihydroxyvitamin D₃ have not been fully characterized, however evidence has been presented in support of its role in both bone formation and resorption. Receptors for 1,25(OH)₂D₃ have been detected only in osteoblasts and preosteoblasts (Kream et al., 1977; Manologas et al., 1979). Using isolated osteoclasts, Chambers and colleagues (Chambers and Fuller, 1985; Chambers et al., 1985) have shown no effect of 1,25(OH)₂D₃ on cytoplasmic motility or bone resorption. However, 1,25(OH)₂D₃ has been shown to stimulate bone
resorption in vivo and in bone organ cultures. Administration of 1,25(OH)$_2$D$_3$ to 1,25(OH)$_2$D$_3$ deficient rats increased the number of osteoclasts (Yoshiki et al., 1974). As was the case with PTH, the effects of 1,25(OH)$_2$D$_3$ on osteoclast function may be mediated by another cell in the bone microenvironment, possibly the osteoblast.

A number of studies on several tumor cell lines have shown that 1,25(OH)$_2$D$_3$ induces differentiation along the monocyte/macrophage pathway (Abe et al., 1981; Tanaka et al., 1983; Bar-Shavit et al., 1983; McCarthy et al., 1983). Differentiation promoting properties include enhanced adhesion to substrates, increased phagocytic activity including degradation of bone particles, expression of monocyte/macrophage morphology, enzymes and cell surface antigenic determinants. Administration of 1,25(OH)$_2$D$_3$ to mouse alveolar macrophage cultures promoted the formation of macrophage derived multinucleated giant cells (Abe et al., 1981). If these data can be extrapolated, based on similarities between progenitors of monocyte/macrophage and osteoclasts, the results suggest that 1,25(OH)$_2$D$_3$ may promote the differentiation and fusion of osteoclast precursors.

Long term bone marrow cultures with and without 1,25(OH)$_2$D$_3$ administration have been shown to promote MNGC formation (Allen et al., 1981; Testa et al., 1981; Ibbotson et al., 1984; Roodman et al., 1985; MacDonald et al., 1986, 1987). Originally the MNGCs formed in these cultures were believed to be osteoclasts however, the results from this system were easily misinterpreted since macrophage-derived MNGCs, which resemble osteoclasts, were also formed. Standard criteria for identi-
...cation is essential, however osteoclasts generated in vitro may not necessarily express the same characteristics as those in vivo. Fuller and Chambers (1987) reported $1,25(OH)_2D_3$ was essential for osteoclast differentiation but not for macrophage-derived MNGC formation in this system. Criteria for identification of osteoclasts in this investigation included staining with an osteoclast specific monoclonal antibody and the most discriminatory characteristic, excavation of bone slices. The cells that met both criteria were often mononuclear or had few nuclei and were not the MNGCs that had been described in previous long term bone marrow investigations (Testa et al., 1981; Pharaoh and Heersche, 1985; Roodman et al., 1985).

Bone formation and mineralization is also affected by the administration of $1,25(OH)_2D_3$. Continuous infusion of the hormone in normal, young mice promoted calcification of bone matrix (Reynolds et al., 1976; Marie et al., 1985). However, the opposite results have been reported in rats (Wronski et al., 1986). Rickets, a childhood disease of reduced mineralization due to reduced $1,25(OH)_2D_3$, is characterized by low levels of serum calcium and phosphate which are insufficient to promote mineralization. $1,25(OH)_2D_3$ supplements which elevate serum calcium and phosphate to normal levels have been shown to correct the disease. The effects of $1,25(OH)_2D_3$ on osteoblast activity are conflicting. In vivo infusion studies and organ cultures of fetal bone indicated decreased matrix apposition rates and inhibition of collagen synthesis respectively (Marie et al., 1985; Raisz et al., 1978). Investigations using cultures of osteoblast or osteoblast-like...
cells have indicated both stimulation and inhibition of activity (Haneji et al., 1983; Manolagas et al., 1981; Chen et al., 1983; Wong et al., 1977). Along with its effects on bone cells, 1,25(OH)$_2$D$_3$ may also play an immunoregulatory role which could indirectly influence bone resorption by regulating the synthesis and release of immune cell products known to affect bone remodelling (Bhalla et al., 1986; Holder et al., 1985). The complex effects of 1,25(OH)$_2$D$_3$ suggest its possible role in the linking mechanism between bone formation and resorption.

Unlike PTH and 1,25(OH)$_2$D$_3$ which have stimulatory effects and act in an indirect fashion on osteoclast activity and formation, calcitonin (CT) has been shown to have a direct inhibitory effect on the osteoclast. To substantiate the direct mechanism, CT receptors have been identified on the rat osteoclast in situ using $^{125}$I labelled salmon CT (Warshawsky et al., 1980). Within minutes of administration of CT to isolated osteoclasts, morphological changes have been exhibited (Chambers and Magnus, 1982). Anderson et al. (1982) have shown CT specifically affects carbonic anhydrase activity, and Hunter et al. (1988) have demonstrated a general reduction in cellular acidity in response to CT. Akisaka and Gay (1986) demonstrated detachment of the osteoclast from the bone surface with CT-inhibited Na$^+$,K$^+$-ATPase activity localized in the ruffled border. In osteoclasts, CT acts by inhibiting cell movement and membrane ruffling (Baron and Vignery, 1982). The effects of the hormone on mature osteoclasts either PTH-stimulated or unstimulated are profoundly inhibitory, however a transient nature of the inhibitory effect on bone resorption in vitro, a
phenomenon termed escape, has been documented (Raisz et al., 1967; Wener et al., 1972; Tashjian et al., 1978; Feldman et al., 1980). These investigators suggested the escape mechanism may be due to a change in responsiveness to CT of the bones themselves. When CT and PTH are administered to bone organ cultures a reduced number of osteoclasts and reduced $^{45}$Ca release was observed within the first 24 hours. However, after another 24 hours, the values for both parameters recovered to an elevated level similar to cultures treated with PTH alone (Feldman et al., 1980; Tashjian et al., 1978). The transient inhibition may be at the level of recruitment and fusion.

The inhibitory effects of CT on bone resorption have been well documented, although CT has also been shown to influence bone formation. The hormone has been localized in rat and mouse osteoblasts (Morel et al., 1985; Boivin et al., 1987). Using a demineralized bone matrix implant as a model for chondrogenesis and osteogenesis, CT promoted osteogenesis if administered during the initial phases of bone formation due to increased proliferation of cartilage and bone precursors. However, when CT was administered after the onset of osteogenesis, subsequent bone formation was suppressed (Weiss et al., 1981).

Local Control of Bone Cell Function

Regulation of bone function as previously described has been of systemic origin. More recently, attention has been turned to the subject of local control by cytokines. Cytokines are soluble proteins produced by a variety of cells that can modulate the activity of the
cells that produce them and other cells. The effects of cytokines in bone became apparent after the number of osteoclasts was increased in rat bone organ cultures treated with a factor produced by mitogen-activated peripheral blood mononuclear cells (Horton et al., 1972; Raisz et al., 1975). Originally thought to be one factor, it was determined that osteoclast activating factor (OAF) is a family of cytokines that possess OAF activity, including interleukin-1 (IL-1), the most potent, tumor necrosis factor-α (TNFα), and TNF-β.

IL-1 has been shown to be a potent stimulator of bone resorption in two organ culture systems, the fetal rat long bone system and the neonatal mouse calvariae system. Bone resorption in both assays is quantified by $^{45}$Ca release into the medium from prelabelled bones. In both assays system, $^{45}$Ca release increased with increasing concentrations of IL-1 (Dewhirst et al., 1987; Sato et al., 1986; Gowen and Mundy, 1986; Heath et al., 1985). Bone resorption occurred in response to IL-1 in organ culture systems, but not when isolated osteoclasts were treated with IL-1 (Thomson et al., 1986). IL-1 stimulated isolated osteoclasts only when osteoclasts were cultured with osteoblasts, osteoblast-like cells or the supernatant of stimulated osteoblasts. Like other mediators of osteoclastic bone resorption, IL-1 stimulation of osteoclasts occurs indirectly. IL-1 has also been shown to have synergistic bone resorptive effects with PTH and other cytokines (Stashenko et al., 1987; Dewhirst et al., 1987; Lorenzo et al., 1988). This synergistic response between cytokines or the combination of cytokine and hormone suggests complex regulation is mediated by both
local and systemic factors.

In pathologic conditions that feature chronic inflammation, such as rheumatoid arthritis and periodontal disease, increased bone resorption occurs. Horton et al. (1972) proposed the possibility that infiltrating immune cells release cytokines that act locally and stimulate bone resorption. More specifically, Gowen et al. (1983) suggested a role for IL-1 in the pathogenesis of inflammatory disorders in which large numbers of monocytes/macrophages are present at osteolytic sites. IL-1 has been detected in the joint effusions from patients with rheumatoid arthritis where pathological bone loss occurs (Miyasaka et al., 1988). IL-1 may play a critical role in both physiological and pathological bone loss.

Two other cytokines that stimulate and inhibit bone resorption respectively are TNF and interferon-γ. TNF-α and TNF-β have been shown to stimulate osteoclastic bone resorption along with the inhibition of bone formation in the fetal rat long bone and neonatal mouse calvariae (Bertolini et al., 1986; Deitrich et al., 1976). As was the case with IL-1, stimulation of bone resorption by TNF is indirect (Thomson et al., 1987). Interferon-γ does not possess OAF activity and has been shown to inhibit both hormone and cytokine stimulated bone resorption (Gowen et al., 1986).

Growth factors have also been shown to be possible mediators of bone cell development and/or function. Transforming growth factors (TGF)s, which had originally been described as factors that induce neoplastic changes on normal cells, have been shown to affect bone cell
function. TGF-β stimulates bone resorption, DNA and collagen synthesis in cultures of fetal rat calvariae (Centrella et al., 1986) and collagen synthesis and alkaline phosphatase in rat osteosarcoma cells (Pfeilschifter et al., 1987). TGF-β-like activity is produced by bone and released during the resorption process (Pfeilschifter and Mundy, 1987). Release of TGF-β during resorption and its stimulatory effects on osteoblasts, indicate TGF-β may play a role in the coupling of bone formation to resorption.

The Origin of the Osteoclast

The osteoclast can be distinguished from osteogenic bone cells not only by its morphology but also by its origin. A variety of experimental methods, including parabiosis and transplantation of bone marrow and spleen cells, have indicated the local mesenchymal origin of osteogenic cells and the extraskeletal origin of the mononuclear precursors that fuse to form multinucleated osteoclasts (Gothlin and Ericsson, 1973; Walker, 1975; Marks and Schneider, 1978, 1982). The identity of the precursor has not yet been definitely established although three possible precursors have been proposed. The oldest theory is that the monocyte or macrophage are the direct precursors. Various studies have demonstrated labelled osteoclasts after labelling peripheral blood leukocytes (Fischman and Hay, 1962; Jee and Nolan, 1963). Zabonin-Zallone et al. (1984) have also shown fusion of isolated monocytes with multinucleated cells in 5 day cultures of isolated osteoclasts. However, there are significant differences between osteo-
clasts and monocytes and macrophages. Macrophages, monocytes and multinucleated phagocytes do not exhibit the hallmark characteristic of osteoclastic bone resorption, namely the ruffled border (Kahn et al., 1978; Rifkin et al., 1979). Osteoclasts lack the characteristic membrane receptors, Fc and C3, and specific surface antigens characteristics of the mononuclear phagocyte lineage (Hogg et al., 1980; Horton et al., 1984, 1985a, 1985b). Recent organ culture studies indicate that osteoclasts will develop from precultured bone marrow mononuclear phagocytes only in the presence of live bone and will resorb only live bone whereas devitalized bone is most likely resorbed by macrophages which are able to multinucleate in response to a number of stimuli (Burger et al., 1984). Also infusions of macrophage or monocyte/macrophage precursors are incapable of restoring osteoclastic bone resorption in osteopetrotic mutants (Schneider and Byrnes, 1983; Schneider and Relfson, 1988). The monocyte/macrophage would therefore be an unlikely candidate for the osteoclast precursor. Burger et al. (1982) have recently shown that only the young, proliferating, weakly adherent mononuclear cells from the bone marrow can differentiate into osteoclasts. Their conclusion was monoblasts or promonoblasts are the more likely candidates for precursor to the osteoclast.

The last possible theory is that the hemopoietic stem cell gives rise to a separate differentiation line. This is based on the inability murine and human osteoclasts to interact with antibodies specific for monocytes and granulocytes (Loutit and Nisbet, 1982; Horton et al., 1984, 1985a, 1985b). Also, transplantation of normal pluripotent
hemopoietic stem cells into a histocompatible osteopetrotic recipient resulted in removal of the excess bone characteristic of osteopetrosis and the production of phenotypically normal osteoclasts (Schneider et al., 1986).

**The Osteoclast and the Mononuclear Phagocyte System**

The osteoclast, because of a putative common lineage and functional and morphological similarities, has been closely associated with the cells of the mononuclear phagocyte system (MPS). The MPS is composed of hemopoietic precursor cells, promonocytes, circulating monocytes and tissue macrophages and their derivatives including macrophage polykaryons (van Furth et al., 1972). Macrophage polykaryons or multinucleated giant cells (MGC) are commonly found at inflammatory sites. They are formed by fusion of mononuclear phagocytes and they may possess from 2 to more than 200 nuclei. Two types of giant cells can be identified based on nuclear arrangements, however they may be variant phases of the same cell. The foreign body giant cell (FBGC) type has nuclei dispersed throughout its cytoplasm while the Langhans type exhibits a circular or semicircular arrangement of nuclei around the cell periphery (Chambers, 1978). The cell types are not specifically responsive to particular agents and both can be observed at the same inflammatory site. Mariano and Spector (1973) observed both cell types in response to subcutaneously implanted glass coverslips. Initially, only foreign body giant cells were observed but by 7 days the Langhans cells were more frequently seen. Cytochemical and ultrastructural
analysis of the Langhans cell revealed increased levels of acid phosphatase and succinic dehydrogenase and a highly developed Golgi apparatus as compared to FBGCs. A general morphological profile includes oval euchromatic nuclei, abundant cytoplasm, lysosomes and plasma membrane ruffling. Cells of the MPS and osteoclasts are concerned with the removal of biological matter and are equipped with the necessary organellar machinery needed to accomplish their function (Chambers, 1978). From a phylogenetic point of view, the mononuclear phagocyte and its derivatives are a primitive form of defense. Comparatively, the osteoclast is a relatively new cell, developing with the evolution of the internalized bony skeleton. Cells of the MPS perform critical roles such as phagocytosis and antigen presentation in immune and foreign body responses. The osteoclast has not been seen to function in this manner. However, mononuclear phagocytes have been shown to have osteolytic capacity.

The possible role of the mononuclear phagocyte in the process of bone resorption has recently become apparent. Monocytes and macrophages are often seen at the sites of rheumatoid arthritis, periodontal disease, and tumors that metastasize to bone. Although osteoclasts are principally responsible for physiological bone resorption, mononuclear phagocytes may also function directly in bone resorption, or indirectly by stimulating osteoclastic activity. Organ culture studies using fetal rat long bones indicated the products of resorbing bones have a chemotactic effect on human blood monocytes (Mundy et al., 1978). Evidence has also been presented to substantiate the direct ability of
phagocytes to degrade devitalized bone in vitro (Kahn et al., 1978; Mundy et al., 1977; Teitelbaum et al., 1979). Kahn et al. (1978) have shown that the resorbing monocytes develop osteoclastic characteristics, clear zones and the release of acid phosphatase at the bone-cell interface. Fallon et al. (1983) showed macrophage polykaryons exhibited an increased osteolytic capacity compared to their mononuclear counterparts. They proposed the fusion of cells has a physiological significance which in osteoclasts and macrophage polykaryons, is directly related to bone resorption. Another study substantiating the ability of mononuclear cells to resorb bone in vitro indicated an increase in $^{45}$Ca release with the administration of E. coli lipopolysaccharide to bone cultures but no effect with PTH or CT, indicating the mononuclear activity was modulated by inflammatory mediators and not by osteotropic hormones (McArthur et al., 1980). Rifkin et al. (1980) reported similar results in cultures of fetal rat long bones. Mononuclear cells were capable of osteoid ingestion and this ingestion was unaffected by administration of inhibitors of osteoclastic resorption. In vitro studies using monocytes obtained from patients suffering from juvenile rheumatoid arthritis exhibited significantly greater $^{45}$Ca release from cultured calvariae when compared to normal age-matched patients (Key et al., 1986). These studies indicate the possible involvement of monocytes in the bone degradation process of rheumatoid arthritis. Mononuclear phagocytes and their derivatives may play an essential, direct and independent role in the physiological and/or pathological processes of bone resorption.
Although there is no direct evidence of monocyte or macrophage fusion to form osteoclasts or a relationship between multinucleated phagocytes and osteoclasts, the putative ontogenic relationship and morphological and functional similarities between these cells have provided a basis for the use of macrophages, monocytes and multinucleated phagocytes in studies of osteoclastic bone resorption (Teitelbaum and Kahn, 1979). However, along with the similarities that exist, there are important differences between osteoclasts and the cells of the MPS that prohibit the use of macrophages, monocytes and multinucleated phagocytes as osteoclast surrogates in experimentation. The ability of mononuclear and multinuclear phagocytes to degrade bone matrix may suggest a role, separate and distinct, from the osteoclast in the normal versus the pathological processes of bone resorption.

The Ectopic Bone Implant System

Implantation or transplantation of live mineralized bone or devitalized, demineralized bone at an ectopic site has become a common model system for osteogenesis, or bone formation (Reddi and Huggins, 1972). The effect of the substrate specificity became apparent with the development of an in vivo implantation model for bone resorption using devitalized, mineralized bone matrix (Glowacki et al., 1981). Devitalized, mineralized bone matrix which was implanted into calvarial defects or a subcutaneous site resulted in a rapid response of cell-mediated bone resorption. Histological evaluation of the implant site revealed mononuclear and multinuclear phagocytes surrounding the bone
particles. Functional analysis indicated bone removal had occurred. Therefore, the mineral content of the implant determines the initiation of either bone resorption or bone formation and may have clinical implications in reconstructive or corrective surgery where bone implants are readily used. In the calvarial defect model, the elicited cells seen may have migrated from the neighboring bone but in the subcutaneous model the implant is isolated from the normal bone cell populations. Although it is proposed that the multinucleated giant cells surrounding the bone surface are indeed osteoclasts (Glowacki et al., 1981; Glowacki and Cox, 1986; Glowacki et al., 1986), similar studies are not able to substantiate these results and characterize these cells as multinucleated giant cells or macrophage polykaryons (Walters and Schneider, 1985, 1987, 1988; Dvonch and Schneider, 1986; Popoff and Marks, 1986). Multinucleated giant cells are commonly found at inflammatory sites. Chronic inflammation or granulomatous inflammation is a response induced by infective agents, foreign bodies or physical irritants. These responses, characterized by a collection of mature mononuclear phagocytes, result because of the persistent and often particulate nature of the foreign body or irritant that has resisted destruction by an acute inflammatory reaction. In this model system the particulate and indestructable nature of the bone implant may provoke a granulomatous inflammatory response. Therefore, further investigation is needed to characterize these multinucleated giant cells at the site of the implant before this subcutaneous bone implant can be used as a model for osteoclastic bone resorption or monocyte/
macrophage bone destruction.
CHAPTER III

QUANTIFICATION OF TARTRATE RESISTANT ACID PHOSPHATASE ACTIVITY USING A COMPUTERIZED IMAGE ANALYSIS SYSTEM

ABSTRACT

Tartrate resistant acid phosphatase (TRAP) is a hydrolytic enzyme that has been associated with bone resorption and has been accepted as a marker for identification of osteoclasts. The ease with which this enzyme can be demonstrated, combined with specific morphological features, creates a quick method for identifying osteoclasts in normal, pathological and experimental conditions. Recently, the specificity and reliability of the enzyme as a marker has been questioned since other cells have been shown to express TRAP. In this study I have developed a method for quantitating TRAP using an image analysis system and have identified a level of the enzyme specific to osteoclasts which can be used to differentiate osteoclasts from other cells capable of TRAP expression. TRAP expression characteristic to the osteoclast was compared with that expressed by multinucleated giant cells (MNGC)s recruited to the site of subcutaneously implanted mineralized bone matrix. Two weeks post-implantation, the pellets were removed and processed for the demonstration of TRAP along with rat proximal tibiae. Using image analysis, the density and distribution of the
enzyme could be determined. A high level of TRAP was consistently expressed by the *in situ* osteoclasts. The MNGCs associated with the mineralized bone implants expressed little if any TRAP reaction product. TRAP levels from the mineralized implant associated MNGCs were significantly different (*p* < 0.05) from the level of expression by tibial osteoclasts. Using this system, the amount of TRAP reaction product or any other enzyme reaction product expressed can be objectively and reproducibly quantitated. In this investigation, I have developed a method which identified a high level of TRAP expression specific to the osteoclast and this can be used to differentiate osteoclasts in other normal and experimental situations.
INTRODUCTION

Tartrate resistant acid phosphatase (TRAP) has been widely accepted as a cytochemical marker for the osteoclast and its precursors (Scheven et al., 1986; van de Wijngaert and Burger, 1986; van de Wijngaert et al., 1987; Hammerstrom et al., 1971). The presence of the enzyme and other morphological features like multiple nuclei and the cell's location along the bone surface allow for quick and easy identification of the osteoclast in the normal microenvironment of bone. TRAP is relatively specific for osteoclasts but the enzyme has been identified in osteoblasts, osteocytes, hairy cells, alveolar macrophages and activated macrophages in normal and pathological states (Bianco et al., 1987; 1988; Cole and Walters, 1987; Ketchum et al. 1985; Efstratiadis et al., 1985; Razdun et al., 1983; Snipes et al., 1986). Osteoclasts are not a product of macrophage or osteoblast fusion and the presence of TRAP in macrophages, macrophage derived multinucleated giant cells (MNGC)s or osteoblasts is probably not an indication of their ability to differentiate into osteoclasts. Morphological and histochemical similarities between the osteoclast and MNGCs and/or macrophages may create problems in identification of osteoclasts in experimental systems where both may be present. The precise function of TRAP is unknown but it is a hydrolytic enzyme related to the process of bone resorption.

In an attempt to study the ontogeny, behavior and function of the osteoclast, in vitro and in vivo model systems have been developed
which employ TRAP as a means of osteoclast identification. Two in vitro systems have been extensively studied. In the first system, periosteal free, 17 day old fetal metatarsals from the mouse, which do not develop osteoclasts on their own, can serve as a live substrate to induce development of osteoclasts when cocultured with osteoclast precursors (Burger et al., 1982). In this model system osteoclasts and their immediate mononuclear precursors have been identified using TRAP and morphological criteria.

In a second popular in vitro model system, large multinucleated cells, originally thought to be osteoclasts were generated in long term cultures of bone marrow mononuclear cells. These cells were presumed osteoclasts based on multiple nuclei, morphology and presence of TRAP (Ibbotson et al., 1984; Roodman et al., 1985; Takahashi et al., 1987). It has recently been questioned whether these cells are osteoclasts or MNGCs, since both can be generated in these cultures (Fuller and Chambers, 1987; Hattersley and Chambers, 1989). Whether or not osteoclasts can be generated in these cultures is not questioned but whether all MNGCs that are generated are osteoclasts.

Other than transplant studies using mutant animals (Schneider and Byrnes, 1983; Schneider, 1985; Marks and Schneider, 1978), there are few models for the study of osteoclasts in vivo. Subcutaneous implantation of devitalized, mineralized bone particles, which was originally thought to elicit a MNGC response after 12 days, more recently has been proposed as a system to elicit osteoclasts (Glowacki et al., 1981; Glowacki et al., 1986; Glowacki and Cox 1986). Others
believe the response is not osteoclastic but an inflammatory response eliciting foreign body giant cells (Walters and Schneider, 1985, 1988; Holtrop et al., 1982; Popoff and Marks, 1986; Schmitz et al., 1986). In some of these investigations TRAP was employed to aid in proving or disproving the presence of osteoclasts. In a study by Glowacki et al., (1986) not only was the presence of TRAP in MNGCs adjacent to bone particles, polyethylene particles and polymethylmethacrylate particles acknowledged, but the intensity of the reaction product was scored. Walters and Schneider (1988) also scored and compared the intensity of TRAP expressed by osteoclasts and MNGCs elicited by subcutaneously implanted bone and suture. These were the first attempts to use the level of TRAP reaction product expressed to characterize a cell. The purpose of this investigation was to devise a method to objectively and reproducibly quantitate and compare the levels of TRAP expression in multinucleated giant cells elicited by a mineralized bone implant and in situ osteoclasts. By quantitating the level of TRAP expression, the level of intensity characteristic of the osteoclast may be determined. This quantitation of TRAP is a more specific marker than the presence or absence of the enzyme. MNGCs other than osteoclasts may express TRAP but the level of TRAP expression may differ from that of the osteoclast. This is the first attempt to standardize the method of analysis.
MATERIAL AND METHODS

**Animals:** These studies were performed using Norway-hooded Long Evans rats (Harlan Labs, Indianapolis, IN). All animals were maintained and used according to the recommendations in the Guide for the Care and Use of Laboratory Animals and the Guidelines of the Institutional Animal Care and Use Committee at Loyola University Medical Center.

**Mineralized bone implant:** Tibiae and fibulae were removed from 4-8 wk old Norway-hooded Long Evans rats. The soft tissue and cartilagenous epiphyses were removed and the marrow cavities were flushed with distilled water. The cleaned diaphyses were washed overnight in distilled water then extracted with absolute ethanol (30 min) and anhydrous ether (30 min). The bones were dried, pulverized with a mortar and pestle and sieved to yield particles 75-250 μm in diameter. Sterile water was added to samples to create cohesive pellets of bone particles that could be easily implanted subcutaneously. The pellets were air dried and sterilized by U.V. radiation.

**Implants:** Five wk old male Norway-hooded Long Evans rats (n=6) were anesthetized with chloral hydrate (400 mg/kg). Dorsal, upper thoracic incisions were made bilaterally and subcutaneous pockets were created. Each animal received a bone implant. The incisions were sutured and treated with betadine. Two wks post-implantation, the animals were sacrificed via ether inhalation, and the implants were carefully
excised. A 3 wk old rat was also sacrificed and the proximal tibia removed.

**Morphological and Histochemical Analyses:** The implant and a sample of proximal tibia were immediately fixed in cold 2.5% glutaraldehyde, 0.1 M cacodylate buffer (pH 7.2) with 7% sucrose for 3 hrs, dehydrated in ascending concentrations of cold acetone and embedded in JB-4. Three micron sections were cut and allowed to adhere to gel-coated slides in the cold for 5 days. Tartrate resistant acid phosphatase activity was demonstrated using the Cole and Walters (1986) technique and 50 mM (L+) tartaric acid, however, the samples were not decalcified. Control sections were incubated without substrate. Twenty cells from each of sample were analyzed.

**Image Analysis of TRAP:** The level of intensity of the TRAP reaction product in multinucleated giant cells located along the bone surface was analyzed using a computerized imaging system. The image was collected using a Dage MTI 68 series video camera attached to the Leitz Orthoplan 2 light microscope. The image was processed using the Colorado Video digitizer 270A and Priceton Gamma Tech (PGT) Imagecraft System 4+, run on a Digital Equipment Systems PDP11/73 using PGT Imagecraft (Figure 1). The sections were examined and the field of interest was displayed on a television monitor (Figure 2). To ensure overall consistency, a wire mesh grid was used as a standard to create a reproducible range of intensity and contrast which would include the
gray scale range of reaction product and background. Inconsistencies in the counterstained background of the samples were further corrected by standardizing the background in each sample to the intensity of mineralized bone. Using samples of implants and tibia, the lowest level at which TRAP reaction product that could be detected was used as a starting point to establish 4 gray level ranges of TRAP reaction product intensity. On the 256 gray level scale, high intensity reaction product (+++), composed level 0-100; moderate intensity reaction product (++), composed level 102-150; low intensity reaction product (+), 151-175 and absence of reaction product (0), 175-256. Computer generated colors were assigned to each of the pixel ranges (Figure 3). This color tablet was stored and used on all images. An image of the field in view was collected and displayed on the computer screen. Area fraction analysis based on this gray-scale palette was limited to the specific window containing the MNGC (Figure 4 & 5). A ratio of the percentages of the gray scale windows, generated by area fraction analysis was used to determine the final score for each cell. Cells were scored as expressing high levels (+++), moderate levels (+) or absence of reaction product. If the percentage of the TRAP reaction product that scored for moderate intensity (gray level range 102-150) was 1.5x more than the percentage of the TRAP reaction that scored for low intensity (gray level range 151-175), the cell was scored as high intensity. If the cell failed to meet these requirements, the cell was scored as moderate intensity. If the cell exhibited a percentage of less than 1 in the low intensity range (151-175), the cell was scored
as absence of reaction product. The cell was scored as very high intensity if the percentage value for the high intensity range (0-100) was 5x the moderate intensity percentage (102-150). All tibial osteoclasts exhibit a very high to high level of reaction product (+++ or ++) and these ranges were combined. Multiple linear comparisons within the implant group and with the tibial osteoclasts were performed using the Welsch's t'W test for multiple linear comparisons (Wilcox, 1987).
RESULTS

Active osteoclasts from the proximal tibia were identified as multinucleated cells exhibiting vacuolization of the cytoplasm adjacent to the bone surface. Tartrate resistant acid phosphatase reaction product was observed as red granules within the cells and extracellular staining of the bone matrix adjacent to the osteoclasts (Figure 6). All osteoclasts consistently exhibited the highest staining intensity.

Each implant was surrounded by a fibrous connective tissue capsule. At the site of the mineralized implant, large multinucleated cells were found in contact with the bone particles. The majority of the multinucleated cells in contact with the bone particles expressed either a low level of TRAP reaction product or absence of the enzyme (Figure 7). The average number of MNGCs from the implant group expressing high levels of TRAP was significantly different (p<0.05) than the average number of osteoclasts expressing high levels of TRAP the proximal tibiae (Figure 8). The enzyme was generally distributed throughout the cytoplasm but in some cells TRAP was localized along the bone apposed cell membrane. There was also a low level of nonspecific extracellular staining that could easily be distinguished from intracellular reaction product. Other MNGCs that were present at the site and not in contact with bone particles also expressed low levels of TRAP. These cells were not analyzed. The enzyme expression in the population of MNGCs from the mineralized implants analyzed was significantly lower than the osteoclasts (Figure 8).
DISCUSSION

This study describes a technique to assess the level of TRAP activity and localization in MNGCs and osteoclasts using digital analysis. The presence of TRAP has contributed to the identification of osteoclasts in the normal microenvironment of bone and in experimental model systems which have been used to study bone resorption and osteoclast development and function (Burger et al., 1982; Walters and Schneider 1988; Glowacki et al., 1986; Ibbotson et al., 1984). The level of acid phosphatase activity and TRAP in particular, has been evaluated to determine the best fixation and demineralization procedures and effects of various concentrations of inhibitors on various acid phosphatase positive cells (Liu et al., 1987; Hammarstrom et al., 1971). Recently, in studies on bone resorption, the level of TRAP activity in MNGCs has been analyzed to indicate a difference between MNGCs and osteoclasts. Both Walters and Schneider (1988) and Glowacki et al. (1986) evaluated the level of TRAP in MNGCs recruited to subcutaneously implanted bone and other substances. In the investigation by Walters and Schneider (1988), TRAP activity in MNGCs elicited by bone and suture implants was scant and differed from the high levels expressed by tibial osteoclasts. Glowacki et al. (1986) claimed that MNGCs elicited to devitalized bone implants were seen with high levels of TRAP activity suggesting these MNGCs were osteoclasts. The TRAP activity in cells from the bone implant however, was not compared to in situ osteoclasts from bone. Each study was a subjective analysis of
TRAP activity. Regardless of the level of TRAP activity needed for a cell to be identified as an osteoclast, error may result in the reproducible quantitative assessment of enzyme activity by an observer. In this investigation, the level of TRAP expression by the MNGCs from the mineralized bone implant was significantly different from the TRAP expressed by osteoclasts. The morphology of the MNGCs was also nonosteoclastic, (data not shown), indicating the cells elicited by the mineralized bone implants were not osteoclasts.

In the system described here, levels of activity, which comprise a specific category defined by the investigator, are set and analysis is objective and reproducible. The image of the field of interest is collected and can be analyzed immediately or saved for future analysis. Images can be saved and displayed simultaneously with other fields for comparison. This system allows for detection of the enzyme intensity related to distribution. Ultrastructurally, acid phosphatase has been localized within the extracellular channels of the ruffled border of the osteoclast, the bone-cell interspace and the underlying degraded matrix (Lucht 1971; Doty and Schofield 1972). In this study TRAP was also localized in the bone apposed cytoplasm and extracellularly on the bone surface.

Area fraction analysis can be performed on an entire digitized image or any part which is enclosed in a window. A window which is limited to the MNGC is analyzed. The area of the window closely approximates the area of the cell so that the percentages of gray level intensities corresponding to TRAP reaction product are only those from
the cell. This, however, was a close approximation since the shape of the cells is variable. For the type of analysis in this investigation, if a window could have been drawn enclosing a specific area dictated by the shape of the cell, analyzing the entire cell and no other part of the field would be ensured.

This method of analysis may be applied to other investigations where analysis of intensity and distribution of reaction product is necessary. The specificity of TRAP to the osteoclast may need to be redefined in terms of the level of intensity expressed, especially in experimental model systems where other cells are capable of TRAP expression and multinucleation. Although 4 categories were established in this study, the availability of 256 gray levels allows for more categories consisting of smaller ranges and more specific levels of analysis. The established categories however, were able to distinguish between levels of TRAP activity expressed by osteoclasts and implant-elicited MNCCs. TRAP is demonstrated using a variety of substrates and tissue preparation methods, although this analysis system would not standardize the method of demonstration it may standardize the method of assessment so that future investigations using similar techniques may be compared.
Figure 1.

Computerized image analysis system for the quantification of intracellular TRAP reaction product.
A - Dage MTI 68 series video camera
B - Leitz Orthoplan 2 light microscope
C - General Electric model 4MV-09 video monitor
D - Colorado Video Inc. video digitizer 270A
E - Princeton Gamma Tech System 4+
   Princeton Gamma Tech Imagecraft Software
   Digital Equipment Corp. PDP 11/73 computer

Figure 1
Figure 2.
Digitized image of rat proximal tibia stained for TRAP activity. Osteoclast (arrows) along the bone (b) surface demonstrating intense reaction product.

Figure 3.
Color enhanced image of rat proximal tibia from Figure 2. Color enhanced gray level ranges highlight intensity and distribution of TRAP reaction product within the osteoclasts (arrows).

Figure 4.
Area of analysis is limited to osteoclast by creating a window around the cell.

Figure 5.
Color assigned gray level ranges (upper left) and histogram of gray level distribution from which area fraction analysis is computed. A numerical value is computed for amount of area occupied by each gray level range.
Figure 6.
Section of a 3 week old rat proximal tibia demonstrating osteoclasts (arrows) along the bone (b) surface stained intensely with TRAP reaction product. x400

Figure 7.
Section of mineralized bone implant demonstrating absence of TRAP reaction product in MNGC (arrows) adjacent to bone particles (b). x425
Figure 8.

TRAP expression in MNGCs elicited by mineralized bone implant (2wk post-implantation) and in situ osteoclasts. TRAP reactivity scored as high level (++), moderate level(+), or absence of reaction product (0). (mean ±SEM).

*, significantly different than tibia (++), p<0.05.
Figure 8

Level of TRAP expression

- ++ high
- + moderate
- 0 absence

Number of MNGC's

Mineralized Implant

Tibia

Figure 8
CHAPTER IV

A MORPHOLOGICAL AND HISTOCHEMICAL COMPARISON
OF THE CELLS ELICITED BY
ECTOPIC BONE IMPLANTS AND TIBIAL OSTEOCLASTS

ABSTRACT

Pellets of mineralized, demineralized and a composite mixture of mineralized and demineralized, devitalized bone particles were subcutaneously implanted on the dorsal body wall of young adult rats. Two wks post-implantation, the pellets were removed and processed for histochemical and morphological analyses. Rat proximal tibia was also processed for evaluation. The levels of tartrate resistant acid phosphatase (TRAP) activity in the multinucleated giant cells (MNGC) from each of the three implants and osteoclasts were assessed using an image analyzer. The osteoclasts from the proximal tibia and the majority of MNGCs from the demineralized implants demonstrated high levels of TRAP activity. MNGCs from the mineralized implants showed either a low level or absence of TRAP activity. Most MNGCs from the composite implants exhibited a low level of TRAP activity, however there was a population of cells that demonstrated a high level of reaction product, similar to that seen in the tibia and demineralized implant. Morphologically, osteoclasts from the proximal tibia and from
the osteogenic demineralized implant exhibited ruffled borders. A small population of MNGCs from the composite implant also revealed osteoclastic features. In summary, MNGCs from the mineralized implant did not exhibit a level of TRAP reaction product or morphology similar to osteoclasts, while the majority of cells from the demineralized implant and a subpopulation of the MNGCs elicited by the composite implant did demonstrate TRAP expression and morphology similar to osteoclasts. The ability to elicit cells expressing osteoclasts at an ectopic site may be more dependent on the presence of demineralized matrix than mineralized matrix.
INTRODUCTION

Subcutaneous or intramuscular implantation of devitalized, demineralized bone matrix can induce osteogenesis at an extraskeletal site (Reddi and Huggins, 1972; Urist and Strates, 1970). The reproducible sequence of events that occurs in response to the implants is analogous to endochondral bone formation with new bone formation occurring on days 10-11 and remodelling by osteoclasts, occurring on days 12-18. Implantation of devitalized, mineralized bone matrix or a composite of mineralized/demineralized matrices at a subcutaneous or intramuscular site inhibits this inductive ability (Urist and Strates, 1970). Early studies by Reddi and Huggins (1972) identified multinucleated giant cells, rich in organic acids presumably for mineral dissolution, at the site of a mineralized matrix implant and proposed the presence of the mineralized matrix either alone or in combination with demineralized matrix inhibited osteogenesis. Resorption of implanted bone fragments was quantitated by histomorphometry and radioactive calcium release assays to indicate a cell-mediated osteolytic response did in fact occur (Glowacki, 1982; Walters and Schneider, 1985). Whether the osteolytic response was osteoclast-mediated was questionable.

Implantation of devitalized, mineralized bone into a cranial defect produced a similar cell-mediated osteolytic response, and the multinucleated giant cells were identified as foreign body giant cells (Glowacki et al 1981; Glowacki, 1982; Holtrop et al., 1982). None of
the cells were characterized as osteoclasts because they did not demonstrate a ruffled border, the hallmark morphological feature of the osteoclast. It is probable that mononuclear and multinuclear phagocytes were responsible for the removal of bone, since in vitro studies have indicated that monocytes and macrophages are capable of resorbing devitalized mineralized bone (Mundy et al., 1977; Kahn et al., 1978; Teitelbaum et al., 1979; McArthur et al., 1980). In these investigations, mononuclear phagocytes and their derivatives did not exhibit ruffled borders which are functionally linked to the export of hydrolytic enzymes and the uptake of degraded matrix components. Teitelbaum et al. (1979) proposed mononuclear phagocytes internalize and digest bone particles, a different mechanism of osteolysis than the osteoclastic resorption of bone extracellularly, which would explain the absence of the ruffled borders in these cells. Epithelioid and foreign body giant cells are mildly phagocytic and are seen in contact with the bone particles giving the impression of an osteoclastic mode of bone resorption.

Even though osteoclasts were not identified in the ectopic bone matrix implantation system, it was pursued as a model for the study of bone resorption (Glowacki, 1982; Glowacki, 1983). This was in part due to the lack of model systems to study the osteoclast and the acceptance of cells of the mononuclear phagocyte system as surrogates because of their presumed common, if not direct, lineage (Kahn et al., 1978; Teitelbaum et al., 1979). Since these investigations a decade ago, a number of differences have been demonstrated between cells of the mono-
nuclear phagocyte system and osteoclasts (Chambers and Magnus, 1982; Horton et al., 1984; Hogg et al., 1980). Because of these differences and improvements in isolation techniques, the surrogate studies were abandoned and the osteoclast itself is used for study. The in vivo bone implant model however, was not abandoned and in a later study Glowacki and Cox (1986) generated osteoclasts that demonstrated tartrate resistant acid phosphatase and ruffled borders. Other investigations have not substantiated these results (Walters and Scheider, 1985, 1988; Popoff and Marks, 1986). Therefore, there is conflicting evidence on the nature of the multinucleated giant cells (MNGCs) recruited to the subcutaneously implanted mineralized bone particles. In this investigation, I have examined the morphology and level of TRAP expressed by the multinucleated giant cells recruited to devitalized mineralized, demineralized and a composite of mineralized/demineralized matrices implants 2 weeks post-implantation, and compared the elicited cells to osteoclasts from rat proximal tibia.

The purpose of this investigation is to assess the ability of each of these three implants to generate osteoclasts that are similar in morphology and expression of TRAP to normal osteoclasts found in the proximal tibia. Although the implant composed of both mineralized and demineralized bone matrices is unable to induce osteogenesis the presence of both matrices may have an effect directly or indirectly on osteoclast recruitment. Not only will the presence or absence of TRAP be evaluated, but also the intensity of the reaction product, to further discriminate between the high level of TRAP expressed by function-
al osteoclasts and the low level of TRAP that has previously been demon­
strated in multinucleated giant cells generated at the site of bone and suture implants (Walters and Schneider, 1988). If the multi-
nucleated giant cells recruited to the mineralized bone particles, in fact are osteoclasts, they should resemble in situ osteoclasts from the proximal tibia.
MATERIAL AND METHODS

Animals

These studies were performed using Norway-hooded Long Evans rats (Harlan Labs, IN). All animals were maintained and used according to the recommendations in the Guide for the Care and Use of Laboratory Animals and the Guidelines of the Institutional Animal Care and Use Committee at Loyola University Medical Center.

Bone Powder Preparation

Mineralized bone implant: Tibiae and fibulae were removed from 4-8 wk old Norway-hooded Long Evans rats. The soft tissue and cartilagenous epiphyses were removed and the marrow cavities were flushed with distilled water. The cleaned diaphyses were washed overnight in distilled water then extracted with absolute ethanol (30 min) and anhydrous ether (30 min). The bones were dried, pulverized with a mortar and pestle and sieved to yield particles 75-250 μm in diameter. Sterile water was added to samples to create cohesive pellets of bone particles that could be easily implanted subcutaneously. The pellets were air dried and sterilized by ultraviolet (U.V.) radiation.

Demineralized bone implant: The mineralized bone powder prepared above was treated with 0.5 N HCl, 25 meq/gm (demineralizing agent) for 3 hrs. followed by repeated washings of cold deionized water to thoroughly remove the acid, absolute ethanol (30 min) and anhydrous ether. (30
min). Sterile water was added to samples of demineralized bone to create pellets. The pellets were air dried and sterilized by U.V. radiation.

Composite implant: A mixed bone powder implant was created from 25 mg mineralized bone powder and 5 mg demineralized bone powder. Sterile water was added to the mixture of the two powders to create cohesive pellets which were air dried and sterilized by U.V. radiation.

Implants

Six 5 wk old male Norway-hooded Long Evans rats were anesthetized with chloral hydrate (400 mg/kg). Dorsal, upper thoracic incisions were made bilaterally and subcutaneous pockets were created. One bone powder pellet was placed in each subcutaneous pocket and the incisions were sutured and treated with betadine. Each animal received one of each of the three types of bone implants. Two wks. post-implantation, the animals were killed via ether inhalation, and the implants were carefully excised.

Morphological and Histochemical Analyses

One half of the specimen was immediately fixed in 4.0% glutaraldehyde, 0.1 M cacodylate buffer (pH 7.2) for 24 hrs, post-fixed in 1% osmium tetroxide, dehydrated in ascending concentrations of ethanol, and embedded in Epon 812. One micron sections were cut and stained with toluidine blue and evaluated by light microscopy. Thin sections
were cut and stained with uranyl acetate and lead citrate for ultrastructural analysis using the Hitachi H600 electron microscope.

The remaining half of each specimen was immediately fixed in cold 2.5% glutaraldehyde, 0.1 M cacodylate buffer (pH 7.2) with 7% sucrose for 4 hrs, dehydrated in ascending concentrations of cold acetone and embedded in JB-4. Proximal tibiae were removed from 3 wk old Norway black-hooded Long Evans rats and also prepared as above. Three micron sections were cut and allowed to adhere to gel-coated slides in the cold for 5 days. Alternate sections were stained for tartrate resistant acid phosphatase activity using 50 mM L(+)-tartaric acid or for mineral content using the von Kossa technique (Humason, 1962). Tartrate resistant acid phosphatase activity was demonstrated using the Cole and Walters (1986) technique but sections were not calcified. Control sections were incubated without substrate or without tartaric acid. Twenty MNGC from each of the samples were evaluated. The level or intensity of the TRAP reaction product in multinucleated giant cells located along the bone surface was analyzed using the computerized imaging system, Princeton Gamma Tech (PGT) Imagecraft System 4+ run on a Digital Equipment Systems PDP 11/73, and PGT Imagecraft software (Figure 1). The technique is described in detail in Chapter III. Comparisons of the average number of cells expressing high TRAP activity were made among the three implant types and the tibia. Multiple linear comparisons among the implant groups and tibiae were performed using the Welch's t'W test for multiple
linear comparisons (Wilcox, 1987).
RESULTS

Active osteoclasts from the proximal tibia were identified as multinucleated cells exhibiting vacuolization of the cytoplasm and a striated border adjacent to the bone surface (Fig. 2a). At the ultrastructural level, osteoclasts were identified by ruffled borders limited by an organelle free, clear zone and a highly vacuolated area adjacent to the ruffled border (Figure 2b). Tartrate resistant acid phosphatase reaction product was observed as red granules within the cells and extracellular staining of the bone matrix adjacent to the osteoclasts (Figure 2c). All osteoclasts consistently exhibited the highest TRAP staining intensity.

Each implant was surrounded by a fibrous connective tissue capsule. At the site of the mineralized implant, large multinucleated cells were found in contact with the bone particles. Some cells exhibited vacuolization at the bone surface, but most cell-bone contacts were unremarkable (Figure 3a,b). The cell membrane adhered to the particle surface and often extended processes into the irregularities on the surface. The non-bone apposing surface of the cells exhibited cytoplasmic folds, often quite elaborate. The nuclei were present most often in a linear arrangement, at a random distance from the bone-apposed cell surface. The majority of the multinucleated cells in contact with the bone particles expressed either a low level of TRAP reaction product or absence of the enzyme (Figure 3c). The number of MNGCs from the mineralized implants expressing high level of enzyme...
expression was significantly different from the osteoclasts from the tibia and demineralized implants and MNGCs from the composite implants (p<0.05) (Figure 4).

Large multinucleated cells were present at the osteogenic site of the demineralized bone particle implant. The osteogenic site or area of mineral deposition was determined using the von Kossa technique and only the MNGCs at that site were analyzed. The cells were not as large as those seen in the mineralized implant and did not surround the bone particles but were present at discrete sites on the newly mineralized bone surface (Figure 5a). The ultrastructure of the cell membrane in contact with the particle exhibited extensive infoldings bound by smooth organelle-free areas (Figure 5b). Adjacent to the membrane infoldings was an area of vacuolization. Rough endoplasmic reticulum and mitochondria were the most abundant organelles. These cells were similar to the osteoclasts of the proximal tibiae in both morphology and TRAP expression (Figure 4, 5b). Almost all MNGCs expressed high levels of TRAP reaction product intracellularly. Weak extracellular staining was noticed in areas of adjacent bone also had extracellular TRAP staining.

The composite implant recruited a population of multinucleated cells similar in size, morphology and expression of TRAP reaction product to those recruited to the mineralized implant. These large multinucleated cells were found in contact with the mineralized particles (Figure 6a). A small population of the MNGCs demonstrated clear zones and infoldings of the cytoplasm along the bone-apposed
surface (Figure 6b). A population of cells which expressed heavy amounts of TRAP reaction product was also present along the mineralized particles (Figure 6c). These cells were located in discrete areas within the implant site, and concentrated the enzyme in the area of the cell adjacent to the bone. These cells were not as large and had fewer nuclei, compared to the majority of MNGCs exhibiting low levels of TRAP reaction product. Ultrastructural examination revealed the majority of MNGCs made contacts with the mineralized matrix particles but showed no membrane specializations except occasional clear zones or vacuolization. The majority of cells exhibited moderate to no TRAP reactivity with a small subpopulation exhibiting high levels of reaction product (Figure 4). The number of cells exhibiting a high level of TRAP expression was significantly different from the number of MNGCs from the mineralized, demineralized or tibial osteoclasts, (p<0.05). The lack of osteoblasts and the lack of patchy von Kossa staining, which is characteristic of newly mineralized bone, indicated osteogenesis had not occurred.
DISCUSSION

Tartrate resistant acid phosphatase and the specific morphological features associated with bone resorption allow for the characterization and discrimination of the osteoclast from the remaining population of bone cells responsible for matrix deposition and maintenance and cells of the mononuclear phagocyte system, specifically the macrophage polykaryon which exhibits some similar morphological features. In the present study, these two parameters, TRAP activity and morphology, were used to evaluate cells generated at the sites of subcutaneously implanted bone matrix. The osteoclasts from the proximal tibiae exhibited ruffled borders and clear zones in contact with the bone surfaces and were consistently heavily stained with TRAP reaction product. TRAP has been isolated from bone (Andersson et al., 1984, 1986; Andersson and Toverud 1979; 1982) and is generally accepted as a cytochemical characteristic of the osteoclast (Minkin, 1972; Hammerstrom et al., 1971; 1983) and its mononuclear precursors (Baron et al., 1986; van de Wijngaert and Burger, 1986). The remaining populations of bone cells, mononuclear phagocytes and virtually all other cells except erythrocytes express primarily, the tartrate sensitive form of acid phosphatase. Recent studies have shown that rat osteoblasts and osteocytes at or near the growth plate express low levels of punctate cytoplasmic TRAP reactivity (Bianco et al., 1988; Cole and Walters, 1987). Limited expression of the enzyme by these cells and the sensitivity to tartrate by other cells in the bone microenvironment allow TRAP to be used as a
tool for identification of osteoclasts. In some pathological states, other cells can express TRAP. Bianco et al. (1987) reported that in disease states where macrophage hypersensitivity is expected like, chronic granulocyte leukemia and metastasis of carcinoma in the bone marrow, a large proportion of bone marrow macrophages express TRAP activity. Bianco et al. suggested the expression of the enzyme may be due to immunologically mediated activation and that TRAP expression in cells of the mononuclear phagocyte system may be a marker of cell activation. In an in vitro study, Razdun et al. (1983) reported stimulated monocytes exhibited TRAP activity and concluded that expression of TRAP activity may indicate macrophage differentiation. In this investigation, the ability of the MNGCs to express low levels of TRAP activity may reflect a stimulated or activated state as in the above cited studies.

The number of MNGCs that express a level of TRAP activity and morphology similar to that of osteoclasts increased with an increase in the amount of demineralized matrix in the subcutaneous bone implant. The mineralized implant generated MNGCs that expressed weak TRAP activity and no ruffled border—a response similar to a chronic inflammatory response. A chronic stimulus results in an infiltration of mononuclear phagocytes which differentiate into epitheloid cells and fuse to form MNGCs (Adams et al., 1976). At 14 days the particles were surrounded by mononuclear and MNGCs which have been shown to be osteolytic or less specifically, able to digest foreign substances including mineralized matrix (Walters and Schneider, 1985; Glowacki, 1982). This indicates
the implantation of mineralized matrix does not create an osteolytic response that is mediated by osteoclasts but does create an inflammatory response that is osteolytic. Popoff and Marks (1986) supported this conclusion and reported implant generated MNGCs were morphologically distinct from osteoclasts. Walters and Schneider (1988), implanting chips of mineralized bone, reported 52% of bone induced MNGC were mildly TRAP positive and differed considerably from that observed in osteoclasts.

Glowacki and Cox (1986) however, reported MNGCs surrounding mineralized bone particles stained for TRAP activity and expressed osteoclast morphology. In the initial qualitative study, MNGCs with osteoclastic morphology and TRAP reactivity were described. The level of TRAP activity in comparison to active osteoclasts or MNGCs was not described. In a subsequent study (Glowacki, 1988), the MNGCs surrounding mineralized bone particles from composite implants of bone and polyethylene or polymethylmethacrylate were quantitated for levels of TRAP activity and shown to stain strongly or very strongly compared to MNGCs adjacent to plastic particles but again they were not compared to in situ osteoclasts. Our work does not support these findings. Acid phosphatase and enzymes in general, may be altered by tissue processing (Burstone, 1958). The use of 2.5% glutaraldehyde in this study instead of 2.0% paraformaldehyde used in the Glowacki studies, may have decreased the intensity of the enzyme reaction product. However, when compared to tibial osteoclasts fixed in the same manner, the relative difference remains and the MNGCs from the mineralized implant still
demonstrate a lower level of activity.

The osteoclast is a highly specific cell which has been shown to be under strict control of a variety of cytokines and hormones, both directly and indirectly. The substrate, mineralized bone, and mechanism of action are specialized. Although the mononuclear phagocyte and its derivatives have osteolytic capabilities among its repertoire, the osteoclast does not share in a wide range of capabilities of the macrophage. Implantation of mineralized bone presents the required substrate for osteoclasts but indicates by their absence, that all requirements for the generation of osteoclasts have not been met.

Implantation of demineralized bone served as a control for generation of osteoclasts at an ectopic site since implantation of demineralized bone matrix initiates a highly reproducible sequence of events that leads to bone formation and subsequent remodelling by osteoclasts (Reddi and Huggins, 1972). In this investigation, demineralized bone matrix was subcutaneously implanted and at 14 days, the majority of MNGCs expressed intense reaction product and osteoclast morphology. In the demineralized implant, osteoclasts are present at an ectopic site, but only where osteogenesis had been initiated. Recruitment of osteoclasts seems to be dependent on (1) the early stages of chemotaxis, chondogenesis and osteogenesis and (2) formation of a mineralized matrix. Burger et al (1984) co-cultured bone marrow mononuclear phagocytes with live mineralized, periosteum and osteoclast-free fetal bone rudiments and reported that the activity of skeletal cells was a prerequisite for osteoclast recruitment. Rodan
and Martin (1981) also suggested that osteoblasts may be mediators of osteoclast activity and need to be present. Implantation of demineralized bone has most frequently been used to study the initial phases of osteogenesis however it can also be used to study recruitment of the osteoclasts and the initial phases of bone remodelling.

Approximately 2/3 of the MNGCs elicited from the mixed implant exhibited moderate to no TRAP reactivity and macrophage-derived MNGC morphology, which was similar to the population profile from the mineralized implant. However, the remaining 1/3 of the total MNGCs population sampled, exhibited high levels of enzyme reactivity and osteoclast-like features. Osteogenesis was not occurring as in the demineralized implant. Reddi and Huggins (1972) first reported the inability of a mixed sample of demineralized bone and bone ash to induce osteogenesis. They reported the presence of osteoclastastic giant cells which had blocked the transformation of fibroblasts and subsequent bone formation. This cell response from the composite implant is similar to the mineralized implant but the subpopulation of MNGCs that resemble osteoclasts makes it unique. The most obvious reason for the unique response is the presence of the demineralized bone matrix. Although osteogenesis was not occurring, the presence of demineralized bone may have had an impact either directly on osteoclast recruitment or indirectly by affecting cells at the site which stimulate recruitment or expression of the osteoclast phenotype. The mineralized matrix may impair the full expression and matrix producing function of the differentiating progenitor cell although, these cells still may be able
to provide the appropriate signals for osteoclast precursor recruitment and differentiation. The highly TRAP positive cells were not distributed uniformly throughout the implant but were present in discrete areas which may indicate a localized response within different parts of the implant. The osteoclast-like cells were most often found adjacent to the mineralized particles which indicates a preference for a mineralized substrate. If the response to stimulate osteoclast-like cells can be augmented in the mixed implant, or if the recruitment and/or expression of the osteoclast phenotype could be stimulated in the mineralized implant, these may be feasible in vivo models for the study of osteoclast function at a nonskeletal site.
Figure 1.

Color enhanced digitized image composite of 4 MNGCs (arrows) adjacent to bone (b), representing the 4 levels of TRAP reaction product intensity (a, no reaction product; b, low intensity; c, moderate intensity; d, high intensity).

Figure 2.

a. Scattered multinucleated osteoclasts (arrows) from rat proximal tibia, present along the trabeculae of bone (b). x413

b. Electron micrograph of multinucleated osteoclast surrounding bone (b). Hallmark morphological features of ruffled border (arrows) and clear zone (arrow heads) are demonstrated. Note smooth appearance of bone surface associated with clear zone (area of attachment) and rough appearance of bone surface under ruffled border (area of resorption). x3719

c. Osteoclasts (arrows) from rat proximal tibia containing high concentrations of TRAP reaction product. (bone,b) x400
Figure 3.

MNGCs elicited by the mineralized bone implant, 2 wks. post-implantation:

a. MNGCs (arrows) surrounding mineralized bone particles (b). x272

b. Stained for TRAP reactivity, MNGCs (arrows) in contact with the bone surface (b) exhibit mild reaction product. x425

c. Electron micrograph of MNGC in contact with a mineralized surface. x7500
Figure 4.

TRAP expression in MNGCs elicited by mineralized, demineralized and composite bone matrix implants (2 wks. post-implantation) and in situ osteoclasts. TRAP reactivity scored as high level (++), moderate level (+) or absence (0) of reaction product. (mean ±SEM)

a. mineralized (+++) significantly different than tibia (++), p<0.05.

b. mineralized (+++) significantly different than
demineralized (++), (p<0.05.

c. mineralized (+++) significantly different than composite
(++) , p<0.05.

d. demineralized (+++) significantly different than composite
(++) , p<0.05.

e. composite (+++) significantly different than tibia (++), p<0.05.
Figure 4
Figure 5.
Osteogenic response elicited by implantation of demineralized bone matrix, 2 wks. post-implantation:

a. Active multinucleated osteoclasts contacting newly mineralized bone matrix (b). x640

b. Electron micrograph of osteoclast exhibiting a ruffled border (rb), in contact with the bone surface (b). x6000

c. Osteoclasts stained intensely with granular TRAP reaction product. (bone,b) x413
Figure 6.

MNGCs elicited by a composite implant, 2 wks. post-implantation:

a. MNGCs (arrows) surrounding bone particles (b). x272

b. MNGC in contact with bone particles (b) demonstrating membrane specialization at areas of contact. Cell-bone contact is unlike that seen in Fig. 3b. x6000

c. MNGCs (arrows) in contact with bone particles (b), stained for TRAP reactivity. Note the range of TRAP reaction product concentrations in MNGCs. x264
CHAPTER V

THE EFFECTS OF INTERLEUKIN 2 INFUSION
ON CELLS ELICITED BY ECTOPIC BONE IMPLANTS

ABSTRACT

Investigations into the effects of interleukin 2 (IL-2) on bone cell functions are limited. Based on the hypothesis of immune-bone cell interactions, the ability of IL-2 to stimulate bone resorption has been tested in vitro and the results are conflicting (Gowen and Mundy, 1986; Ries et al., 1989). In an attempt to further analyze the effects of IL-2 on bone cell function, human rIL-2 (3 x 10⁴ U/day) was infused at the site of 3 types of subcutaneous bone implants (mineralized, demineralized and a composite mixture of both types). Implantation of pulverized mineralized bone matrix elicits an inflammatory response while a demineralized bone implant elicits an osteogenic response and a composite implant elicits an inflammatory response with a minor population of cells exhibiting osteoclastic characteristics. Tartrate resistant acid phosphatase (TRAP) and ultrastructural analyses were used as criteria to assess whether IL-2 could stimulate differentiation of these cells at the site into osteoclasts or modulate the cells at the site of the implants to express the osteoclast phenotype. Multinucleated cells elicited by the IL-2 treated mineralized implants exhibi-
ited weak TRAP activity and no osteoclastic features. There was no change in the multinucleated cells elicited by the IL-2 treated mineralized implants compared to control. Osteoclasts from the treated and control demineralized implants demonstrated high levels of TRAP activity and osteoclastic morphology. The osteogenic response seemed to be enhanced by IL-2. An increase in the number of highly TRAP positive multinucleated cells and cells exhibiting clear zones and ruffled borders was present in the IL-2 treated composite implants versus controls, however the increases were not significant. From these implant studies, it has been shown that IL-2 has no effect on the inflammatory multinucleated cells elicited by the mineralized implant and it was unable to promote osteoclast differentiation or expression. In the demineralized, IL-2 may have an effect on bone cell function recognized by an enhancement of the osteogenic response. IL-2, like other cytokines, may have a profound direct or indirect effect on bone cell function.
Cytokines, as local mediators of bone cell function, have recently been shown to play an integral role in the complex functions of bone formation, growth, remodelling and repair in health and disease. One means by which the relationship between the immune and skeletal systems has been recognized is through the study of osteopetrosis, a congenital disease resulting in accumulation of bone matrix. Functional defects in both immune cells and osteoclasts have been shown which may be the result of a common defect in development since immune cells and osteoclasts are of hemopoietic origin. Transplantation of bone marrow or splenocytes has been shown to cure the disease in some mutant rodents (Walker 1975; Ash et al., 1980; Schneider 1985). Another aspect of the relationship between the immune system and the skeletal system is the modulatory effects of immune cell products on bone cell function. An effect was first recognized when products of mitogen-activated peripheral blood mononuclear cells stimulated bone resorption by increasing the number and function of osteoclasts in bone organ cultures (Horton et al., 1972). Osteoclast activating factor, as it was first described, has now been characterized as a family of cytokines including interleukin-1 (IL-1) and tumor necrosis factors (TNF α) and (TNFβ). These stimulators of bone resorption may function in both health and disease. In chronic inflammatory diseases such as rheumatoid arthritis and periodontitis, the infiltrating immune cells release products that may contribute to osteolysis and tissue destruction.
Analysis of joint fluid taken from patients with rheumatoid arthritis indicate the presence of osteoclast activating factors (Nouri et al., 1984b; DiGiovani et al., 1988; Saxne et al., 1988).

Cyclosporin A is a potent immunosuppressive agent that is frequently used to control graft rejection after organ transplant surgery. Among a number of effects, it has been shown to inhibit T-cell proliferation and activation, B-cell differentiation, and secretion of cytokines (specifically IL-1 and IL-2) (Thomson et al., 1984; Bunjes et al., 1981). Cyclosporin has also been shown to inhibit normal bone resorption in vivo (Orcel et al., 1989) and parathyroid hormone, prostaglandin E\(_2\), 1,25-dihydroxyvitamin D\(_3\) and osteoclast activating factor induced resorption of fetal rat limb bones in vitro (Stewart et al., 1986; 1989). It seems possible that the inhibitory effects on bone resorption may be an indirect effect of suppressed immune cell activity. Normal bone cell function may be dependent on normal immune system function.

The effects of isolated cytokines on bone have been evaluated in vitro and have been shown to have a variety of effects. The conclusions from these investigations however, may not address the complex interactions and profound indirect effects of cytokines, the immune cells that produce them, systemic hormones and bone cell function. Interleukin 2 (IL-2) is one such cytokine that may have a complex indirect mechanism of action. IL-2 was first functionally described as thymocyte stimulating factor due to its ability to enhance the thymocyte mitogenic response to phycohemagglutinin and concanavalin
A (Morgan et al., 1976). It is also able to regulate the production of cytokines (Numerof et al., 1988; Nedwin et al., 1985), play a role in wound healing (Barbul et al., 1986) and act as an antineoplastic agent in cancer therapy (Rosenberg et al., 1985; Lotze et al., 1986). IL-2 therapy produces toxic side effects including fever, hypotension, increased plasma acute-phase proteins and increased stress-related pituitary hormones (Mier et al., 1988; Mier et al., 1987).

Investigations into the effects of IL-2 on the skeleton are limited. Based on the premise that other immune cell products may contribute to osteoclast activating factor activity, Gowen and Mundy (1986) tested IL-1, IL-2 and interferon-γ for their activity in the mouse calvarial resorption assay. IL-2 failed to stimulate bone resorption. In a similar study by Ries et al. (1989), IL-2 was shown to stimulate osteoclastic activity by increasing acid production and calcium release. Other studies have focused on the role of IL-2 in the connective tissue destruction of chronic inflammatory conditions, including rheumatoid arthritis. Elevated levels of IL-1 and IL-2 in the joint fluid of patients with rheumatoid arthritis have been reported (Nouri et al., 1984a; Nouri et al., 1984b). The effects of this cytokine on bone cell function or its mechanism of action, whether direct or indirect, are unknown. IL-2 does seem to indicate another link between the immune and skeletal systems.

A system that may evaluate the effects of IL-2 in vivo is the subcutaneous bone matrix implant model. Implantation of pulverized mineralized matrix produces a chronic inflammatory response that is
osteolytic but not osteoclastic. Using the presence of tartrate resistant acid phosphatase (TRAP) and ultrastructural morphology of the osteoclast as criteria, the ability of IL-2 to stimulate the differentiation of osteoclasts at this ectopic site can be evaluated. Implantation of a demineralized matrix produces an osteogenic response. The effects of IL-2 on the promotion of an osteogenic site and subsequent osteoclastic activity can be evaluated using this demineralized matrix implant. A composite of both mineralized and demineralized bone matrix has been shown to generate a large population of non-osteoclastic multinucleated giant cells and a small population of osteoclast-like cells. The effects of IL-2 on these populations will be evaluated.
MATERIAL AND METHODS

Animals

These studies were performed using Norway-hooded Long Evans rats (Harlan Labs, IN). All animals were maintained and used according to the recommendations in the Guide for the care and use of Laboratory Animals and the Guidelines of the Institutional Animal Care and Use Committee at Loyola University Medical Center.

Bone Powder Preparation

Mineralized bone implant: Tibiae and fibulae were removed from 4-8 wk old Norway-hooded Long Evans rats. The soft tissue and cartilagenous epiphyses were removed and the marrow cavities were flushed with distilled water. The cleaned diaphyses were washed overnight in distilled water then extracted with absolute ethanol (30 min) and anhydrous ether (30 min). The bones were dried, pulverized with a mortar and pestle and sieved to yield particles 75-250 um in diameter. Sterile water was added to samples to create cohesive pellets of bone particles that could be easily implanted subcutaneously. The pellets were air dried and sterilized by ultraviolet (U.V.) radiation.

Demineralized bone implant: The mineralized bone particles prepared above were treated with 0.5 N HCl, 25 meq/gm (demineralizing agent) for 3 hrs followed by repeated washings of cold deionized water to thoroughly remove the acid, absolute ethanol (30 min) and anhydrous ether (30 min). Sterile water was added to samples of demineralized
bone to create pellets. The pellets were air dried and sterilized by U.V. radiation.

Composite implant: A composite bone particle implant was prepared from 25 mg mineralized bone powder and 5 mg demineralized bone powder. Sterile water was added to the bone particle mixture to create cohesive pellets which were air dried and sterilized by U.V. radiation.

IL-2 Administration

Human rIL-2 was administered by constant infusion at a dose of $3 \times 10^4$ U/day for 14 days (Piquet et al., 1986). Infusion of rIL-2 at the site of the bone implants was achieved by subcutaneous implantation of the Alzet osmotic minipump (Alzet Corp., Palo Alto, CA; model 2002) at the site. Human rIL-2 was kindly provided by Hoffmann-LaRoche, Nutley, N.J. rIL-2 and was diluted in 0.9% NaCl, 5.0 mg/ml mannitol and 75 mg/ml SDS; the control rats received pumps loaded with vehicle only.

Implants

Five wk old male Norway-hooded Long Evans rats were anesthetized with chloral hydrate (400 mg/kg). Dorsal, upper thoracic incisions were made bilaterally and subcutaneous pockets were created. One pocket was extended anteriorly and medially. The bone powder pellets were placed in the subcutaneous pockets, one pellet placed as far anteriorly as possible, in the cervical region; the remaining two pellets were placed in the left and right scapular regions. Each animal received one of each of the three types of bone implants. A third
midline, lower thoracic incision was made in each animal and a subcutaneous pocket was created anteriorly. An Alzet minipump was inserted into the pocket, with the delivery outlet in the vicinity of the three bone implants thus concentrating the cytokine at the site of the bone implants. The incisions were sutured and treated with betadine. Two wks post-implantation, the animals were sacrificed via ether inhalation, and the implants and proximal tibia were carefully excised. The minipumps were removed and analyzed for proper delivery.

**Morphological and Histochemical Analyses**

One third of the specimens and a sample of proximal tibia were immediately fixed in 2.5% glutaraldehyde, 0.1 M cacodylate buffer (pH 7.2) for 48 hrs., post-fixed in 1% osmium tetroxide, dehydrated in ascending concentrations of ethanol, and embedded in Epon 812. One micron sections were cut and stained with toluidine blue and evaluated by light microscopy. Thin sections (60-70nm) were cut and stained with uranyl acetate and lead citrate for ultrastructural analysis using the Hitachi H600 electron microscope.

Another third of each specimen and samples of proximal tibia were immediately fixed in cold 1% paraformaldehyde, 0.1 M cacodylate buffer (pH 7.2) with 7% sucrose for 3 hrs, dehydrated in ascending concentrations of cold acetone and embedded in JB-4. Three micron sections were cut and allowed to adhere to gel-coated slides in the cold for 5 days. The sections were then stained for alkaline phosphatase. Alkaline phosphatase was demonstrated using Burstone's complete
media (Pearse, 1968) containing Napthol AS-BI phosphate as a substrate and Red-violet LB diazonium salt as the capture agent in 0.1 M Trizma buffer, (pH 8.5). The substrate was omitted from control media.

The remaining third of each specimen and samples of proximal tibiae were stain for tartrate resistant acid phosphatase activity using 50 mM L(+)-tartaric acid, and a modified Cole and Walters (1986) technique (samples were not decalcified), or for mineral content using the von Kossa technique. Control sections were incubated without substrate. The level or intensity of the TRAP reaction product in multinucleated giant cells located along the bone surface was assessed using the PGT System 4+ image processor, DEC PDP 11-73, and PGT Imagecraft software. The cells were categorized into 3 groups according to the level of TRAP expression. All tibial osteoclasts exhibit a high to moderate level of reaction product (+++ or ++) and these ranges were combined. The IL-2 treated cell population profiles were compared to the controls for each of the three implant types and the tibiae. Comparisons of the number of cells expressing high levels of TRAP reaction product were also made among the implant groups and the tibiae. Multiple linear comparisons among the three implant groups and tibial osteoclasts were performed using the Dunn-Bonferroni t-test and the Welch's t'W test for multiple linear comparisons (Wilcox, 1987).
RESULTS

Active osteoclasts from the proximal tibia in both IL-2 treated and controls were identified as multinucleated cells exhibiting vacuolization of the cytoplasm adjacent to the bone surface. Tartrate resistant acid phosphatase reaction product was observed as red granules within the cells and extracellular staining of the bone matrix adjacent to the osteoclasts. All osteoclasts consistently exhibited the highest staining intensity. At the ultrastructural level, osteoclasts were identified as multinucleated cells with ruffled borders limited by an organelle free, clear zone along the bone surface and a highly vacuolated area adjacent to the ruffled border.

Each implant was surrounded by a fibrous connective tissue capsule. At the site of the mineralized implant, large multinucleated cells were found in contact with the bone particles. Von Kossa staining indicated the particles were mineralized. Some cells exhibited vacuolization of the membrane at the bone surface, but most cell-bone contacts were unremarkable (Figure 1a). The cell membrane adhered to the particle surface and often extended processes into the irregularities on the surface. The non-bone apposing surface of the cells exhibited cytoplasmic folds, often quite elaborate. The majority of the multinucleated cells in contact with the bone particles expressed either a low level of TRAP reaction product or absence of the enzyme (Figure 1b). Other MNGCs that were present at the site and not in contact with bone particles also expressed low levels of TRAP. These
cells were not quantitated. No cells were stained with alkaline phosphatase. The enzyme expression in the population of MNGCs analyzed was significantly lower than the osteoclasts (p<.05) but there was no difference in control mineralized implant versus IL-2 treated mineralized implant (Figure 2).

Large multinucleated cells were present at the osteogenic site of the demineralized bone particle implant. The osteogenic site or area of mineral deposition was determined using the von Kossa technique and by the presence of alkaline phosphatase containing cells. Only the MNGCs in that site were analyzed. The cells were not as large as those seen in the mineralized implant and did not surround the bone particles but were present at discrete sites on the newly mineralized bone surface. The ultrastructure of the cell membrane in contact with the particle exhibited extensive infoldings bound by smooth organelle-free areas (Figure 3a). Adjacent to the membrane infoldings was an area of vacuolization. Rough endoplasmic reticulum and mitochondria were the most abundant organelles. These cells were morphologically similar to the osteoclasts of the proximal tibiae. In these areas of the implant almost all MNGCs expressed high levels of TRAP reaction product intra-cellularly (Figure 3b). The TRAP expression in the MNGCs from the IL-2 treated and control demineralized implants and tibial osteoclasts was not significantly different.

The composite implant recruited a population of multinucleated cells similar in size, morphology and expression of TRAP reaction product to those recruited to the mineralized implant except for a
subpopulation of cells exhibiting osteoclastic features (Figure 4a). The majority of cells exhibited moderate to no TRAP reactivity with a subpopulation exhibiting high levels of reaction product (Figure 2). These cells were present on the surface of the bone particles and were not as large and had fewer nuclei, than the majority of MNGCs exhibiting low levels of TRAP reaction product (Figure 4b). Image analysis revealed nonspecific extracellular staining and TRAP concentrated along the bone-apposed cell surface. Statistical analysis did not reveal a significant difference between TRAP expression in the IL-2 treated versus control composite implant however a shift in TRAP expression was evident. Von Kossa and alkaline phosphatase staining revealed no evidence of osteogenesis at these implant sites.
DISCUSSION

The role of IL-2 in these models for osteolysis and osteogenesis is uncertain. The data indicates osteoclasts were not recruited by infusion of IL-2 at the implantation site of mineralized bone particles, based on the lack of TRAP expression and osteoclast morphology. The response to implantation of mineralized bone particles can be characterized as a chronic inflammatory response. Initially lymphocytes and monocytes are recruited to the site. The monocytes, macrophages and multinucleated giant cells surround the particles and attempt to degrade them while fibroblasts attempt to isolate the irritant by proliferating and producing a fibrous capsule. Lymphocytes are present during these events and their role which may be significant, is not well defined. Lymphocytes may act as modulators of macrophage and fibroblast activity. Cytokines, like IL-2, which have a stimulatory effect on lymphocytes may therefore have an indirect role in the inflammatory response.

Wound healing, which may be the endpoint of an inflammatory response, has been shown to be significantly augmented by chronic IL-2 administration (Barbul et al., 1986). One mechanism of action proposed by the authors is that IL-2 stimulated lymphocytes to release factors which promote macrophages to produce IL-1. In this case, IL-1 may stimulate fibroblastogenesis but in the case of the mineralized bone implant, it may also stimulate osteolysis. At 14 day post-implantation, the osteoclasts from the demineralized implant expressed high
levels of TRAP reaction product. There was no significant difference between the TRAP expressed by the osteoclasts from the experimental and control demineralized implants indicating that IL-2 did not inhibit the recruitment or activity of osteoclasts during demineralized matrix induced bone formation. By 14 days after implantation, the development of a marrow cavity has begun. As in normal bone development, bone cells are in close proximity to the bone marrow cells and specialized environment. Infusion of IL-2 may have an effect on the developing hemopoietic cells. Chronic administration of IL-2 to osteopetrotic mutant rats has produced both changes in natural killer cell activity and bone resorption (Schneider et al., 1988, 1990).

Enhanced bone formation was observed in two samples. Recently a study has shown the mitogenic activity of IL-1 for fibroblasts appears to be indirect and mediated by the platelet derived growth factor (PDGF)-A chain gene which has a paracrine or autocrine effect (Raines et al., 1989). PDGF is member of the growth factor family that is responsible for regulating the proliferation of connective tissue cells. PDGF has been shown to enhance induction of bone formation by demineralized bone matrix implants (Howes et al., 1988). Since IL-2 can stimulate the production of IL-1, the accelerated osteogenesis may be due to IL-1 stimulated PDGF production. In patients with intra-abdominal cancers, IL-2 may be administered directly into the abdominal cavity by Technokoff catheter. A side effect of intraabdominal administration lymphokine activated killer (LAK)/IL-2 therapy is fibrosis. The fibrosis may be due to cytokines produced by the LAK
cells or the induction of cytokines produced by host cells stimulated by IL-2. Wound healing factors like TGF-β and PDGF, which may be produced, could create the fibrosing response which may be similar to the enhancement of the osteogenic response in the demineralized implant (Urba et al., 1989).

The composite implant showed a subpopulation with osteoclast-like TRAP expression and morphology whose number increased with IL-2 administration. From the demineralized bone implant, it was shown that IL-2 did not inhibit recruitment or osteoclast activity. IL-2 promoted osteoclast-like TRAP expression and the morphological features of the multinucleated giant cells from the mixed implant. I was unable to determine if the effect was direct or via the production of other cytokines. I was also unable to determine from this study if the increase in the number of cells expressing high levels of TRAP reaction product and osteoclast-like morphology was due to promoting osteoclast phenotype in cells that had not expressed it previously by recruiting new cells to the implant site. Further studies are needed to address the specific effects of IL-2 on osteoclast function and/or development.

The effects of IL-2 on this chronic inflammatory response was not evaluated in this investigation but may be significant. Cells extracted from inflamed periodontal lesions are capable of producing both IL-2 in vitro as assessed by the method of Warren and Pembrey (1981), and bone resorbing factors, as assessed by calcium release from cultured mouse calvariae (Seymour et al., 1985). IL-2 has also been detected in the synovial fluid of patients with rheumatoid arthritis,
alkylosing spondolysis and psoriatic arthritis, immunoinflammatory
diseases and osteoarthritis, a degenerative disease (Nouri et al.,
1984a). The presence of IL-2 in the synovial fluid of patients with
immunoinflammatory disease is not surprising because activated lympho-
cytes which produce IL-2 are present. However, the presence of IL-2 in
the joint fluid of osteoarthritics is surprising because few activated
lymphocytes are present. Regardless of the nature of the disease,
these finding indicate lymphocytes and IL-2 which facilitates their
expansion, may contribute to connective tissue degenerative diseases.

The most relevant effect of IL-2 to this investigation is its
ability to stimulate the expression of cytokines which may play a role
in bone remodelling. Human peripheral blood mononuclear cells (PBMCs)
were induced by recombinant IL-2 to secrete TNF-\(\alpha\) and TNF-\(\beta\) (Nedwin et
al., 1985). Production of TNF-\(\alpha\) and TNF-\(\beta\) was augmented by combining
IL-2 with recombinant interferon-\(\gamma\) (rINF-\(\gamma\)). IL-2 has also been shown
to stimulate the production of IL-1\(\alpha\) and IL-1\(\beta\) by human PBMCs in
vitro (Numerof et al., 1988). The results indicated that the IL-1 is
largely derived from direct stimulation of monocytes.

Both TNF and IL-1 have been shown to have effects on bone cell
function. TNF-\(\alpha\) stimulates DNA synthesis which resulted in an increase
number of collagen synthesizing cells, but it has a direct inhibitory
effect on osteoblast function (Canalis, 1987; Centrella et al., 1988).
Bertolini et al. (1986) have demonstrated human rTNF-\(\alpha\) and rTNF-\(\beta\)
induced osteoclastic bone resorption in organ cultures of fetal rat
calvariae. The effects of TNF appear to be mediated by the osteoblast
(Thomson et al., 1987). Its ability to stimulate osteoclastic resorption and its presence in the synovial fluid from patients with rheumatoid arthritis provides evidence that TNF-α may play a significant role in connective tissue destruction and more specifically the pathogenesis of arthritis (Di Giovine et al., 1988; Saxne et al., 1988).

IL-1 has also been shown to affect both bone formation and resorption. Using the calvarial resorption assay, IL-1 has been shown to stimulate DNA, collagen and noncollagenous protein synthesis but exposure to IL-1 at high doses or for long periods of time results in inhibition of collagen synthesis (Canalis, 1987). Using the osteoblastic cell line MC3T3-E1, IL-1β stimulated cell proliferation but inhibited alkaline phosphatase and collagen synthesis (Ikeda et al., 1988). Both studies imply the importance of IL-1β in the local regulatory mechanism of bone remodelling. The effects of IL-1β on bone formation seem to be secondary to its potent effects on bone resorption. Gowen and colleagues have shown that highly purified IL-1 and recombinant IL-1 resorbs bone in vitro (Gowen et al., 1983; Gowen and Mundy, 1986). The effect is indirect and mediated by the osteoblast (Thomson et al., 1986). It is now thought that IL-1 is major constituent of OAF and is 1000 times more potent than TNF (Thomson et al., 1986). IL-1 has also been shown to have synergistic effects with other cytokines and hormones and its role in physiological and pathological bone loss may be significant (Dewhirst et al., 1987; Lorenzo et al., 1988; Tatakis et al., 1988).
The use of IL-2 therapy as an antineoplastic agent in cancer treatment has proven effective in some cases. It does however, produce toxic side effects. The effects on the skeleton have not been investigated. In this investigation, it was shown that IL-2 has an effect on the expression of TRAP and osteoclast morphology. The role of IL-2 in bone cell function may be a complex one. Further investigation into the role of IL-2, both direct and indirect, and the role of immune cells in the mechanisms of bone formation, growth, and remodelling is needed to further understand relationship between the immune and skeletal systems.
Figure 1.
Sections of IL-2 treated mineralized bone implant 2 wks. post-implantation:

a. MNGC adjacent to bone particle (b). The MNGC shows no membrane specializations at the area of contact with the bone surface. x6500

b. Mild TRAP reactivity in MNGCs (arrows) contacting the bone particles (b). x264
Figure 2.

TRAP reactivity in MNCCs elicited from mineralized, demineralized and composite bone implants (2 wks. post-implantation) and in situ osteoclasts from IL-2 treated (t) and control (c) rats. TRAP expression scored as high level (++), moderate level (+), and absence (0) of reaction product. (mean ±SEM).

a. mineralized (T) (++) is significantly different than tibia (C) (++) p<0.05.

b. mineralized (T) (++) is significantly different than demineralized (T) (++), p<0.05.
Figure 2

Level of TRAP expression

- ++ high
- + moderate
- 0 absence

Number of MNGC's

Tibia

Mineralized Implant
Demineralized Implant
Composite Implant
Figure 3.

Sections of IL-2 treated demineralized bone implant:

a. Multinucleated osteoclast displaying a ruffled border (rb) at the bone surface (b). x4875

b. Osteoclasts (arrows) at the osteogenic site, stained intensely with granular TRAP reaction product. (bone,b) x438
Figure 4.
Sections of IL-2 treated composite bone implant:

a. MNGC adjacent to bone (b) demonstrating clear zone (cz) and ruffled border (rb). Note the degraded appearance of the bone surface subjacent to the ruffled border area. x6125

b. MNGCs (arrows) contacting bone particles (b). Note the intensity of the intracellular reaction product along the area of cell-bone contact.  x413
CHAPTER VI

THE EFFECTS OF 1,25 DIHYDROXYVITAMIN D₃, PARATHYROID HORMONE AND CALCITONIN ON THE CELLS ELICITED BY ECTOPIC BONE IMPLANTS

ABSTRACT

The nature of the cells recruited to the site of subcutaneously implanted, devitalized bone matrix are dependent on the mineral content of the implant. The difference between macrophage derived-multinucleated giant cells (MNGCs) and osteoclasts can be determined on the basis of morphological features and level of tartrate resistant acid phosphatase (TRAP) expression. Two wks post-implantation, MNGCs are present at the site of mineralized bone matrix particles and fail to exhibit osteoclast morphology or a high level of TRAP, while MNGCs similar to osteoclasts are present at the site of demineralized and composite implants. In an attempt to recruit, stimulate differentiation and expression of the osteoclast phenotype, or inhibit these effects, osteotropic hormones, 1,25 dihydroxyvitamin D₃ (1,25(OH)₂D₃) (2.08 pmol/hr), parathyroid hormone (PTH) (1.24 U/hr) and calcitonin (CT) mU/hr) were infused locally into the area of 3 types of bone matrix implants. Control animals received the 3 types of bone implants and vehicle. Two wks post-implantation, the implants were removed along with proximal tibiae from each animal and processed for histochemical
and ultrastructural analyses. The level of TRAP activity in the MNCCs from each of the three implants and tibial osteoclasts were assessed using an image analyzer. No MNCCs from any treated or control mineralized groups expressed high levels of TRAP or membrane specializations, characteristic of the osteoclast. The 1,25(OH)₂D₃-treated composite implants had more MNCCs expressing higher levels of TRAP activity compared to controls (p<.05). There were no changes in TRAP expression in the PTH or CT-treated composite groups. Ultrastructurally, both treated and control composite groups showed MNCCs with osteoclastic morphology. In the PTH stimulated demineralized group, the demineralized implant was not osteogenic. There was no difference between treated and control in the 1,25(OH)₂D₃ and CT treated demineralized implants. In summary, the hormones did not appear to effect the MNCCs elicited by the mineralized implant since they are probably not osteoclastic. 1,25(OH)₂D₃ which is thought to exert its effect on immature osteoclasts, in contrast to PTH which stimulates mature osteoclasts, was effective in increasing the number of MNCCs expressing high level of TRAP in the composite implants. PTH completely inhibited the osteogenic process in the demineralized treated implant. Because of the presence of inflammatory and osteoclast-like cells in the composite implant, and its possible cellular modulation by hormones, the composite implant may be a valuable model for inflammatory bone loss.
INTRODUCTION

Ectopic bone implant systems have been developed to study both bone resorption and bone formation using mineralized and demineralized bone matrix implants, respectively. Although the response to the mineralized bone matrix is osteolytic, there is some discrepancy as to whether the cells responsible for bone removal are osteoclasts. Glowacki and Cox (1986) claimed osteoclasts, which were identified morphologically and histochemically, are present 12 days post-implantation. Walters and Schneider (1985,1987) and Popoff and Marks (1986), however, dispute this claim and describe the multinucleated giant cells (MNGCs) as osteolytic but non-osteoclastic, due to lack of ruffled borders and low tartrate resistant acid phosphatase (TRAP) activity. I substantiate these results in this study and in an earlier investigation (Chapter IV). The identity of these MNGCs is critical as to whether this mineralized bone implant system can be used as a model to study osteoclast function and normal bone remodelling. Whether osteoclasts can be found at the site of ectopically implanted mineralized bone matrix remains in question.

The osteogenic response elicited by demineralized bone particles is similar to endochondral bone formation, including development of a marrow cavity at 21 days post-implantation. Although this model system has been extensively used to study bone formation, this type of implant can also be used to study bone remodelling. The fact that osteoclasts
are recruited to the osteogenic site and are responsible for remodeling the newly mineralized matrix has not been disputed. Functional osteoclasts can be elicited at this site however only after the appearance of osteoblasts and the formation of mineralized matrix. The osteoclasts present at this implant may be used for comparison to the MNGCs elicited at the mineralized site.

Although not as extensively investigated, implanting a composite of mineralized bone matrix and demineralized bone matrix results in the appearance of MNGCs and a subpopulation of cells which exhibit osteoclastic morphology. This is a unique response since a population of cells with osteoclastic characteristics are present without the occurrence of osteogenesis as in the demineralized implant. The ability of the demineralized matrix to induce bone formation is inhibited, however it seems to be able to elicit osteoclast-like cells along with the MNGCs that are elicited by the mineralized matrix.

In this investigation, the relationships between MNGCs and osteoclasts, the ability of three types of bone matrix implants to elicit specific populations of cells, and the use of these implants as models for physiological and/or pathological bone resorption will be addressed. Specifically, the MNGCs will be assessed morphologically for classic osteoclastic features, and histochemically for expression of TRAP, and compared with multinucleated giant cells and tibial osteoclasts. TRAP has been proposed as a marker for the osteoclast and its precursor and can easily be demonstrated (Minkin, 1982; Cole and Walters, 1987). A high level of cytoplasmic TRAP expression and the
morphological feature of the ruffled border provide a quick and easy method for osteoclast identification and have been extensively used in
*in vitro* investigations (Ibbotson et al., 1984; MacDonald et al., 1987; Roodman et al., 1985). Comparison among the MNGCs elicited by each of the three types of bone implants will reveal morphological and histochemical similarities and differences which are important to determine which implants can be used for the study of osteoclastic bone resorption.

Hormone infusion will be used to further stimulate or inhibit the systems to recruit osteoclast precursors and their subsequent fusion into functional osteoclasts and/or stimulate the expression of the osteoclastic phenotype and function in the cells already present. The hormones include parathyroid hormone (PTH) and 1,25 dihydroxy-vitamin D₃ (1,25(OH)₂D₃), stimulators of osteoclastic function and calcitonin (CT) an inhibitor of osteoclastic function. The ability of the cells at the ectopic site to respond to these osteotropic hormones will provide another means to assess the true nature of their identity.
MATERIALS AND METHODS

Animals

These studies were performed using Norway-hooded Long Evans rats (Harlan Labs, IN). All animals were maintained and used according to the recommendations in the Guide for the Care and Use of Laboratory Animals and the guidelines of the Institutional Animal Care and Use Committee at Loyola University Medical Center.

Bone Implant Preparation

Mineralized bone implant: Tibiae and fibulae were removed from 4-8 wk old Norway-hooded Long Evans rats. The soft tissue and cartilagenous epiphyses were removed and the marrow cavities were flushed with distilled water. The cleaned diaphyses were washed overnight in distilled water then extracted with absolute ethanol (30 min) and anhydrous ether (30 min). The bones were dried, pulverized with a mortar and pestle and sieved to yield particles 75-250 um in diameter. Sterile water was added to 50 mg samples to create cohesive pellets of bone particles that could easily be implanted subcutaneously. The pellets were air dried and sterilized by ultraviolet (U.V.) radiation.

Demineralized bone implant: The mineralized bone powder prepared above was treated with 0.5 N HCl, 25 meq/gm (demineralizing agent) for 3 hrs followed by repeated washings of cold deionized water to thoroughly remove the acid, absolute ethanol (30 min) and anhydrous ether (30
min). Sterile water was added to 10 mg samples of demineralized bone to create pellets. The pellets were air dried and sterilized by U.V. radiation.

Composite bone implant: A composite bone powder implant was created from 25 mg of mineralized bone matrix and 5 mg of demineralized bone matrix. Sterile water was added to the mixture of the two powders to create cohesive pellets which were air dried and sterilized by U.V. radiation.

Hormone Administration

Bovine PTH fragments 1-34 (10,000 U/mg) was purchased from Sigma Chemical Co., St. Louis, MO. It was dissolved in a vehicle of $10^{-3}$ HCl in 0.9% NaCl containing 20 mg/ml cysteine·HCl (Rasmussen, 1959) and delivered at a rate of 1.24 U/hr (Obie and Cooper, 1979) (n=4). 1,25(OH)$_2$D$_3$ was a gift from Dr. Uskokovic at Hoffmann LaRoche, Nutley, N.J. It was dissolved in a vehicle of propylene glycol and delivered at at rate of 2.08 pmol/hr (Parfitt et al., 1984; Brommage et al., 1983) (n=5). Salmon CT (4500 U/mg) was a gift from Dr. J. Bastian at Armour Chemical Co., Kankakee, IL. It was dissolved in a vehicle of 1% gelatin, pH adjusted to 3.2 with concentrated HCl and delivered at a rate of 42 mU/hr (Bastian et al., 1978) (n=4). Infusion of the hormones at the site of the bone implants was achieved by subcutaneous implantation of the Alzet osmotic minipump, Model 2002 (Palo Alto, CA) at an adjacent site. Control pumps, containing the appropriate vehi-
cles, were also prepared for each of the three hormone studies. The length of infusion was 14 days.

**Implants**

Five wk old male Norway-hooded Long Evans rats (n=16) were anesthetized with chloral hydrate (400 mg/kg). Dorsal, upper thoracic incisions were made bilaterally and subcutaneous pockets were created. One pocket was extended anteriorly and medially. The bone powder pellets were placed in the subcutaneous pockets, one pellet placed as far anteriorly as possible, in the cervical region; the remaining two pellets were placed in the left and right scapular regions, respectively. Each animal received one of each of the three types of bone implants. A third midline, lower thoracic incision was made in each animal and a subcutaneous pocket was created anteriorly. An Alzet minipump, containing one of the hormones or their respective vehicles, was inserted into the pocket, with the delivery outlet in the vicinity of the three bone implants, concentrating the hormone at the site of the bone implants. The incisions were sutured and treated with betadine. Two wks post-implantation, the animals were sacrificed via ether inhalation, and the implants and proximal tibiae were carefully excised. The minipumps were removed and analyzed for proper delivery.

**Morphological and Histochemical Analyses**

One third of the implant specimens and samples of proximal tibia were immediately fixed in 2.5% glutaraldehyde, 0.1 M cacodylate buffer
(pH 7.2) for 48 hrs., post-fixed in 1% osmium tetroxide, dehydrated in ascending concentrations of ethanol, and embedded in Epon 812. One micron sections were cut and stained with toluidine blue and evaluated by light microscopy. Thin sections (60-70nm) were cut and stained with uranyl acetate and lead citrate for ultrastructural analysis, using the Hitachi H600 electron microscope.

Another third of the specimens and samples of proximal tibia were prepared for demonstrating alkaline phosphatase activity. Samples were immediately fixed in cold 1% paraformaldehyde, 0.1 M cacodylate buffer (pH 7.2) with 7% sucrose for 3 hrs, dehydrated in ascending concentrations of cold acetone and embedded in JB-4. Three micron sections were cut and allowed to adhere to slides in the cold for 5 days. The sections were incubated for 60 min in Burstone's complete media (Pearse, 1968) containing Napthol AS-BI phosphate as substrate and Red-violet LB diazonium salt as the capture agent in 0.1 M Trizma buffer (pH 8.5). Control sections were incubated without substrate.

The last third of the specimens and samples of the proximal tibiae were prepared for demonstration of tartrate resistant acid phosphatase activity using a modified Cole and Walters (1986) method (sections were not decalcified). In brief, samples were immediately fixed in cold 2.5% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.2) with 7% sucrose for 3 hr., dehydrated in ascending concentrations of cold acetone and embedded in JB-4. Three micron sections were cut and allowed to adhere to gel-coated slides in the cold for 5 days. The sections were stained for TRAP activity using 50 mM L(+)-tartaric acid.
Control sections were incubated without substrate. Alternate sections were stained for mineral content using the von Kossa technique.

The level or intensity of the TRAP reaction product in multinucleated giant cells located along the bone surface was analyzed using a computerized image analysis system, Princeton Gamma Tech System 4+ image processor, DEC PDP 11/73 and PGT Imagecraft software. The technique is described in detail in Chapter III. Comparisons were made among the average number of cells expressing different levels of TRAP activity within each hormone-treated implant type. Comparisons were also made between the average number of cells expressing high levels of TRAP activity for each hormone-treated implant types (mineralized, demineralized and composite), their appropriate controls and the average number of osteoclasts expressing high levels TRAP from the proximal tibiae. Statistics were performed using the Dunn-Bonferroni t-test and Welch's t'W test for multiple comparisons (Wilcox, 1987).
RESULTS

TRAP localization by this technique, results in a granular red-maroon reaction product nonuniformly distributed throughout the cytoplasm. Extracellular staining was also noticed along the bone particles and in the dense connective tissue, often near MNGCs. No differences were noticed among the MNGCs from each of the three hormone control implant groups. The cells from each control implant group were similar in morphology and TRAP reactivity indicating the vehicles had no effect on the parameters analyzed.

The majority of the MNGCs from the mineralized implants exhibited low levels or absence of TRAP reactivity, regardless of hormone treatment (Figures 1,2,3). Low level reaction product could be characterized by a diffuse pink staining throughout the cytoplasm (Fig. 4). The reaction product was not often seen in granular form. These cells were in contact with the bone particles and often elongated in shape to cover the surface of the particle. Ultrastructural analysis revealed the area of the bone-cell interface was unremarkable in terms of membrane specializations (Fig. 5). Nuclei were usually linearly situated, parallel and at a distance from the bone surface. The non-opposing cell membranes could be found exhibiting nonspecific membrane ruffling.

MNGCs stained with intense TRAP activity were found at the osteogenic site of the demineralized implant. Areas of osteogenesis were determined by the presence of cells exhibiting alkaline phospha-
ease and von Kossa staining for mineralized matrix. Fourteen days represented the early stages of remodelling. There were areas of developing cartilage and mineralized matrix, which were covered by a layer of osteoblastic cells or scattered osteoclasts (Fig. 6). The osteoclasts were irregular in shape and made discrete contacts with the mineralized matrix. The multinucleated cells along with some mononuclear cells stained strongly for TRAP. Only the multinucleated cells in contact with the bone matrix were evaluated (Fig. 7). In some cells the reaction product was unevenly distributed and localized in the cytoplasm adjacent to the areas of bone contact. Treatment with 1,25(OH)\(_2\)D\(_3\) and CT showed no major changes in development of the osteoclasts at this ectopic site. CT seem to be more stimulatory in terms of osteogenesis. PTH administration however, completely inhibited osteogenesis and osteoclast development in the demineralized implant samples. The PTH treated implants elicited mononuclear cells with little TRAP activity to the implant site. MNGCs similar to those seen at the mineralized site of other implants were not seen. Half of the samples from the 1,25(OH)\(_2\)D\(_3\) treated animals did not generate an osteogenic site. In the 1,25(OH)\(_2\)D\(_3\) samples that were osteogenic and CT treated demineralized implants, normal bone remodelling occurred. Ultrastructural analysis of the MNGCs from 1,25(OH)\(_2\)D\(_3\) and CT treated demineralized implants revealed normal osteoclast morphology. Cells in contact with the bone surface exhibited abundant mitochondria, clear zones and ruffled borders. The nonopposed surface did not exhibit intense membrane ruffling as seen in the MNGCs from the mineralized
The composite implant was able to elicit a mixed population of MNGCs based on morphological and histochemical criteria. Von Kossa staining revealed most MNGCs were in contact with mineralized particles. Osteogenesis occurred in one 1,25(OH)₂D₃ treated implant and one CT treated implant. In these implants, the MNGCs exhibited intense TRAP activity and ruffled borders and resembled those from the osteogenic demineralized implants. Most MNGCs from the hormone-treated composite implants resembled those from the mineralized implants, exhibiting little if any TRAP reactivity and no ruffled borders (Fig. 8). However, a subpopulation, which stained more intensely for TRAP and exhibited osteoclastic features including ruffled borders were also present (Fig. 9). The number of these cells increased significantly (p<.05) with 1,25(OH)₂D₃ treatment (Fig. 1). These cells, could be identified at the light microscopic level, on the basis of smaller size, discrete contacts with the bone surface and membrane specializations at the bone/cell interface.
DISCUSSION

This investigation was designed to assess the effects of local hormone infusion on the morphology and enzyme expression in populations of multinucleated cells elicited by mineralized, demineralized and composite bone implants. Each implant elicited a unique cellular response, evidently dependent on the presence or absence of mineralized matrix. The effects of the hormones on cells elicited by the mineralized implants were of particular interest because infusion of the stimulators of bone resorption was an attempt to generate osteoclasts at an ectopic site. In previous investigations by this and other laboratories, osteoclasts have not been elicited by mineralized matrix, as claimed by Glowacki and Cox (1986). Gowacki and Cox stated the only stimulus necessary for osteoclast development was the implanted mineralized matrix. I do not deny the importance of mineralized matrix as a stimulator of osteoclast development and function and as the substrate for these cells. However, in this investigation, the presence of the mineralized matrix alone or together with local infusion of either PTH or 1,25(OH)\(_2\)D\(_3\), did not promote the development of osteoclasts at the site of ectopically implanted mineralized bone matrix. Instead, as documented by the studies of Walters and Schneider (1985, 1988) and Popoff and Marks (1986) the implanted mineralized matrix elicited an inflammatory response characterized by the infiltration of osteolytic, non-osteoclastic, slightly TRAP positive MNGCs. At the light microscopic level, the location and morphology of these MNGCs
are similar to those of osteoclasts however, ultrastructurally, ruffled borders, the hallmark morphological feature of the osteoclast, were not seen in treated or control implants.

PTH and 1,25(OH)₂D₃ have been shown to stimulate osteoclastic formation and function. PTH-stimulated bone resorption is associated with increased synthesis and secretion of hydrolytic enzymes including TRAP and expansion of the ruffled border (Vaes, 1965, 1968; Miller, 1978; Miller et al., 1984). Like other stimulators of bone resorption, PTH and 1,25(OH)₂D₃ do not affect the osteoclast directly but are thought to act indirectly via the osteoblast (Rodan and Martin, 1981; Chambers et al., 1985; McSheehy and Chambers, 1986a). In the mineralized implants the absence of alkaline phosphatase staining indicates the lack of osteoblasts and osteogenesis at this site. The ineffectiveness of PTH and 1,25(OH)₂D₃ may be due to the lack of osteoblasts at the site. However, it has been shown that other cells like lymphocytes, which are present at the site of implantation, have receptors for these hormones and may play a role as modulator of osteoclast development and function (Whitfield et al., 1970).

CT has also been shown to be a direct modulator of bone activity, specifically as an inhibitor of osteoclast activity (Nicholson et al., 1986; Chambers et al., 1986). Chambers and Magnus (1982) showed the quiescent effects on isolated osteoclasts and no effects on isolated osteoblasts, peritoneal macrophages or inflammatory giant cells. By the parameters evaluated, there was no effect of CT on the MNGCs elicited by the mineralized bone matrix implant.
The question still remains, are osteoclasts or their precursors present at the site of the implanted mineralized matrix? I believe that osteoclasts are not present and cannot be recruited to this site, therefore, the hormones would be ineffective in stimulating an increase in TRAP activity or expression of osteoclastic morphology. The osteotropic hormones have not been shown to affect non-osteoclastic osteolysis which is characteristic of some inflammatory pathologies (Chambers and Magnus, 1982). The data from this investigation indicate osteoclasts are not present either with or without local infusion of osteotropic hormones at the site of the mineralized bone implant.

Osteoclasts can be elicited at the site of ectopically implanted demineralized matrix after the formation of bone. The demineralization process unlocks the osteoinductive potential of bone morphogenic protein, which initiates the complex cascade of bone formation (Urist and Strates, 1970). Reddi and Huggins (1972) described the osteoinductive nature of the demineralized bone matrix as transformability, because the matrix, the factors present in it and/or released by it, induces fibroblasts to undergo differentiation toward the osteoblast phenotype. Osteogenesis does not occur at the site where mineralized bone is implanted.

The phase of the osteogenic cascade relevant to this investigation is remodelling. The implants were removed at 14 days to correspond to previous studies and to the lifetime of the pumps. The experiments were designed so that the hormone would be infused from the time of implantation. At 14 days, the remodelling phase had just
began. Osteoclasts were present and demonstrated ruffled borders and intense TRAP activity. The effect of the hormones was therefore on the process of osteoclast recruitment and subsequent function. Mononuclear TRAP positive cells were present in the area of remodelling and these cells may be osteoclast precursors. There was a marked difference in the multinuclear cells elicited by the demineralized and mineralized matrices. The osteoclasts from the demineralized implant were smaller in size and made discrete contacts with the newly mineralized bone. This was in sharp contrast to the MNGCs from the mineralized implants which were large cells that almost totally surrounded the particles. All osteoclasts from controls, 1,25(OH)$_2$D$_3$ and CT treated implants exhibited moderate to high TRAP activity and ruffled borders. 1,25(OH)$_2$D$_3$ and CT have opposing effects on bone resorption, but they may have similar, stimulatory effects on bone formation (Parfitt et al., 1984; Farley et al., 1988; Weiss et al., 1981). I saw no change in TRAP activity since the control osteoclasts were classified at the highest level. To determine if a change was present, the scale of enzyme activity would have to be reevaluated.

The PTH treated demineralized implants failed to generate either an osteogenic or inflammatory reaction. Few MNGCs were seen at the implant site indicating again, the presence or absence of the mineralized matrix had profound effects on eliciting an inflammatory reaction. PTH has been shown to decrease bone formation by inhibiting synthesis and secretion of matrix proteins, and alkaline phosphatase (Kream et al., 1980; Majeska and Rodan, 1982). Chronic high doses of PTH have
been shown to have an inhibitory effect on bone formation, which may explain what was seen in this investigation (McGuire and Marks, 1974; Tam et al., 1982).

Mineralized matrix is essential for osteoclast function. Osteoclasts preferentially chose mineralized matrix as a substrate although it has been shown they are capable of resorbing a nonmineralized surface (Reid 1986). Krukowski and Kahn (1982) grafted particles of devitalized, mineralized bone; devitalized, demineralized bone; hydroxyapatite and egg shell onto chorioallantoic membranes, and showed the only substrate to elicit osteoclast-like giant cells was the mineralized bone. They concluded factors responsible for osteoclast differentiation lie within the matrix, specifically associated with the mineral phase; in response to mineralized matrix, osteoclast can differentiate at an ectopic site. This study does not substantiate the conclusion from Krukowski and Kahn (1982). The role of the live, mineralized matrix in osteoclast development is emphasized in the fetal metatarsal coculture system. Osteoclasts developed when marrow mononuclear cells were cocultured with these bone rudiments but not with devitalized fetal metatarsals or devitalized, mineralized bone particles (Burger et al., 1982; Burger et al., 1984). Mineralized matrix is not the only signal for osteoclast formation.

In the composite implant, the dominant reaction to the bone particles was an inflammatory response, similar to that seen in the purely mineralized implant. This response is probably due to the presence of the mineralized matrix since an inflammatory response is
seldom seen when demineralized bone is implanted. The ability of this type of implant to elicit osteoclastic cells seems to be more dependent on the presence of the demineralized bone matrix than the mineralized matrix. As in the case with bone morphogenic protein, the demineralization process unlocks its inductive capabilities. Other cytokines which stimulate osteoclastogenesis may also be associated with the mineralization phase of the matrix and are therefore incapable of being released. These cytokines which can be released from the demineralized matrix may influence osteoclast development directly or indirectly. In the purely demineralized bone implants osteoclasts were not present until mineralized matrix was formed indicating that mineralized matrix may be needed for recruitment, maturation and/or activation. In the composite implant the mineralized matrix is already present and inhibits the osteoinductive potential of the demineralized matrix simply by its presence or by the inflammatory reaction it elicits. However, just because osteogenesis did not occurred, does not mean that the demineralized matrix had no effect on the cells at the implant site. The only parameter of osteogenesis that was evaluated in this investigation was alkaline phosphatase. The demineralized matrix in the presence of the mineralized matrix may not induce osteogenesis but it may continue to cause some cellular differentiation or expression of factors to elicit osteoclasts. This type of implant may serve as a model of pathological bone loss because it is able to elicit osteoclasts and inflammatory phagocytes capable of osteolysis. Both may play equally important roles directly or indirectly, in bone destruction.
1,25(OH)₂D₃ was the only hormone which affected the TRAP activity of the MNGCs in the composite implant. The number of intensely staining cells increased with 1,25(OH)₂D₃ treatment. From this investigation, I am unable to determine if (1) 1,25(OH)₂D₃ stimulated an increase in TRAP expression in cells which had previously expressed low levels of the enzyme or, (2) more cells that express high levels of TRAP were recruited to the site. Recruitment rather than of induced expression seems more likely the case because more TRAP positive mononuclear cells were seen with 1,25(OH)₂D₃ infusion. If 1,25(OH)₂D₃ acted as a fusogen, more TRAP positive multinucleated cells may have been formed. Few if any osteoclasts analyzed expressed low levels of TRAP indicating, a high level of TRAP activity is normally seen in osteoclasts.

The purpose of this investigation was to determine if local hormone infusion could influence the recruitment of osteoclasts to ectopic bone implants. Osteoclasts could not be recruited to the site of the mineralized bone particle implants with or without stimulation from 1,25(OH)₂D₃ or PTH. Osteoclasts were recruited for remodelling of the new bone in the demineralized implants except in the PTH treated implant, where osteogenesis and subsequent remodelling were absent. 1,25(OH)₂D₃ had a stimulatory effect on the number of MNGCs expressing TRAP activity in the composite implants. Osteogenesis was absent except in one 1,25(OH)₂D₃ treated sample and one CT treated sample. CT had no effect on the MNGCs elicited by any of the implants in this model system.
Although a number of other parameters could have been included to evaluate the hormonal effects on formation of MNGCs, the cellular populations elicited by the different bone implants, osteogenesis and remodelling, this study was limited to the morphology and histochemistry of the MNGCs. The results indicate a stimulatory role for 1,25(OH)$_2$D$_3$ in the development of osteoclasts in the composite implant. This study also indicates that demineralized matrix may function as a storehouse of inducing factors which may affect osteoclast development either directly or indirectly. The matrix is important in the development and function of bone cells.
Figure 1.

TRAP expression in MNGCs from mineralized, demineralized and composite bone implants (2 wks. post-implantation) and in situ osteoclasts from 1,25(OH)₂D₃ treated (T) and control (C) rats. TRAP expression scored as high level (++), moderate level (+++) and absence (0) of reaction product. (mean ±SEM).

a. mineralized (T) (+++) significantly different than tibia (C) (++), p<0.05.

b. mineralized (T) (+++) significantly different than demineralized (T) (++), p<0.05.

c. mineralized (T) (+++) significantly different than composite (T) (++), p<0.05.

d. composite (T) (+++) significantly different than composite (C) (++), p<0.05.
Figure 1
Figure 2.
TRAP expression in MNGCs from mineralized, demineralized and composite bone implants (2 wks. post-implantation) and in situ osteoclasts from PTH treated (T) and control (C) rats. TRAP expression scored as high level (++), moderate level (+), and absence (0) of reaction product. (mean ±SEM).

a. mineralized (T) (++) significantly different than tibia (C) (++), p<0.05.

b. demineralized (T) (++) significantly different than demineralized (C) (++), p<0.05.

c. demineralized (T) (++) significantly different than tibia (C) (++), p<0.05.
Figure 2
Figure 3.

TRAP expression in MNGCs from mineralized, demineralized and composite bone implants (2 wks. post-implantation) and in situ osteoclasts from CT treated (T) and control (C) rats. TRAP expression scored as high level (++), moderate level (+), and absence (0) of reaction product. (mean ±SEM).

a. mineralized (T) (++) significantly different than tibia (C) (++), p<0.05.
Figure 3
At 14 days, MNGCs elicited by a mineralized bone implant from a 1,25 Dihydroxyvitamin D$_3$ treated rat:

Figure 4.
Low levels of TRAP are demonstrated in MNGCs (arrows) adjacent to mineralized bone particles (b). x413

Figure 5.
MNGC in contact with a mineralized bone particle. Note the absence of membrane specialization at the cell-bone interface. x12000
At 14 days, osteoclasts at demineralized bone implant from a calcitonin treated rat:

Figure 6.
Osteoclasts (arrows) are present along the newly mineralized matrix (b) and remodelling is in progress at the osteogenic site. x438

Figure 7.
Osteogenic site stained for TRAP reaction product. Osteoclasts (arrows) along the bone (b) containing high concentrations of TRAP reaction product. x413
At 14 days, MNGCs elicited by composite bone implant from a parathyroid hormone-treated rat:

Figure 8.
MNGCs (arrows) at or near the bone (b) surface demonstrating a range of TRAP reaction product intensities. In the highly TRAP postive cells note the distribution of TRAP reaction product and the intensity of the enzyme along the bone-cell interface. x660

Figure 9.
MNGC in contact with the bone (b), demonstrates a clear zone (c) and ruffled border (rb), membrane specializations of osteoclasts. x7500
CHAPTER VII

DISCUSSION

The skeleton defines the shape of an organism. It facilitates locomotion, protects vital organs and functions as a storehouse for calcium and phosphate. It is under constant reconstruction. Although the gross shape remains relatively constant throughout adult life, the matrix microstructure is being destroyed and replaced by a complex sequence of cell-mediated events. The osteoclast is mainly responsible for the destructive phase of the remodelling process. Because of its life-threatening potential, the osteoclast is highly regulated, mainly through the osteoblast, the effector cell of bone formation. The roles of the osteoblast in both bone formation and resorption provides a link between the two interdependent processes. Whether the osteoblast is necessary for osteoclast differentiation and function is one question addressed by the studies of this dissertation.

In this dissertation, the bone implant system, using three different matrix preparations, has provided three models to study the events in normal and pathological bone cell function. The focus of the studies have been on the bone resorption process and whether any or all of the bone implants would provide a good model to study osteoclast differentiation and function.

TRAP and classic morphological features were used to identify
osteoclasts in these implant studies. TRAP has been accepted as a marker of osteoclast function and has been extensively used to identify the differentiation of osteoclasts in vivo and in vitro. It is easily demonstrated and seemed to be a reliable marker. However, the specificity of this form of acid phosphatase to the osteoclast has been questioned after its demonstration in other cells. Its specificity became particularly important since TRAP was being used to assess the differentiation of osteoclasts in bone marrow cultures where nonosteoclastic multinucleated cells which are capable of TRAP expression were also present. An objective method of quantitation had never been established to determine if a particular level of TRAP reaction product was specifically associated with the osteoclast. A specific level of enzyme expression could be used to identify osteoclasts instead of the presence or absence of the enzyme. From the development of one such method of assessment, described in detail in Chapter III, a high level of TRAP reaction product was determined to be a characteristic of osteoclast. The osteoclasts consistently expressed high levels of TRAP reaction product. With the ability to standardize the method of analysis and determine a level which could characterize the osteoclast, TRAP could again be considered an effective criteria for osteoclast identification in normal and experimental situations.

Three different bone matrix preparations were used to elicit three different responses. Implantation of mineral bone particles had been shown to elicit an osteolytic response. It seemed possible that osteoclasts may be recruited to the site to remove bone matrix since
mineralized bone stimulates the formation and is the specific substrate of osteoclasts, but it seemed equally possible that the bone implant would elicit an inflammatory response like other particulate foreign matter and be removed by phagocytic cells. Evidence has previously been reported to support both the presence of osteoclasts in one case and phagocytic cells in another. Results from this study indicate the lack of osteoclasts at the site of mineralized bone implants. The multinucleated giant cells present 14 days post-implantation which resemble osteoclasts in some ways, fail to exhibit ruffled borders or a high level of TRAP reaction product.

One explanation for these findings is that the devitalized mineralized bone particles do not provide the appropriate cues necessary for the recruitment and differentiation of osteoclasts at this ectopic site. Mineralized bone has been shown to be a stimulator of osteoclast formation and function however in this model, the presence of mineralized bone alone is insufficient to promote osteoclast formation. Burger et al. (1984) have suggested live bone, particularly the osteoblasts population, is necessary for the differentiation of osteoclasts. Others have also shown that osteoblasts are required for osteoclast formation and activation in vitro (McSheehy and Chambers, 1986; Rodan and Martin, 1981). It is evident osteoclasts are dependent on the physical and cellular microenvironment in which they function. Absence of factors at this subcutaneous site, which are normally present in the microenvironment of bone, may have limited the recruitment of mononuclear precursors, their fusion, expression and/or function as
mature osteoclasts.

Another explanation for the findings is that the inflammatory response is the dominant response inhibiting the ability of the matrix to stimulate osteoclastic recruitment. The bone particles are almost completely surrounded by multinucleated giant cells at 14 days, which would prevent exposure of the matrix to osteoclasts. If osteoclasts were present, they would be in competition for contact with the bone matrix particles.

In the final attempt to stimulate the recruitment of osteoclast precursors and/or differentiation of osteoclasts at this ectopic site, stimulatory hormones and interleukin 2 (IL-2) was infused. Direct, constant infusion of 1,25 dihydroxyvitamin D$_3$ (1,25(OH)$_2$D$_3$), parathyroid hormone (PTH) and IL-2 for 14 days failed to produce osteoclasts at the mineralized bone implant. If osteoclasts had been present at the site but were unable to express ruffled borders or a high level of TRAP, the hormones may have been able to stimulate their expression. If osteoclasts were not present at the implant site, the hormones would be ineffective. With these results from the hormone studies, it is unlikely that the devitalized, mineralized implant with or without stimulation, is able to recruit precursors or stimulate formation of osteoclasts. Use of this system for the study of osteoclast recruitment and function would be inappropriate. This system however, could be used to evaluate the potential role of macrophages and macrophage-derived cells in the inflammatory osteolytic process. Mononuclear phagocytes have been identified at sites of inflammatory osteolysis.
and have been shown to be active participants. Their direct contribution to the osteolytic process and their role in health and disease may be significant. The mineralized matrix implant may be the appropriate model to analyze the potential of the nonosteoclastic populations in matrix degradation and develop means to control this inflammatory bone loss.

Implantation of a demineralized matrix elicits an osteogenic response and has been extensively used to study the process of bone formation. The amazing ability to stimulate the differentiation of cells responsible for the synthesis of a mineralized bone matrix at an extraskeletal site reveals the undifferentiated state of mesenchymal cells, even in the adult organism, and the impact of the matrix on these cells. The sequence of events is highly predictable and is similar to endochondral bone formation. By day 14 post-implantation, osteoclasts are recruited to remodel the mineralized matrix which would indicate this system a model appropriate for the study of both bone formation and resorption. The appearance of osteoclasts at the site of the demineralized implants, expression of high levels of TRAP and ruffled borders were expected. The data from the demineralized implants were in agreement with earlier studies however, the morphology and histochemistry of the osteoclast had never been the focus of attention. The differences between the osteoclasts recruited to this ectopic site with the multinucleated cells recruited to the mineralized site further reinforced the conclusions that the mineralized implant could not be used as a model for osteoclastic bone resorption.
PTH was the only hormone that affected the ability of the demineralized implant to recruit osteoclasts by inhibiting its osteoinductive capacity. Since the osteoclasts had been expressing high levels of the enzyme without hormone treatment, it was difficult to conclude if the hormones enhanced TRAP expression. The ability of the demineralized implant to recruit osteoclast precursors and stimulate their differentiation, and the potential ability to modulate these responses again indicates the ectopic bone implant system may be a useful model to study osteoclastic formation and function.

The results from the composite implant system proved to be the most interesting of the three implants. The mixture of mineralized and demineralized bone matrices produce a nonosteogenic, inflammatory response. The recruited cell population was similar to that from the mineralized implant except for a minor population expressing high levels of TRAP and membrane specializations reminiscent of ruffled borders. The dominant response as seen in the mineralized implant is the inflammatory reaction. In this case, the presence of the mineralized matrix and/or the inflammatory cells elicited by it, may inhibit osteogenesis. Even though there are no signs of bone formation, as monitored by the expression of alkaline phosphatase, it cannot be concluded that the demineralized bone particles had no effect. The results from the other 2 types of matrix implants indicates the demineralized implant may have more of an effect on bone cell differentiation than the mineralized matrix. Urist and Strates (1970) stated the demineralization process allows the release of factors that stu-
late bone formation. It is now known that the bone matrix is a storehouse for factors that affect both bone formation and bone resorption and stimulators of the bone resorption process may also be freed by the extraction of the mineral phase. The signal for osteoclast recruitment and/or differentiation may be weak which would explain the small number of osteoclasts that were identified.

Treatment with 1,25(OH)$_2$D$_3$ and IL-2 was shown to increase the expression of the enzyme while all treatments continued to produce cells with osteoclastic characteristics. Unlike PTH and calcitonin (CT) which are effectors of osteoclastic function, 1,25(OH)$_2$D$_3$ is thought to be an effector of osteoclast differentiation, which may indicate in this investigation, that 1,25(OH)$_2$D$_3$ is stimulating the formation of osteoclasts at this ectopic site. IL-2 may also have a direct or indirect effect on osteoclast formation.

In order to effectively study the formation and function of osteoclasts, investigators are in search of the perfect model system. One in which an isolated pure population of osteoclasts can be used for study. Attempts have been made to preferentially induce the formation of osteoclast in vitro and to isolate mature osteoclasts from the surface of the bone. These systems have provided important information, however they are far from ideal. The bone implant system may provide an in vivo approach to the study of bone cell function. From its uncertain hemopoietetic origins, to the complexity of its function and interactions with other cells, the osteoclast is a challenging subject of intriguing investigation.
In summary, the work of this dissertation has:

1. developed an objective method to quantitate intracellular TRAP activity and determined that there is a level of expression specific to the osteoclast
2. provided evidence to reject the use of the mineralized matrix implant as a model for bone resorption due to the inability to generate osteoclasts at the site with or without stimulation
3. provided evidence to substantiate the presence of osteoclasts at the site of the demineralized bone implant and propose the use of the system to study the osteoclast
4. introduce the composite implant as a model for inflammatory bone loss due to the presence of osteoclasts and inflammatory giant cells.


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APPENDIX A

A MORPHOLOGICAL, HISTOCHEMICAL AND FUNCTIONAL COMPARISON OF
THE CELLS ELICITED BY MINERALIZED BONE IMPLANTS
24 HOURS AND 14 DAYS POST-IMPLANTATION
Cells recruited to the site of ectopically implanted mineralized bone particles can be characterized functionally as osteolytic cells but morphologically they do not demonstrate the hallmarks of active osteoclasts. Pellets of mineralized bone powder (particle size 75-250 um), were implanted subcutaneously on the dorsal body wall of young adult rats. Two weeks post-implantation, the connective tissue encapsulated pellets were removed and processed for morphological, histochemical and functional analyses. Implanted pellets removed 24 hours post-implantation served as controls. At one day after implantation, the bone particles were surrounded by mononuclear inflammatory cells, which exhibited no TRAP activity (Fig 1a). Ultrastructurally, the mononuclear cells also failed to exhibit ruffled borders, the hallmark morphological feature of the osteoclast (Fig 1b). In the 2 week implant samples, the bone particles were surrounded by large multinucleated cells. Unlike normal osteoclasts which express intense TRAP activity, the multinucleated cells of the 2 week implants expressed weak or total absence of TRAP activity (Fig 2a). Ultrastructurally, these cells were seen contacting the bone particles however, ruffled borders or other membrane specializations were absent (Fig. 2b). To evaluate the functional capabilities of these multinucleated cells, the per cent surface area occupied by bone and the number of particles per field were determined. Three micron sections were stained with von Kossa for mineral content. The total surface area occupied by the bone particles, 24 hrs. post-implantation versus 2 wk.
post-implantation, was calculated using the Bioquant software package, run on an Apple 2 computer. The number of bone particles per field was also calculated. The implants exhibited a 20% reduction in bone surface area (p<0.05), and a 38% reduction in particles per field compared to controls (p<0.05). In conclusion, although these cells appear to be osteolytic, they do not appear to be osteoclasts. These results indicate the implantation of mineralized bone promotes a response similar to that elicited by other particulate foreign substances. It does not differentially recruit osteoclasts and exposure of mineralized matrix may be one of many signals needed to stimulate osteoclast recruitment, differentiation, and/or function.
Figure 1.

24 hrs. post-implantation of mineralized bone particles:

a. Lack of MNGCs at the site of the implant at this early time point. Lack on TRAP reactivity in mononuclear cells present. x425

b. Mononuclear cell in contact with a bone particle (b). x12000
Figure 2.

2 wks. post-implantation of mineralized bone particles:

a. Lack of TRAP reactivity in MNGCs (arrows) in contact with mineralized bone particles (b). x425

b. MNGC in contact with a bone surface (b). Note the absence of membrane specializations. x10000
APPENDIX B

ELEMENTAL ANALYSIS OF A COMPOSITE IMPLANT AS DETERMINED BY ENERGY DISPERSIVE SPECTROSCOPY
Elemental analysis for calcium, phosphorus and zinc superimposed on a scanning electron micrograph of a composite implant prior to implantation as determined by energy dispersive spectroscopy (EDS). Particles (75-250 um diameter) were prepared per Material and Methods, Chapter IV, coated with gold and analyzed using the Princeton Gamma Tech System 4+ and JEOL JSM-840A scanning electron microscope. The horizontal line (at arrow) represents the scan line for the collection of X-ray counts. The superimposed spatial distribution of counts within the energy window for phosphorus (a) and calcium (b) indicates high levels of these elements in the bone particle (B1) low levels in bone particle (B2). The spatial distribution of counts within the energy window for zinc was used to assure the changes in above counts were unrelated to the geometric profile of the particles at the level of the scan line.
This dissertation submitted by Janet Dvonch Kelly has been read and approved by the following committee:

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The dissertation is therefore accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

4/11/90
Date

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