

**A FUNCTIONAL, MORPHOLOGICAL AND HISTOCHEMICAL
CHARACTERIZATION OF CELLS ADJACENT TO
ECTOPIC BONE IMPLANTS**

by

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DEDICATION

To my husband, Eric
and our children, Abby and Matt

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VITA

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The author is married to D. Eric Walters. They have two children, Abigail and Matthew.

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CHAPTER I

INTRODUCTION

Bone resorption involves the degradation and removal of both the mineral and organic components of the bone matrix. Physiologic bone resorption, which occurs in normal bone development and remodeling, is considered to be the primary responsibility of the osteoclast (Holtrop and King, 1977; Marks, 1983). This multinucleated cell contains the specialized structural and enzymatic machinery necessary to affect bone resorption, and its resorbing activities are regulated in a predictable manner by known osteotropic hormones (Holtrop et al., 1974; Bonucci, 1981). Observations that macrophages and monocytes accumulate near areas of bone resorption in vivo (Rifkin and Heigl, 1979; Stanka and Bargsten, 1983), respond chemotactically to the products of normal bone resorption and components of bone matrix (Mundy et al., 1978; Malone et al., 1982), and appear capable of bone resorption in vitro (Mundy et al., 1977; Kahn et al., 1978; Teitelbaum et al., 1979) have prompted speculation that mononuclear phagocytes may also play a role in normal bone resorption. In addition, mononuclear phagocytes, specifically macrophages and monocytes, have been proposed as osteoclast precursors (Owen, 1980; Loutit and Nisbet, 1982). Based on functional similarities, a putative ontogenic relationship, and their

ready availability for experimentation, monocytes, macrophages, and macrophage polykaryons have been advocated as appropriate investigational surrogates for the study of osteoclasts (Teitelbaum and Kahn, 1980). Viewed in this way, osteoclasts would then be considered as macrophage polykaryons specialized to resorb a specific substrate-bone, implying that the mechanisms of osteoclastic and macrophage-monocyte-induced bone resorption may be the same.

The formation of macrophage polykaryons can be elicited by the introduction of a variety of materials into the subcutaneous tissues of experimental animals (Mariano and Spector, 1974; van der Rhee et al., 1979). One approach to the investigation of osteoclast-macrophage polykaryon relationships involves the in vivo implantation of bone matrix into ectopic sites followed by the recovery and evaluation of the morphological and resorptive characteristics of the cells found adjacent to the implants. Devitalized bone powder implanted into calvarial defects (Glowacki et al., 1981; Glowacki, 1982; Holtrop et al., 1982) resulted in the formation of multinucleated cells surrounding the bone fragments. The resorptive activity of the cells within the calvarial defects was evaluated by morphometric analysis. Results indicated that resorption had occurred in this system. Ultrastructural assessment of the multinucleated cells, however, failed to reveal the presence of ruffled borders, the hallmark characteristic of the active osteoclast, in areas of bone-cell contact. Despite this deficit, the use of this implant system

was advocated as a model for osteoclastic lineage studies on the basis of the similarities between osteoclasts and macrophage polykaryons (Holtrop et al., 1982).

The primary purpose of this series of investigations was to evaluate the premise that multinucleated cells elicited by ectopically implanted bone matrix are cells functionally, morphologically and histochemically equivalent to the osteoclast, and therefore, appropriate cells for the study of osteoclastic differentiation and function. Studies were conducted at subcutaneous sites in both normal and osteopetrotic (ia) rats using normal and osteopetrotic bone preparations. Mammalian osteopetrosis, a disease characterized by excessive accumulation of bone caused by decreased bone resorption, has provided a model for the study of osteoclastic lineage and function (Marks, 1984). The bone resorbing defect in the ia rat mutation of osteopetrosis is due to faulty osteoclasts. Osteopetrotic osteoclasts are distinguishable from their normal counterparts in vivo by their inability to form ruffled borders, the site of active bone resorption, or to release normal amounts of lysosomal enzymes which accumulate in their cytoplasm (Marks and Walker, 1976; Marks, 1984).

The first and second studies in this dissertation were designed to assess the resorptive and morphological characteristics of cells found surrounding bone implants in ia and normal littermates to determine if the usual functional and structural disparities between ia and normal osteoclasts were mimicked in this system, lending

validity to its use as an osteoclastic model. An evaluation of the amount of bone resorption occurring during a two week implantation period was achieved through ^{45}Ca release assays. Quantitation of ^{45}Ca released from pre-labeled bone into surrounding tissue fluids or culture medium is commonly used as an indirect index of bone resorption. We developed a new release assay, permitting more direct assessment of ^{45}Ca loss, for use in our bone implant system. This technique employs the individual members of uniformly labeled bone chip pairs as control and experimental implant halves. Recovery of the implant half and subsequent comparison of its ^{45}Ca content with that of the control was used to assess the total label loss over the implant period. Label content of bone chips sealed in diffusion chambers prior to implantation indicated the quantity of passive or non-cell mediated ^{45}Ca release over a similar two week interval. The amount of cell-mediated label release was determined by subtracting the release from diffusion chambers from that of direct implantations.

The first two studies also included a light and electron microscopic evaluation of the cell populations found at the bone surface at 3, 7 and 14 days post-implantation focusing on the morphology of bone-induced multinucleated cells. In addition, the second study compared the structures of suture-elicited macrophage polykaryons and bone-induced multinucleated cells with those of normal and osteopetrotic osteoclasts.

The final portion of the dissertation examined the tartrate-

resistant acid phosphatase (TRAP) staining characteristics of the osteoclast, the suture-elicited macrophage polykaryon and the bone-induced multinucleated cell. Tartrate-resistant acid phosphatase has been proposed as a histochemical marker for the osteoclast (Minkin, 1982; Hammarstrom et al., 1983) and, as such, TRAP has been used for the cytochemical identification of proposed osteoclastic precursors (Roodman et al., 1985; Baron et al., 1986; Jilka, 1986). Appendix B outlines the development of an improved technique for the localization of tartrate-resistant acid phosphatase in skeletal tissues. This procedure permits glutaraldehyde fixation, decalcification and plastic embedding. The description of the technique also includes an evaluation of two isomers of tartaric acid as effective inhibitors of tartrate-sensitive acid phosphatases. In the third study, this histochemical procedure was used to compare TRAP localization in the cells surrounding bone and suture implants with that of normal and osteopetrotic osteoclasts at 3, 7 and 14 days after implantation. Osteopetrotic osteoclasts have been shown to possess increased acid phosphatase activity when compared to their normal counterparts (Handelman et al., 1964; Schofield et al., 1974); most of this increased activity is expressed as tartrate-resistant acid phosphatase (Hammarstrom et al., 1983). Reproduction of this pattern of TRAP localization within bone-induced multinucleated cells in ia rats would support the use of these cells as osteoclastic surrogates.

Because access to bone mineral may be necessary for the induc-

tion and activation of osteoclasts (Chambers et al., 1984; Chambers and Fuller, 1985), we included within these studies an evaluation of the effects of increased mineral exposure on the recruitment of cells to the bone implant surface. The calvarial and trabecular bone present in rat pups is undergoing extensive turnover and remodeling. Because mineralization of organic bone-matrix lags behind its formation, bone surfaces undergoing remodeling in young rats are characterized by the presence of an osteoid seam (8-10 μ m) between the bone lining cells and the mineralized bone (Jee, 1983; Jaworski, 1983). Quiescent or "resting" bone also exhibits a similar, though thinner, osteoid layer (1 μ m) (Jee, 1983). Chambers et al. (1985) have proposed that this osteoid layer acts as a barrier to osteoclastic contact with the underlying bone mineral and that exposure of the osteoclast to bone mineral may be important for the initiation of osteoclastic resorptive behavior. We evaluated this hypothesis by using freeze-thawed, bleached or collagenase-treated bone chips as implant substrates. Bone chips which were harvested and subjected to devitalization only (freeze-thawed) were used to represent primarily osteoid (unmineralized or organic) exposed bone surfaces, while bleached and collagenase treated bone chips represented primarily mineral-exposed bone surfaces. Abe et al (1983) and Abe et al (1984) employed 5% sodium hypochloride (bleach) to remove organic material from bone surfaces during SEM studies of bone resorption. Incubation of bone chips with collagenase also removes the organic (osteoid)

phase of bone, providing a primarily mineral-exposed substrate for implantation (Green et al., 1985; Chambers et al., 1985). In the first study, both bleached and collagenase-treated bone chips tended to demonstrate more ^{45}Ca release than did freeze-thawed implants. Because the results of functional studies were very similar and the use of collagenase as a mineral-exposing agent is more frequently documented in the current literature, incubation in bleach was eliminated from the second and third studies to simplify the the number of treatment groups. Osteopetrotic bone served as another type of mineral altered implant matrix. Metaphyseal bone isolated from ia rat tibiae exhibits elevated hexosamine levels and mineral content when compared to normal age-matched controls (Boskey and Marks, 1985). Similar, though less significant findings are noted between normal and ia calvarial bone (Boskey and Marks, 1985). Through the implantation of normal and osteopetrotic trabecular or calvarial bone chips (freeze-thawed, bleached or collagenase-treated), we evaluated the effects of substrate composition on the differentiation of cells found adjacent to the bone matrix in an effort to define the most effective implant matrix for the induction of osteoclast-like multinucleated cells.

In summary, this dissertation describes an in vivo model for the study of cell-mediated bone resorption. An evaluation of the functional, morphological and histochemical features of the cell populations attracted to various preparations of devitalized bone implants

was conducted to delineate the relationship between the implant-induced cells, the osteoclast and the mononuclear phagocyte system. This work also evaluates the effects of substrate composition on the differentiation and functional capabilities of the cells elicited to the implanted matrix. This model may reflect true osteoclastic resorption and may, therefore, be used to study osteoclastic lineage and function or, alternatively, it may define a system for the study of macrophage-monocytic bone resorption which has been implicated in chronic inflammatory disorders involving pathological osteolysis i.e. periodontal disease, some forms of osteoporosis, osteomyelitis, and rheumatoid arthritis.

CHAPTER II

REVIEW OF LITERATURE

Osteoclast Biology - General Review

Bone resorption and its relationship to the osteoclast have been surrounded by controversy since the nineteenth century. Tomes and de Morgan, 1853, were the first to propose a cellular mechanism for bone resorption, although they were unable to cytologically characterize the bone resorbing cell itself. While making no suggestions as to function, Robin in 1864 was able to differentiate between two types of giant cells inhabiting bone--one belonging to the marrow (megakaryocyte) the other associated with the bone itself which he termed "myeloplaques" (Hancox, 1949a, 1972). Koelliker, 1873, named and identified the osteoclast as the active agent of bone resorption. Basing his predictions on the presence of a "brush border" in areas of cell-bone contact and on observing bone-like material inside the cells, he proposed that the osteoclast resorbed bone by releasing chemical substances capable of bone degradation into the region of the brush border with subsequent phagocytosis of the degradative products (Kroon, 1954). This hypothesis, however, was just one of many proposed to explain bone resorption (Abrey, 1920; Jaffe, 1930; Kirby-Smith, 1933) and its validation had to await the arrival of technical

advancements such as the electron microscope and autoradiography. Pioneering studies by Scott and Pease (1956) on the ultrastructure of the epiphyseal plate in young kittens and by Arnold and Jee (1957) on plutonium incorporation and removal from osseous surfaces into osteoclasts provided definitive evidence that osteoclasts are active agents of bone resorption. More recently, Takagi et al. (1982) and Blair et al. (1986) have demonstrated that rodent and avian osteoclasts are capable of degrading both the organic and mineral components of bone, biochemically confirming the bone resorptive status of the osteoclast.

Time-lapse cinematography of cultured avian and murine osteoclasts reveals that they are highly mobile cells with an active and extensive undulating peripheral membrane capable of exploring its environment by extending and retracting cytoplasmic processes. These cells also exhibit vigorous pinocytotic activity as evidenced by the appearance, fusion, and discharge of vacuoles of various sizes by the undulating membrane. Bone matrix can be seen to dissolve beneath the osteoclasts providing additional evidence of their active participation in bone resorption (Hancox, 1949b; Gaillard, 1959; Hancox and Boothroyd, 1961).

Observations made by scanning electron microscopy (SEM) reveal a wide range of osteoclastic appearances which may reflect different functional states. At one end of the spectrum are flat osteoclasts with fimbriated borders and a microvillus covered dorsal surface. Because osteoclasts displaying this structure are difficult to dissect

from the bone, it is postulated that they are involved in active bone resorption (Jones and Boyde, 1977). In contrast, a second group of elongated and extensively branched osteoclasts with smooth dorsal surfaces can be removed from the bone quite easily. Osteoclasts with these features are considered to be resting or inactive with regard to bone resorption. Both phenotypes have been observed at different locations on the same cell implying varying functional states may exist within the same cell (Jones and Boyde, 1977). Osteoclasts are presumed to dissolve bone as they move, etching their paths into its surface. Bone resorption surfaces, as identified by SEM, demonstrate a variety of roughened areas, trenches and concavities--three dimensional illustrations of osteoclastic activity (Abe et al., 1983).

Under the light microscope, osteoclasts can be located in shallow depressions of the bone or investing a bony trabecula. They vary in size from 20 to 100um in diameter. True multinucleated cells, osteoclasts can contain as many as 100 or as few as 2 nuclei. These nuclei are round or oval and tend to cluster away from the bone apposed surface. Mitotic figures are not usually observed in osteoclasts (Gonzales and Karnovsky, 1961; Hancox, 1972; Gothlin and Ericsson, 1976; Chambers, 1978). The osteoclastic cytoplasm is variable in appearance from moderately basophilic to acidophilic and at times exhibits a "foamy" or highly vacuolated region adjacent to the bone. The characteristic histological feature of the osteoclast at the light microscopic level is the presence of a "brush or striated"

border at the bone-cell interface (Gothlin and Ericsson, 1976; Chambers, 1978; Bonucci, 1981). Kroon (1954), in a thorough investigation of changes in the staining properties of parathyroid hormone (PTH) stimulated avian osteoclasts and the surrounding bone matrix, concluded that the striated border represented a region in which the osteoclast was actively penetrating the bone with cytoplasmic processes and that its degree of development was a reflection of the bone resorbing status of the cell.

Ultrastructurally, osteoclasts exhibit areas of nuclear concentration. Osteoclastic nuclei are seen to vary in shape from smooth regular ovals to highly irregular outlines. They possess normal nuclear membranes with dense chromatin concentrated along the inner leaflet and one prominent nucleolus (Gonzales and Karnovsky, 1961; Gothlin and Ericsson, 1976). Ribosomes are abundant throughout the cytoplasm while rough endoplasmic reticulum is relatively scarce and located in regions distant from the bone surface. Other prominent cytoplasmic inclusions are: multiple perinuclear Golgi apparatus; high concentrations of mitochondria often containing crystalline granules and tubular or fenestrated cristae; numerous primary and secondary lysosomes and a variety of dense core vacuoles (Scott, 1967a; Cameron, 1972; Holtrop and King, 1977; Marks, 1983). A giant centrosphere containing multiple pairs of centrioles can often be identified in the nucleus-free areas of the cytoplasm (Matthews et al., 1967; Lucht, 1973). Although it has been proposed as an exclu-

sive feature of the osteoclast, Sapp (1976) reported the presence of similar giant centrospheres in a variety of multinucleated cells including foreign body giant cells, casting doubt on its use as a definitive osteoclastic marker. The significance of this centrosphere, however, lies in the fact that centrioles located in a common pool some distance from the nuclei would not be readily available for nuclear division, providing an explanation for the absence of mitotic figures in the osteoclast.

Actively resorbing osteoclasts can be differentiated ultrastructurally from inactive osteoclasts and other types of multinucleated cells by the presence of a ruffled border at the bone-cell interface (Gothlin and Ericsson, 1976; Bonucci, 1981; Marks, 1983, 1984). The ruffled border, a complex series of cytoplasmic infoldings, is considered to be the site of osteoclastic bone resorption (Scott and Pease, 1956; Dudley and Spiro, 1961; Bonucci, 1974; Holtrop, 1975). The bone underlying the ruffled border is disrupted as illustrated by disaggregation of collagen exposing individual fibrils. Free crystals of bone mineral are found between the infoldings and channel expansions of the ruffled border as well as within cytoplasmic vacuoles of the osteoclast (Dudley and Spiro, 1961; Gonzales and Karnovsky, 1961; and Lucht, 1972). The exocytosis of lysosomal enzymes and hydrogen ions necessary for bone degradation as well as the endocytosis of degradation products are believed to occur at the ruffled border (Vaes, 1968; Holtrop and King, 1977; and Marks, 1983). Baron et al.

(1985) have localized a 100-kD protein within the membranes of the ruffled border and secondary lysosomes of the osteoclast. Antibodies to the 100-kD protein cross-react with a proton pump ATPase from pig gastric mucosae, suggesting participation of this protein in acidification of both intracellular organelles and extracellular compartments. The authors propose that an acidified extracellular compartment is produced between the plasma membrane of the ruffled border and the underlying bone matrix by exportation of hydrogen ions from inside the osteoclast. Directional secretion of lysosomal enzymes (acid hydrolases) by the osteoclast into this compartment may then create the functional equivalent of a secondary lysosome containing the appropriate environment for matrix degradation. The authors go on to suggest that the products of this extracellular digestion could diffuse through the ruffled border plasma membrane as they would through lysosomal membranes intracellularly and may not require endocytosis for further degradation inside the osteoclast. However, the presence of a coated membrane in the region of the ruffled border implies that endocytosis may also play a role in the resorptive process (Kallio et al., 1971).

The relative activity of the osteoclast is reflected by the degree of development of its ruffled border. In bone organ cultures stimulated with parathyroid hormone (PTH), 64% of the osteoclasts exhibited ruffled borders, while in non-stimulated cultures only 11% of the osteoclasts had ruffled borders, and these consisted of a few

shallow infoldings. Calcitonin administration to the PTH-stimulated cultures decreased the proportion of ruffled area significantly by one hour; this was followed by a decrease in ^{45}Ca release indicative of decreased resorption (Holtrop et al., 1974). Similarly, in vivo studies of the egg laying cycle in Japanese quail indicate that medullary bone osteoclasts modulate their activity to accommodate the changing calcium requirements imposed by egg shell formation. The osteoclasts appear to be active only during the period of shell calcification and inactive during the rest of the cycle. Active osteoclasts exhibit ruffled borders apposed to the bone surfaces; their cytoplasm is rich in mineral containing vacuoles. At the completion of the egg shell calcification the ruffled borders and vacuoles move away from the bone surface, although the osteoclast may remain attached to the bone along a clear zone. Associated with the disappearance of the ruffled border is the appearance of extensive interdigitated cell processes along the peripheral surfaces of the osteoclast away from the bone (Miller, 1977, 1981). PTH stimulation of inactive osteoclasts during the non-calcifying period of the cycle results in ruffled border formation within 15 minutes. By 30 minutes the ruffled borders are well developed and large endocytic vacuoles appear (Miller et al., 1984).

The clear zone, an actin containing (King and Holtrop, 1975) organelle free area surrounding the ruffled border, is believed to function in the adhesion of the cell to the bone and in limiting the

area of osteoclastic bone resorption (Malkani et al., 1973; Holtrop and King, 1977; and Ryder et al., 1981). Vimentin-like intermediate filaments, vinculin, actinin and fimbrin have been localized within paramarginal adhesion sites, probably clear zones, in cultured avian osteoclasts, supporting the proposal that clear zones may serve as specific adhesion devices in osteoclasts in vivo (Marchisio et al., 1984). Clear zones can also be seen in actively resorbing human monocytes and mouse macrophages cultured with devitalized bone particles (Teitelbaum and Kahn, 1980). A "transitional region", relatively free of organelles, has been identified in lamellopodia and in areas of cell adhesion to the substratum in foreign body giant cells (Sutton and Weiss, 1966; Papadimitriou and Archer, 1974). However, ruffled borders have not been observed in any of these other cell types.

Indicative of their resorptive function, osteoclasts are metabolically active cells exhibiting high levels of oxidative and hydrolytic enzyme activity. Metabolic pathways are well developed for utilization of NADP and NADPH, succinic, malic, lactic, and isocitric acids, beta-hydroxybutyrate and glucose-6-phosphate, the reactions mediated by diaphorases and dehydrogenases. The activities of hydrolytic enzymes, such as acid and neutral phosphatases, non-specific esterases, and leucine naphthylamidase are high in these cells (Addison, 1978). Acid phosphatase was the earliest of the hydrolytic enzymes to be demonstrated histochemically in bone tissue (Burstone,

1959a) and localized to osteoclasts (Schajowicz and Cabrini, 1958; Burstone, 1959b). Ultrastructurally, it was found in the lysosomes, cytoplasmic vacuoles, and ruffled border of the osteoclast and within the extracellular space between the osteoclast and the underlying bone (Lucht, 1971; Doty and Schofield, 1972). Doty et al., 1968, demonstrated that the amount of acid phosphatase activity increased under the influence of parathyroid extract and decreased with calcitonin, correlating with known osteoclastic activity (Cameron, 1972). On the basis of sensitivity or resistance to tartrate inhibition, two types of bone acid phosphatase have been identified biochemically (Wergedal, 1970; Anderson and Toverud, 1979). Histochemically, tartrate-resistant acid phosphatase can be localized predominantly within osteoclasts while tartrate-sensitive activity is associated primarily with bone cells other than osteoclasts (Hammarstrom et al., 1971; Minkin, 1982). Consequently, tartrate-resistant acid phosphatase has been proposed as a biochemical marker for the study of osteoclasts (Minkin, 1982). The enzyme carbonic anhydrase is also being explored as a suitable biochemical probe for osteoclastic studies (Anderson et al., 1982). Highly sensitive immunohistochemical methods reveal the presence of the C isoenzyme of carbonic anhydrase exclusively in rat calvarial and human trabecular osteoclasts (Vaananen and Parvinen, 1983; Vaananen, 1984). While its exact function is unknown, carbonic anhydrase may act to facilitate CO₂ diffusion from the cell or to provide hydrogen ions for secretion into the surrounding bone matrix

to create the acidic environment necessary for lysosomal enzyme activation and mineral resorption (Cao and Gay, 1985; Hall and Kenny, 1985).

Origin of the Osteoclast

Osteoclasts originate through the fusion of postmitotic mononuclear cells. The exact nature of the osteoclastic precursor cell has been, and is, the source of much controversy. Koelliker regarded the osteoblast as the cell source; Jordan (1921) advocated the marrow reticulum; Mallory and Haythorn in 1911 and 1929 respectively proposed the monocyte as the precursor (Hancox, 1946). From this debate two hypotheses arose to explain osteoclastic lineage--the monophyletic and the polyphyletic theories (Krukowski et al., 1983).

The monophyletic or skeletal scheme proposed that all the cells responsible for the formation and resorption of bone and cartilage arose from a common progenitor or stem cell population and through modulation could transform from one cell type to another, even rejoining the stem cell pool. According to this theory, the osteoblast and osteoclast were physiological expressions of the same undifferentiated stem cell, and the course of stem cell differentiation was regulated by the local environment surrounding it (Rasmussen and Bordier, 1974; Krukowski et al., 1983). Evidence in support of the monophyletic theory was based on interpretations of the size and shape of cells seen in histological sections at the light level. Autoradio-

graphic studies of the uptake of tritiated thymidine by rodent bone cells were also used to bolster the monophyletic view (Tonna and Cronkite, 1961; Young, 1962). Following the injection of tritiated thymidine, the first bone cells to be labeled were osteoprogenitor cells, followed by osteoblasts. Labeled osteoclasts were not observed until 36-40 hours post injection and usually only 1 or 2 nuclei were involved. The authors concluded that osteoprogenitor cells can divide while osteoblasts and osteoclasts do not and osteoclasts and osteoblasts arise by modulation from the osteoprogenitor pool. Hall (1975) in his review on the origin and fate of the osteoclast, however, points out that both studies involved the injection of label systemically which would label dividing cells all over the body, some of which could have migrated to the bone providing an extraskeletal source of label. He also points out that these experiments do not exclude the possibility of more than one type of osteoprogenitor cell within the bone--one for osteoblasts and another for osteoclasts--either of which could have incorporated label while in the S phase of the cell cycle. These criticisms illustrate a shortcoming in the monophyletic theory: it viewed bone as a "closed cell system" presuming that labeled cells were bone bound; thus cells could possibly leave the bone, but no potential precursor cells could enter it. With the realization that bone is not an isolated cell system, the monophyletic scheme was abandoned in lieu of the polyphyletic theory of bone cell lineage.

The polyphyletic theory of bone cell lineage proposes that different stem cells give rise to osteogenic cell lines and to osteoclasts (Krukowski et al., 1983). Osteogenic cells are derived from the marrow stromal populations while the osteoclast originates from a hemopoietic stem cell via a blood-borne mononuclear cell (Owen, 1980). There appears to be no transformation between the cells of the hemopoietic and stromal systems, nor a pluripotent stem cell capable of giving rise to both tissues (Owen, 1980). The first definitive evidence of an extraskeletal source for osteoclastic precursors was provided by Fischman and Hay (1962) who studied the incorporation of tritiated thymidine into regenerating newt limbs. This model system was useful because suspected precursors could be labeled selectively by administration of the isotope at different intervals before or during regeneration. When animals were injected and their regenerating limbs fixed the same day, no radioactive osteoclasts were observed. When animals were injected at 5, 10, or 15 days of regeneration and their limbs fixed at daily intervals, labeled mesenchymal cells were present, but no labeled osteoclasts were seen. In animals injected one day prior to amputation and the limbs fixed at intervals during regeneration, labeled osteoclasts were seen in the limb stumps between 10 and 20 days post-amputation. Prior to the appearance of labeled osteoclasts, the only radioactive cells present in the inner limb tissues were macrophages and monocytes. From these findings, the authors concluded that osteoclasts do not divide by mitosis and that

osteoclasts were not formed by the fusion of mesenchymal elements of the limb, but rather from circulating mononuclear leukocytes, probably monocytes. Jee and Nolan (1963) observed that charcoal particles, injected into rats, appeared at 4 days in the macrophages and spindle shaped mesenchymal cells of the bone, and after 15 days in osteoclasts. They, like Fischman and Hay, proposed that osteoclasts form by fusion of mononuclear phagocytes.

Scott (1967b) described two types of labeled precursor cells through the use of tritiated thymidine electron microscopic autoradiography. Type A cells (preosteoblasts) were spindle-like with characteristics associated with matrix production--large well developed endoplasmic reticulum and large intracellular accumulations of glycogen. Type B cells (preosteoclasts) had rounded profiles and resembled mononuclear leukocytes and osteoclasts in their cytoplasmic inclusions: an abundance of free ribosomes and mitochondria, complex Golgi apparatus associated with dense specific granules and morphologically identifiable primary lysosomes.

Gothlin and Ericsson (1973) in studies of fracture repair in parabiotic rats, obtained further evidence that local progenitor cells of the host produce osteoblasts whereas a separate migratory cell population produces osteoclasts. Rats were joined in pairs by establishing cross-circulation through their body walls via skin flaps. The rat pairs were then lethally irradiated except for the left hind-limb of one animal which was shielded. The right femur of each animal

was fractured one day post irradiation and tritiated thymidine administered to the protected rat at intervals of 4 days. Results of these experiments showed that all bone cells were labeled in the fracture callus of the protected rat whereas only the osteoclasts were labeled in the unprotected animal, confirming the origin of osteoclasts from migratory cells and the separate origin of osteogenic cells from local precursors.

Bone cell lineage was also studied through the use of quail-chick chimeras. The interphase nucleus of the quail possesses one or more large nucleolus-associated heterochromatic masses while that of the chick has diffuse heterochromatin. These two types of nuclei can easily be identified and used as markers to follow cell migration. Quail embryonic limb rudiments were harvested and grafted onto chick chorioallantoic membranes. After an appropriate period of time, the explants were removed and the nuclear characteristics of the cells surrounding it were evaluated. In this implant system, osteoclasts were found to be predominantly of host (chick) origin indicating a vascular migration of precursor cells, whereas osteoblasts were derived primarily from local grafted (quail) tissues (Kahn and Simmons, 1975; Jotereau and LeDouarin, 1978; Simmons and Kahn, 1979). Kahn et al. (1981), using quail bone explants grafted onto younger chick embryos, demonstrated that chick osteoclastic precursor cells are present in the circulation and can be induced to form osteoclasts in the graft before osteoclasts are formed in the chick embryo itself.

Similarly, Thesingh and Burger (1983) found osteoclastic precursors present in mouse mesenchyme 6 days before osteoclasts actually appeared. These findings indicate that osteoclastic precursors are present in the circulation long before they migrate into the mesenchymal tissues to become osteoclasts.

Osteopetrosis, a disease characterized by reduced bone resorption, has also been used as a model for the study of osteoclastic lineage. Mammalian osteopetrosis is most commonly inherited as an autosomal recessive trait in a number of species including man. The clinical manifestation of the disease in each mutant is an increase in skeletal mass associated with retarded bone growth and abnormal bone shape. Marrow cavities fail to develop and anemia is often present despite increases in extramedullary hematopoiesis. The skeletal abnormalities can interfere with dentition, cause neurological defects and may be associated with pigmentation changes (Marks and Walker, 1976). The bone resorbing defect in some forms of osteopetrosis is due to faulty osteoclasts. Osteoclasts from ia rats, mi and oc mice, and os rabbits do not form ruffled borders (Marks, 1984). Histochemical studies have provided evidence of reduced production of lysosomal enzymes by osteoclasts from gl, mi, oc, and op mice (Marks, 1984). In contrast, ia osteoclasts are capable of normal lysosomal enzyme production, but are unable to release these enzymes which accumulate in the cytoplasm (Handelman et al., 1964; Marks, 1973). In addition, the clear zones of ia osteoclasts, unlike those of their

normal counterparts, are extensive, covering most of the bone-cell interface, preventing lysosomal contact with the bone surface (Marks, 1983).

Walker (1972, 1973) was able to restore bone resorption in osteopetrotic mice by parabiotic union with normal siblings, suggesting a blood-borne factor for the cure of osteopetrosis. As a result, hematopoietic transplants from the spleens of osteopetrotic (mi) into lethally irradiated normal littermates and, conversely, transplants of normal spleens or bone marrow into irradiated osteopetrotic littermates were performed. Normal mice that had received osteopetrotic spleen cells gave evidence of skeletal sclerosis indicative of osteopetrosis, while osteopetrotic mice that received infusions of normal spleen or bone marrow cells showed restoration of resorptive activities with clearing of marrow cavities and near normal bone remodeling (Walker, 1975). Similarly, spleen cells were also found to cure the disease in the ia rat mutation (Marks, 1976). This restoration of bone resorption in the ia rat by normal spleen cells was accompanied by the transformation of osteoclasts from mutant to normal phenotype (Marks and Schneider, 1978; 1982).

Ash et al. (1980) provided direct evidence of the extraskeletal source of the osteoclast through the use of radiation chimeras. The granular leukocytes, monocytes, and osteoclasts of beige mice contain giant lysosomes which can serve as cytoplasmic markers. Lethally irradiated osteopetrotic (mi) mice received infusions of bone marrow

or spleen cells from non-osteopetrotic beige mice. This resulted in complete regeneration of the lymphomyeloid complex. Osteoclasts displaying giant lysosomes of donor origin were observed. Giant lysosomes were absent from osteogenic cells whose precursors were of host origin. In human chimera studies, female patients cured of juvenile osteopetrosis by bone marrow transplants from HLA matched male siblings demonstrated osteoclasts of donor (male) origin and osteoblasts of host (female) origin as evidenced by fluorescent Y body analysis (Coccia et al., 1980; Sorell et al., 1981).

Mononuclear Phagocyte System (MPS)

In accordance with the evidence provided by these investigations, the origin of the osteoclast from a hemopoietic stem cell has been confirmed. Hemopoietic stem cells give rise to several proliferating precursors each of which is committed to a specific cell line. The cell line to which the osteoclast belongs has not been definitively established. The candidate most often proposed is that of the mononuclear phagocyte series (Owens, 1980; Loutit and Nisbet, 1982).

Cell types included in the mononuclear phagocyte system (MPS) meet the following criteria:

- (1) Common derivation from bone marrow precursor cells
- (2) Strong trypsin resistant adhesion to glass surfaces
- (3) Avid phagocytosis

(4) Characteristic cell structure: a highly convoluted plasma membrane with abundant finger-like projections, numerous primary and secondary lysosomes, mitochondria, variable amounts of endoplasmic reticulum and round, oval, or reniform nuclei with a thin rim of dense chromatin and a prominent nucleolus

(5) Presence of surface receptors for F_c fragment of IgG and the third component of complement (Spector, 1974; Weiss, 1983).

Mononuclear phagocytes can be divided into two groups of cells: the circulating blood monocytes and the tissue macrophages of various organs such as the spleen, lymph nodes, liver, lung, peritoneal cavity and subcutaneous tissues (van Furth and Cohn, 1968). While it may have a role in antigen processing in the immune response, the blood monocyte appears to primarily represent a transit form of macrophage which upon emigration from the peripheral circulation differentiates into the tissue macrophage appropriate to its environment (Krause and Cutts, 1981). The tissue macrophage through phagocytosis is mainly involved in the clearance and destruction of bacteria, foreign materials and damaged tissue cells or debris from its surroundings. Epithelioid cells and multinucleated giant cells are included in the MPS as macrophage derivations (Spector, 1974). The proposed sequence of cell lineage of the MPS is :

Pluripotent stem cells---Committed stem cell---monoblast---
 promonocyte---monocyte---tissue macrophage---multinucleate giant cells
 (Weiss, 1983).

The hypothesis that the osteoclast is derived from the MPS is based on the common characteristics shared by osteoclasts and mononuclear phagocytes. Osteoclasts and mononuclear phagocytes demonstrate mobility, possess an active, undulating plasma membrane, and stain supravitaly with neutral red (Carrel and Ebeling, 1926; Hancox, 1946; Barnicot, 1946; Hancox and Boothroyd, 1961). Osteoclasts and macrophages are both capable of trypsin resistant glass adherence (Chambers, 1979). Zallone et al. (1983) observed that cultured osteoclasts and monocytes have similar arrangement and distributions of cytoskeletal structures as well as similar adhesion patterns to fibronectin free areas. Both osteoclasts and members of the MPS are functionally specialized for the uptake and degradation of biological substances as indicated by cytoplasmic concentrations of mitochondria and lysosomes (Chambers, 1978). Macrophage polykaryons share many of the enzymatic properties of osteoclasts (Cabrini et al., 1962) and while exhibiting less phagocytic potential than their macrophage precursors relative to size, are capable of lysosomal enzyme secretion (Papadimitriou et al., 1975; Papadimitriou and Wee, 1976).

There is mounting evidence for the participation of mononuclear phagocytes in the process of bone resorption. Macrophages are known to secrete collagenase, lysosomal enzymes, and prostaglandins all of which are believed to be fundamental to bone resorption (Teitelbaum and Kahn, 1980). Resorbing bone has been shown to be chemotactic for monocytes (Mundy et al., 1978). Human monocytes, mouse macrophages, and mononuclear cells isolated from avian peripheral blood have been shown to resorb labeled devitalized bone in vitro (Mundy et al., 1977; Kahn et al., 1978; Teitelbaum et al., 1979; McArthur et al., 1980; Chambers, 1981a). Osteoclasts and mononuclear phagocytes appear to exhibit the same functional preference toward the resorption of mineralized bone matrix as opposed to demineralized matrix (Chambers, 1981b; Chambers et al., 1984). Peritoneal macrophages have been reported to possess cell surface receptors for calcitonin and parathyroid hormone (Minkin et al., 1977). However, Perry et al. (1984) report that monocytes do not bind PTH, but are capable of degrading the hormone. Calciferol (vitamin D) has been shown to induce monocytic-macrophage differentiation in the U937 (Rigby et al., 1984) and HL60 monoblastic cell lines and in the latter, enhance the cells' ability to bind and degrade bone matrix (Bar-Shavit et al., 1983). These observations coupled with the already cited studies of Fischman and Hay and Jee and Nolan have prompted the proposal that monocytes, macrophages, and macrophage polykaryons might serve as appropriate

osteoclastic surrogates for studies of cellular bone resorption (Teitelbaum and Kahn, 1980).

Implantation Studies

In vivo implantation of bone matrix into ectopic sites may provide a method to evaluate the relationship between osteoclastic and MPS-mediated bone resorption. Implantation procedures were among the first techniques applied to the study of bone cells, their lineage and functions. While most commonly used as models of bone formation (Rohde, 1925; Sandison, 1928; Levander, 1938; Urist, 1980), they have also been employed to study bone resorption. Koelliker drove ivory pegs into living bones. Upon examination of the recovered material, he found extensive pitting of the pegs and named the multinucleated cells occupying those pits "ostoklasts" (Jordan, 1921). Groves (1918) used implant techniques to study bone formation in adult cats. He found that transplanted bone chips induced bone formation in bony defects while ground bone was resorbed in a few weeks resulting in nonunion. This finding was supported by Keith (1934) through the use of bone shavings as grafting material in dogs. He concluded that it was "fallacious to fragment a bone graft more than is necessary since fragmentation lowers both its supporting and osteogenic functions". Bujard, 1946, using bone powder, and Ham and Gordan (1952) employing bone chips demonstrated the presence of multinucleated cells adjacent to the implants in subcutaneous and intramuscular sites, respectively.

Irving and Handelman (1963) implanted dead decalcified or rachitic osteoid into the subcutaneous tissue of rats in experiments designed to study bone destruction by multinucleated giant cells. On the basis of similar acid phosphatase profiles and evidence of bone resorption as evaluated by light microscopy, the authors classified the giant cells as osteoclasts despite indications that they were nonresponsive to parathyroid extract administration or parathyroidectomy. Buring (1975) combined the techniques of parabiosis, autoradiography, and implantation to provide evidence in support of the polyphyletic origin of bone cells. Implantation of devitalized bone powder into calvarial defects (Glowacki et al., 1981; Glowacki, 1982; Holtrop et al., 1982) or chick chorioallantoic membranes (CAM) (Krukowski and Kahn, 1982) resulted in the formation of multinucleated cells adjacent to the bone fragments. While results indicated that bone resorption had occurred in both of these systems, the presence of ruffled border formation by these multinucleated cells could not be consistently demonstrated. The use of these implant systems as models for the study of osteoclasts was still advocated based on the similarities between osteoclasts and macrophage polykaryons (Holtrop et al., 1982).

Osteoclasts and the MPS

There is, however, growing evidence that osteoclasts and macrophages differ in significant ways and that macrophage-mediated bone resorption may not be equivalent to osteoclastic resorption. Osteo-

clasts, but not macrophages or monocytes, form ruffled borders at the bone surface (Kahn et al., 1978; Rifkin et al., 1979). Members of the mononuclear phagocyte family failed to resorb bone as detected by changes in the bone surface by scanning electron microscopy (Horton et al., 1986), even under the influence of macrophage activators (Chambers and Horton, 1984). Macrophages and macrophage polykaryons do not show a morphological response to calcitonin (Chambers and Magnus, 1982). Osteoclasts lack the F_c and C_3 receptors characteristic of all mononuclear phagocytes (Hogg et al., 1980; Jones et al., 1981) and fail to express monocyte-macrophage, granulocyte or lymphocyte surface antigens (Horton et al., 1984; Horton et al., 1985a; Horton et al., 1985b). While macrophages readily fuse to form polykaryons, formation of osteoclasts from macrophages has not been demonstrated (Marks, 1983). Injection of cell suspensions of macrophages into ia rats does not effect the cure of osteopetrosis, but injections of pluripotent hemopoietic stem cells of bone marrow origin are capable of curing the skeletal sclerosis and results in the formation of normal osteoclasts (Schneider and Byrnes, 1983; Schneider, 1985). In an effort to further define the nature of the osteoclast precursor, Schneider and Relfson (1986) evaluated the ability of cells more mature than pluripotent stem cells to differentiate into normal osteoclasts at 3 weeks post-transplantation into ia rats. They report that granulocyte colony-forming cells and granulocyte-macrophage colony-forming cells were capable of curing the ia resorptive defect while

macrophage colony-forming and GM-cluster forming cells were not. The results of these studies suggest that osteoclast precursors and members of the mononuclear phagocyte system in this osteopetrotic mutant may share a very early common stem cell ancestor, but once definitive MPS maturation has begun, the MPS cells lose the ability to differentiate into osteoclast precursors. Burger et al.(1982) studied the origin of the osteoclast using mouse long bone primordia. This culture system does not contain osteoclasts, nor do they develop in these tissues when cultured. Osteoclasts developed in fetal bones co-cultured with embryonic liver or weakly adherent radiosensitive bone marrow mononuclear cells, but not with strongly adherent cells, resident or elicited peritoneal macrophages or monocytes. These findings also suggest that osteoclast precursors are not mature monocytes or macrophages and support the premise that cells of the monoblast, promonoblast or even earlier stages contain the stem cell population for osteoclastic lineage. Ibbotson et al.(1984) and Roodman et al. (1985) report the formation of osteoclast-like cells from long term cultures of feline and primate marrow-derived mononuclear cells. These multinucleated cells contained large numbers of mitochondria, clear zones, and displayed extensive membrane ruffling in the presence of bone. They exhibited tartrate-resistant acid phosphatase, the activity of which was increased by PTH administration and inhibited by calcitonin. PTH, PGE₂, and 1,25(OH)₂-vitamin D₃ increased formation of the osteoclast-like cells, while calcitonin inhibited the stimulatory

effects of PTH. The bone marrow mononuclear precursors of these cells were nonadherent to plastic, stained heavily with nonspecific esterase and appeared to be immature monocytes histologically. The multinucleated cells were able to resorb bone in vitro as demonstrated by the release of ^{45}Ca from devitalized bone powder, but addition of PTH and calcitonin had little or no effect on the bone resorption. This is in contrast to more recent studies by Burger et al. (1984) who found that osteoclasts developed from precultured bone marrow mononuclear phagocytes (BMMP) in the presence of live, but not devitalized, bone and that only live bone was resorbed by osteoclasts. Devitalized bone powder was resorbed but, according to the authors, most probably by mature macrophages which comprised 50% of the BMMP. They concluded that this type of bone resorption was different from osteoclast-mediated resorption.

While the use of mononuclear phagocytes as osteoclastic surrogates has been called to question, the nature and role of macrophage-monocyte bone resorption remain open to speculation. Localized bone loss is frequently associated with chronic inflammatory conditions such as rheumatoid arthritis, periodontal disease, and chronic osteomyelitis (Deporter, 1979; Minne et al., 1984). The role of the osteoclast in these pathological processes is unclear. In laboratory animals, the inflammation-linked osteopenia appears to occur independent of PTH secretion or vitamin D metabolism and to be mediated by localized accumulations of inflammatory cells (Minne et al., 1984), of

which macrophages and monocytes form a large part. While these cells may participate directly in the resorptive process, macrophage-monocyte contributions to bone loss may also be mediated indirectly through the osteoclast itself. Modulation of osteoclastic activities could occur through a variety of means including: (a) production of prostaglandins, potent stimulators of bone resorption (Dominguez and Mundy, 1980), (b) regulation of the production of osteoclast activating factor by activated lymphocytes, a monocyte dependent process (Yoneda and Mundy, 1979a,b) and (c) phagocytosing bone matrix debris left after osteoclastic bone resorption (Heersche, 1978). Therefore, it appears that there are both direct and indirect means by which mononuclear phagocytes could influence normal and pathologic bone resorption, and that osteoclastic bone resorption and macrophage-monocyte-mediated bone resorption may not be equivalent processes.

CHAPTER III

A FUNCTIONAL AND MORPHOLOGICAL STUDY OF CELLS ADJACENT TO ECTOPIC BONE IMPLANTS IN RATS

ABSTRACT

Subcutaneous implantation of bone chips into normal and osteopetrotic (ia) rats results in the formation of multinucleate giant cells (MNGC's) adjacent to the bone surface. In this study the resorptive and morphological characteristics of the cells surrounding these implants were assessed to determine if the bone resorbing defects seen in ia animals would be mimicked in this implant system, and thus lend validity to its use as a model for the study of osteoclastic lineage and function. Direct measurement of in vivo bone resorption was achieved through the use of ^{45}Ca labeled primarily osteoid exposed, freeze-thawed (FT), and primarily mineral exposed, bleached (B) and collagenase-treated (CT) bone chip pairs. The high degree of uniformity in label content between individual members of each pair permitted one chip to be designated as the implant and the other as the control. Comparison of the ^{45}Ca content of the implant to that of the control (corrected for half-life) indicated the total ^{45}Ca release during a 2 week implantation period. Similar comparison of the ^{45}Ca content of bone chips implanted inside diffusion chambers with that of controls measured the non-cell mediated ^{45}Ca release over the same time period. Results showed normal recipients with mean ^{45}Ca releases of 17.7%, 19.1%, and 21.5% from FT, B, and CT bone chips respectively. Similarly, ia animals had mean releases of 17.4% from FT, 24.4% from B, and 22.4% from CT implants. Both normal and ia rats showed 23% greater total ^{45}Ca release from mineral versus osteoid

exposed matrix. Cellular events occurring on the bony substrate were evaluated by light and electron microscopy. At 3 days, bone chips were surrounded primarily by mononuclear cells. By 14 days, MNGC's were present at the bone surface in both ia and normal animals. In mineral exposed implants, 40 to 50% of the bone surface was covered by MNGC's as compared to 20% of the osteoid exposed surface. These MNGC's possessed occasional clear zones, but did not exhibit ruffled borders; therefore, they could not be classified as osteoclasts. The results of this study indicate that the defects seen in ia mutants were not reproduced in this implant system. The ^{45}Ca release that occurred was probably due to the action of mononuclear phagocytes and macrophage polykaryons rather than by true osteoclastic bone resorption.

INTRODUCTION

An osteoclast is defined as a multinucleate giant cell (MNGC) adjacent to resorbing bone. Ultrastructurally, the osteoclast is differentiated from other types of MNGC's by the presence of both a clear zone and ruffled border at the bone-cell interface (Bonucci, 1981; Gothlin and Ericsson, 1976; Marks, 1983). The ruffled border, a complex series of cytoplasmic infoldings, is considered to be the major site of active osteoclastic bone resorption. The exocytosis of lysosomal enzymes necessary for bone degradation as well as the

endocytosis of degradation products occur at the ruffled border (Holtrop and King, 1977; Marks, 1983). The relative activity of the osteoclast is reflected by the degree of development of its ruffled border. In cultures stimulated with parathyroid hormone, 64% of the osteoclasts demonstrated large and highly developed ruffled borders while in nonstimulated cultures only 11% of the osteoclasts had ruffled borders and these consisted of a few shallow infoldings (Holtrop, et al., 1974). The clear zone, an organelle free area surrounding the ruffled border, is believed to function in the adhesion of the cell to the bone and in limiting the area of osteoclastic bone resorption (Holtrop and King, 1977). Clear zones can also be seen in actively resorbing human monocytes and mouse macrophages cultured with devitalized bone particles (Teitelbaum and Kahn, 1980). A "transitional region", relatively free of organelles, has been identified in lamellopodia and in areas of cell adhesion to the substratum in foreign body MNGC's (Papadimitriou and Archer, 1974; Sutton and Weiss, 1966). However, ruffled borders have not been observed in any of these other cell types.

The osteoclast originates through the fusion of blood-borne mononuclear precursor cells. The exact nature of this precursor cell is still unknown (Marks, 1983). Osteopetrosis, a disease characterized by the excessive accumulation of bone, has provided a model for the study of osteoclastic lineage and function. The primary defect in some forms of osteopetrosis is faulty osteoclasts.

Osteoclasts from osteopetrotic (ia) rats do not form ruffled borders nor are they able to release normal amounts of lysosomal enzymes which accumulate in the cytoplasm. Their clear zones, unlike those of normal osteoclasts, are extensive, covering most of the cell-bone interface. This may prevent lysosomal contact with the bone surface (Marks, 1983). Bone resorption is reduced, resulting in increased skeletal mass associated with abnormal bone growth and shape (Marks and Walker, 1976).

Bone implants have been used in attempts to develop an in vivo model for osteoclastic differentiation and function. Bone powder implanted into calvarial defects (Glowacki, et al., 1981; Holtrop, 1982) or onto chick chorioallantoic membranes (Krukowski and Kahn, 1982) resulted in the formation of MNGC's surrounding the bone fragments. The resorptive activity of the chick MNGC's was indirectly assessed by liquid scintillation counting of samples of amniotic fluid taken from embryos implanted with ⁴⁵Ca labeled bone powder. These studies report that mineralized implants more readily induced the formation of osteoclast-like MNGC's. Recent work on the effects of substrate composition on bone resorption by rabbit osteoclasts implies that contact with bone mineral may be necessary to initiate osteoclastic resorption (Chambers, et al., 1985).

We have found that subcutaneous implantation of recoverable bone chips into osteopetrotic(ia/ia) and normal(ia/+) rats results initially in the migration and attachment of mononuclear cells to the

bony substrate. At seven and fourteen days post-implantation, bone surfaces show increasing numbers of MNGC's. This series of experiments was designed to (1) assess the resorptive capabilities of cell populations adherent to the implanted bone, (2) to determine the effect of presenting mineral versus primarily osteoid exposed bone matrix on the differentiation and resorptive activity of these cells, and (3) to characterize morphologically the cell types adjacent to the bone implant. Implantation into both osteopetrotic and normal animals was performed to determine if the usual disparity between osteopetrotic and normal bone resorption was present, and if so, was this disparity reflected by morphological differences similar to those found between ia and normal osteoclasts. Finding such differences in the resorption of implanted bone matrix and in the morphology of the cells surrounding the implants between ia rats and normal littermates would validate the use of this implant system as a model for the study of osteoclastic lineage and function.

MATERIALS AND METHODS

Animals

Rats were obtained from our colony bred to maintain the osteopetrotic(ia) mutation. Breeding groups were established to produce litters of ia/ia and ia/+ genotypes. The former can be identified on the tenth day after birth by failure of eruption of the

incisors (Greep, 1941). The latter are phenotypically identical with +/+ rats of this stock (Marks, 1973) and were used as normal controls.

Calcium Release Assay

Radioactively labeled bone chips were prepared in the following manner. Ten day old rats were injected with 40uCi ^{45}Ca (in 0.1ml distilled water). To insure adequate label retention in calvaria assigned to mineral exposed studies, other rats received injections of 40uCi at both 7 and 10 days of age. Three days later, the animals were killed by ether inhalation, the calvaria removed and the pericrania stripped. The calvaria were devitalized by freeze-thawing x 3 in liquid nitrogen and sonication in distilled water for 7 minutes. Following devitalization, calvaria designated for mineral exposed matrix studies were either immersed in bleach for one minute or incubated in 5mg/ml crude collagenase (Lot #113F-6831, 245 U/mg dry wt collagenase, .10 U/mg dry wt clostripain, <.01 U/mg dry wt trypsin, 85 U/mg dry wt neutral protease, Sigma, St. Louis, Mo.) in phosphate buffered saline for 2 hours at 37°C. All calvaria were divided into frontal, parietal and occipital sections; these were bisected along the midline suture resulting in three pairs of labelled bone chips per calvarium. The chips were dried at 60°C. for 20 minutes and then weighed. After overnight exposure to ultraviolet light, the three types of bone chip pairs (freeze-thawed, bleached, and collagenase-treated) were allocated to experimental groups.

Group 1 Uniformity of labeling within each bone chip pair

Each member of the bone chip pair was digested in an individual vial with 0.5 ml concentrated formic acid at 100°C. for 30 minutes. After cooling, 10ml of aqueous scintillation cocktail was added to each vial. The vials were vortexed and their radioactivity assessed by liquid scintillation counting. Comparison of the ^{45}Ca content (counts/mg) of the members of each bone chip pair was performed to determine the uniformity of ^{45}Ca label within the pair.

Group 2 Diffusion chamber implants

Materials to be used in preparation of the diffusion chambers were sterilized by overnight exposure to ultraviolet light. A 0.45um Millipore filter was glued to one side of a 13mm plexiglass O ring. One half of each bone chip pair was placed in the chamber after which the chamber was completed by gluing a second Millipore filter to the O ring. Two chambers per animal were implanted into the dorsal subcutaneous tissue of 6 week old normal rats. A total of 15 rats (5 per bone chip preparation) received implants. The remaining bone chip half was digested and the ^{45}Ca content determined. Two weeks following implantation the chambers were removed and their contents examined by light microscopy to determine whether they had remained intact and cells had been excluded. Those chambers which were not intact were eliminated from this study. The devitalization process was repeated, the bone chips digested and the ^{45}Ca content determined.

The implant ^{45}Ca content was then compared to that of the control chip (corrected for natural radioactive decay over the two week period). This theoretical (calculated) ^{45}Ca content of the control chip was accurate to within 1% of the actual measured ^{45}Ca content.

Group 3 Direct bone chip implantation

One member each of 3 freeze-thawed and 3 collagenase-treated bone chip pairs was implanted directly into the dorsal subcutaneous tissue of 6 week old ia or normal rats. Six bleached bone chip halves were placed in a single ia or normal animal. A total of 18 rats (10 ia and 8 normal) received implants. The remaining control calvarial half was digested and ^{45}Ca content determined. Two weeks after implantation, the bone chips were recovered, devitalized, digested, and the ^{45}Ca content measured. Comparison of the ^{45}Ca content of the calculated control versus the implant was done as in the diffusion chamber studies.

Morphology

Two non-radioactive freeze-thawed, bleached, and collagenase-treated bone chips, prepared from 13 day old normal rat calvaria, as previously described, were implanted into the dorsal subcutaneous tissue of 3 ia and 3 normal rats. Bone chips were recovered from 1 ia and 1 normal rat at 3, 7, and 14 days post-implantation. The bone chips were fixed in 2.5% gluteraldehyde in cacodylate buffer (pH 7.2)

for 2 hours, post-fixed in 1% OsO₄ for 1 hour, decalcified in 10% EDTA (pH 7.3) for 48 hours, dehydrated in ethanol, and embedded in Epon. The tissue was sectioned at 2um, stained with toluidine blue, and evaluated by light microscopy. Areas warranting further investigation were thin sectioned, stained with uranyl acetate and lead citrate for viewing with the Hitachi H600 electron microscope.

Morphometric Analysis

A total of 33 toluidine blue-stained sections taken from bone chips recovered from ia and normal recipients were analyzed using a Zeiss videoplan to determine the percentage of freeze-thawed, bleached, or collagenase-treated bone surface lined by MNGC's 14 days post-implantation. Percentage of bone length lined by MNGC's was calculated as follows:

$$\frac{\text{Length of bone covered by MNGC's}}{\text{Total length of bone surface}} \times 100 = \%$$

The statistical comparison of these percentages was performed using the unpaired Students t-test.

RESULTS

Uniformity of label within bone chip pairs

Comparison of the ⁴⁵Ca content of corresponding bone chip halves (counts/mg) showed mean variations in label of 1.7% ± 1.2%, 2.2% ±

1.1%, and $1.7\% \pm 1.6\%$ for freeze-thawed (Table 1), bleached (Table 2), and collagenase-treated (Table 3) pairs respectively. This degree of uniformity in label content between members of individual pairs allowed their use as control and implant chips in subsequent experiments.

Diffusion chamber implants (Figure 1)

These studies were conducted to establish the baseline amount of in vivo non-cell mediated ^{45}Ca release for each type of bone chip preparation. As indicated in materials and methods, chambers were examined by light microscopy to determine whether the chamber seal had been maintained and cells excluded. The results illustrated in Figure 1 were tabulated from chambers found to be cell-free. There was a mean $4.1\% \pm 0.5\%$ ^{45}Ca loss from freeze-thawed pairs over the two week implantation period. In this same period, bleached bone chips showed a mean decrease of $6.7\% \pm 3.0\%$ in ^{45}Ca label and collagenase-treated bone chips a mean of $7.1\% \pm 4.1\%$. This additional ^{45}Ca loss from bleached and collagenase-treated chips may be due to increased mineral exposure to fluids found within the diffusion chambers. In chambers that did contain cells, label loss from implanted bone chips approached that of directly implanted bone chips.

Direct implantation studies

Figure 2 illustrates the results of the direct implantation

studies. Normal recipients had mean ^{45}Ca releases of $17.7\% \pm 4.8\%$, $19.1\% \pm 3.5\%$, and $21.5\% \pm 1.9\%$ from freeze-thawed, bleached, and collagenase-treated bone chips respectively. A mean release of $17.4\% \pm 3.7\%$ from freeze-thawed, $24.4\% \pm 2.1\%$ from bleached, and $22.4\% \pm 1.3\%$ from collagenase-treated bone chips occurred in ia animals. The mean percent ^{45}Ca release reflects the total ^{45}Ca loss from implanted bone chips over the two week period. This percentage minus the appropriate non-cell mediated ^{45}Ca release from diffusion chamber studies reflects the amount of cell mediated ^{45}Ca loss. Both ia and normal animals were capable of ^{45}Ca release over and above that of the baseline. Although the data presented are for normal bone implants, osteopetrotic recipients appeared to be capable of ^{45}Ca release greater than or equivalent to that of their normal counterparts whether ia or normal bone chips were implanted. Appendix A includes the results of uniformity, diffusion chamber and direct implantation studies using osteopetrotic calvarial bone as the implant substrate. Figure 2 also indicates that both normal and ia animals tend to demonstrate greater ^{45}Ca release when presented with a mineral exposed bone surface (bleached or collagenase-treated) rather than a primarily osteoid exposed surface (freeze-thawed).

Morphometric Analysis (Figure 3)

An equivalent percentage of the total freeze-thawed bone chip surface was covered by MNGC's in ia (mean $18.1\% \pm 6.4\%$) and normal

recipients (mean $24.7\% \pm 13.0$). Similar results were found for bleached ($39.4\% \pm 13.0\%$ vs $46.6\% \pm 10\%$) and collagenase-treated ($53.3\% \pm 10\%$ and $46.6\% \pm 10\%$) bone chips. Mineral exposed matrix (bleached and collagenase-treated) showed significantly more surface covered by MNGC's than did primarily osteoid exposed matrix (freeze-thawed) in ia (freeze-thawed to bleached $p \leq .04$ and to collagenase-treated $p \leq .015$) and normal (freeze-thawed to bleached $p \leq .006$ and to collagenase-treated $p \leq .0001$) animals.

Morphology

At three days post-implantation, regardless of bone chip treatment, mononuclear cells could be seen surrounding and aligning themselves along the implanted bone surface in both normal and ia animals (Fig. 4, a,b,c,d,). Ultrastructurally, these cells possessed irregularly shaped nuclei with heterochromatin uniformly dispersed along the nuclear membrane. The cytoplasm was unremarkable with the exception of lipid droplets and what appeared to be phagosomes. Their surfaces were covered by numerous cytoplasmic processes that interdigitated extensively with those of adjacent cells (Fig. 5). The occasional multinucleated cell observed was usually binucleate, possessed a rather uniform cytoplasm and did not display clear zones or ruffled borders.

By fourteen days, much of the bone surface, particularly in mineral exposed implants was covered by large MNGC's. At the light

microscopic level, these MNGC's presented two different morphological appearances. One was an elongated cell extending over much of the bone surface. Its nuclei were usually oval and linearly arranged within the cell. The second type of MNGC presented a more rounded profile and was often seen to lie in shallow depressions on the bone or to surround cut edges of the bone. The nuclei were round or irregular in shape and arranged in clusters (Fig. 6a). Frequently this second type of MNGC exhibited highly vacuolated cytoplasm adjacent to the bone (Fig. 6b). Ultrastructurally no apparent difference, other than nuclear arrangement, was noted between these two types of cells. MNGC's at 14 days were larger, contained more nuclei than 3 day old MNGC's, and displayed the usual complement of cytoplasmic organelles (Fig. 7). Clear zones were seen infrequently adjacent to the bone and at no time was a distinctive ruffled border observed. Samples of implants removed at 7 days revealed cell populations intermediate to those found at 3 and 14 days. There was no apparent morphological difference between cell populations generated in the normal versus ia animals or in mineral versus osteoid exposed implants at the light or electron microscopic level.

DISCUSSION

Bone resorption involves both the removal of bone mineral and the degradation of organic bone matrix. Quantitation of the amount of

^{45}Ca label released from bone into surrounding tissue fluids or culture medium is commonly used as an indirect index of bone resorption. We have found that ^{45}Ca , when injected into 10 day old rat pups, is uniformly incorporated into the calvaria. Labeled calvaria can be divided into three bone chip pairs; individual members of which can be designated as control and experimental halves. These bone chips are large enough to be recovered easily allowing the remaining ^{45}Ca content of each experimental chip to be determined accurately. The comparison of label content of control and experimental halves results in a direct assessment of total ^{45}Ca release over a given period of time. This technique offers a practical alternative to other ^{45}Ca release assays. It was successfully applied in our study of the MNGC's found adjacent to implanted bones in ia and normal animals.

Osteopetrosis is a disease characterized by reduced bone resorption due to defective osteoclasts, yet results from ^{45}Ca release assays over the two week implant period indicate that ia animals are as capable of cell-mediated resorption of the implanted bone as normal animals. Both types of animals show increased ^{45}Ca release when presented with a mineral exposed matrix versus one having a primarily osteoid exposed matrix. Therefore, the usual reduction in bone resorption seen in the ia mutants was not reproduced at this ectopic implantation site.

Morphologically, ia and normal cell populations surrounding the

implants appeared to be similar. Presenting these cells with a mineral exposed matrix increased the amount of bone surface covered by MNGC's in addition to increasing ^{45}Ca release, but it did not alter their structural characteristics.

At three days post-implantation mononuclear cells were aligned along the bone surface. These cells demonstrated the extensive pattern of cytoplasmic processes typical of macrophages and epithelioid cells activated in response to an inflammatory stimulus (Sutton and Weiss, 1966). Although actual cell fusions were not observed, by 14 days MNGC's extensively covered the bone surface. At the light microscopic level, these MNGC's appeared to be actively engaged in bone resorption, however, ultrastructurally, they did not exhibit the typical characteristics of active osteoclasts. Only an occasional clear zone was seen and no ruffled borders were observed despite evidence, ^{45}Ca release, that bone resorption had occurred. Instead, these MNGC's resembled foreign body giant cells which arise from the fusion of mononuclear phagocytes in response to a variety of stimuli (Sutton and Weiss, 1966; Mariano and Spector, 1973; Chambers, 1978). The ^{45}Ca label loss seen in this study may have occurred as a result of a chronic inflammatory reaction involving macrophages and macrophage polykaryons rather than by true osteoclastic resorption. Resorbing bone has been shown to be chemotactic for monocytes, macrophage precursors (Mundy, 1978). Mouse macrophages appear to be able to resorb labeled devitalized bone in vitro (Teitelbaum et al.,

1979). Macrophage polykaryons, while exhibiting less phagocytic potential than their macrophage precursors relative to size, are capable of lysosomal enzyme secretion (Papadimitriou et al., 1975 and Papadimitriou and Wee, 1976). Holtrop, et al. (1982) reached similar conclusions about MNGC's surrounding bone fragments implanted into rat calvarial defects. She proposes that foreign body giant cells release degradative enzymes which exert their action within the canaliculi of dead bone. Macrophages act secondarily to remove the bone fragments produced by the enzymatic action of MNGC's. She suggests the use of this implant system as a model for osteoclastic lineage studies. Marks (1983) in his review of the origin of osteoclasts, provides strong evidence against the use of such a model. He points out that the osteoclast and macrophage differ in significant ways. Osteoclasts, but not macrophages, form ruffled borders at the bone surface. Osteoclasts do not exhibit all the same enzyme or receptor characteristics of monocytes and macrophages; nor do they respond to the same hormonal stimuli (Marks, 1983). Injection of cell suspensions of macrophages into ia rats does not affect the cure of osteopetrosis, but pluripotent hemopoietic stem cells of bone marrow origin are capable of curing the skeletal sclerosis, resulting in the formation of normal osteoclasts (Schneider and Byrnes, 1983; Schneider, 1985). Members of the mononuclear phagocyte family failed to resorb bone, as detected by changes in the bone surface by scanning electron microscopy, even under the influence of macrophage activators

(Chambers and Horton, 1984). Therefore, there appears to be mounting evidence against the use of macrophages, monocytes, and macrophage polykaryons as models for the study of osteoclastic function and lineage.

As a result of our investigation of MNGC's adjacent to subcutaneously implanted bone chips in normal and ia rats we conclude the following:

- (1) The use of uniformly labeled bone chip pairs is a valid and practical method for the assessment of ^{45}Ca release in vivo.
- (2) The osteopetrotic implant recipients were able to release ^{45}Ca via cellular means in equal or greater amounts than their normal counterparts.
- (3) Both ia and normal animals showed an increase in ^{45}Ca release and percent of bone surface covered by MNGC's in the presence of mineral exposed matrix over that of primarily osteoid exposed matrix.
- (4) The cell populations surrounding the bone implants appeared to be morphologically the same in both types of animals.
- (5) The MNGC's adjacent to the bone implants could not be characterized as osteoclasts because they did not display ruffled border formation despite evidence of ^{45}Ca release. Instead, they appeared to be foreign body giant cells.
- (6) Because the resorbing and morphological defects in the ia rat were not mimicked in this study, the ^{45}Ca release probably

occurred as a result of the action of mononuclear phagocytes and polykaryons rather than by true osteoclastic resorption.

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Table 1. Uniformity of ^{45}Ca Label Within Freeze-Thawed Bone Chip Pairs

<u>Bone Chips</u> ^a	<u>Weight (mg)</u>	<u>Counts/mg</u>	<u>Mean</u> ^b	<u>Percent Error</u> ^c
<u>Frontal</u>				
Left	5.5	53,969	54,154 \pm 261	0.5
Right	5.4	54,338		
Left	6.6	33,423	33,188 \pm 333	1.0
Right	6.7	32,952		
Left	5.8	53,575	54,017 \pm 625	1.2
Right	4.9	54,459		
<u>Parietal</u>				
Left	4.8	48,330	49,572 \pm 1756	3.5
Right	5.0	50,814		
Left	7.1	30,566	30,344 \pm 313	1.0
Right	6.4	30,122		
Left	5.5	47,481	47,821 \pm 480	1.0
Right	5.1	48,161		
<u>Occipital</u>				
Left	6.3	59,029	59,850 \pm 1161	1.9
Right	6.0	60,671		
Left	6.6	34,938	34,300 \pm 902	2.6
Right	5.8	33,662		
Left	5.3	55,164	56,537 \pm 1941	3.4
Right	5.1	57,909		

^aSamples taken from three 13-day-old rats.

^bMean \pm standard deviation in counts/mg.

^cPercent error = (standard deviation x 100)/mean.

Table 2. Uniformity of ^{45}Ca Label Within Bleached Bone Chip Pairs

<u>Bone Chips</u> ^a	<u>Weight (mg)</u>	<u>Counts/mg</u>	<u>Mean</u> ^b	<u>Percent Error</u> ^c
<u>Frontal</u>				
Left	2.5	281,934	277,908 \pm 5694	2.0
Right	2.7	273,882		
Left	3.2	305,174	303,581 \pm 2252	0.7
Right	2.7	301,989		
Left	3.1	301,901	309,889 \pm 11,297	3.6
Right	2.3	317,877		
<u>Parietal</u>				
Left	4.6	258,497	254,459 \pm 5711	2.2
Right	5.0	250,421		
Left	4.7	255,264	251,618 \pm 5156	2.0
Right	4.5	247,972		
Left	4.1	271,083	273,403 \pm 3281	1.2
Right	4.0	275,723		
<u>Occipital</u>				
Left	3.3	268,853	265,588 \pm 4618	1.7
Right	3.3	262,322		
Left	2.8	262,908	267,749 \pm 6846	2.6
Right	2.9	272,589		
Left	2.5	334,376	325,222 \pm 12,946	4.0
Right	2.9	316,067		

^aSamples taken from three 13-day-old rats.

^bMean \pm standard deviation in counts/mg.

^cPercent error = (standard deviation x 100)/mean.

Table 3. Uniformity of ^{45}Ca Label Within Collagenase-Treated Bone Chip Pairs

<u>Bone Chips</u> ^a	<u>Weight (mg)</u>	<u>Counts/mg</u>	<u>Mean</u> ^b	<u>Percent Error</u> ^c
<u>Frontal</u>				
Left	3.1	95,369	96,950 \pm 2236	2.3
Right	4.1	98,531		
Left	5.3	95,183	95,076 \pm 152	0.2
Right	5.1	94,968		
Left	3.3	90,926	91,323 \pm 561	0.6
Right	3.2	91,720		
<u>Parietal</u>				
Left	8.4	89,348	89,033 \pm 446	0.5
Right	7.5	88,717		
Left	7.9	74,812	74,829 \pm 23	0.0
Right	8.0	74,845		
Left	6.0	84,072	82,555 \pm 2146	2.6
Right	7.0	81,037		
<u>Occipital</u>				
Left	6.1	123,499	119,325 \pm 5904	4.9
Right	6.0	115,150		
Left	5.9	92,444	91,578 \pm 1225	1.3
Right	6.3	90,712		
Left	5.3	102,341	100,361 \pm 2799	2.8
Right	6.3	98,382		

^aSamples taken from three 13-day-old rats.

^bMean \pm standard deviation in counts/mg.

^cPercent error = (standard deviation \times 100)/mean.

Figure 1.

Mean percent \pm S.D. ^{45}Ca release over a two week period from freeze-thawed, bleached, and collagenase-treated bone chips in diffusion chambers implanted into normal rats.

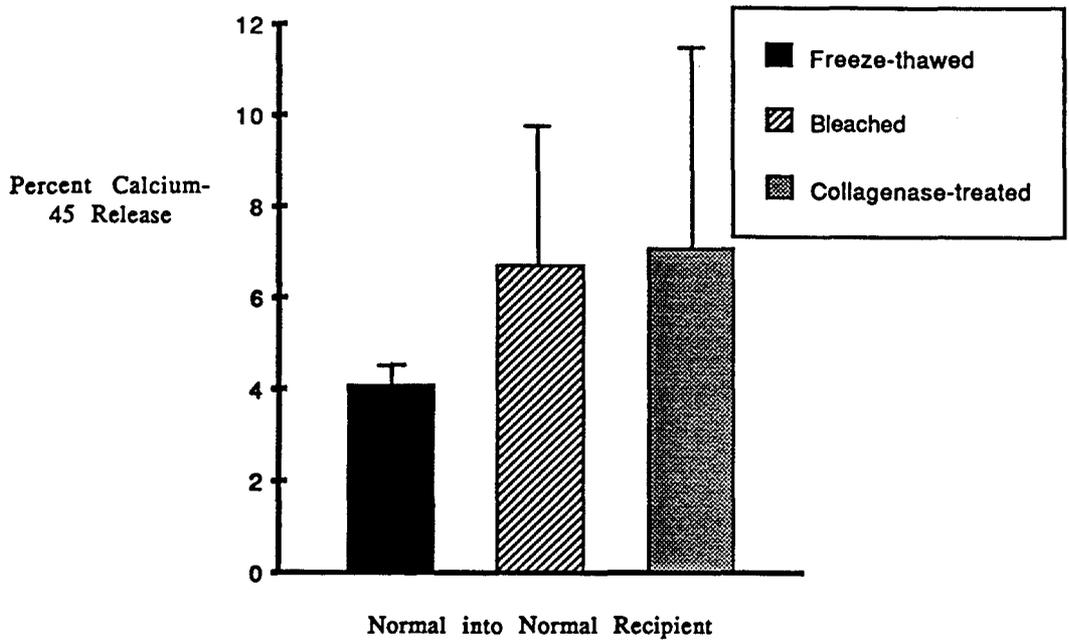


Figure 2.

Mean percent \pm S.D. ^{45}Ca release over a two week period from freeze-thawed, bleached, and collagenase-treated bone chips implanted into normal and ia rats.

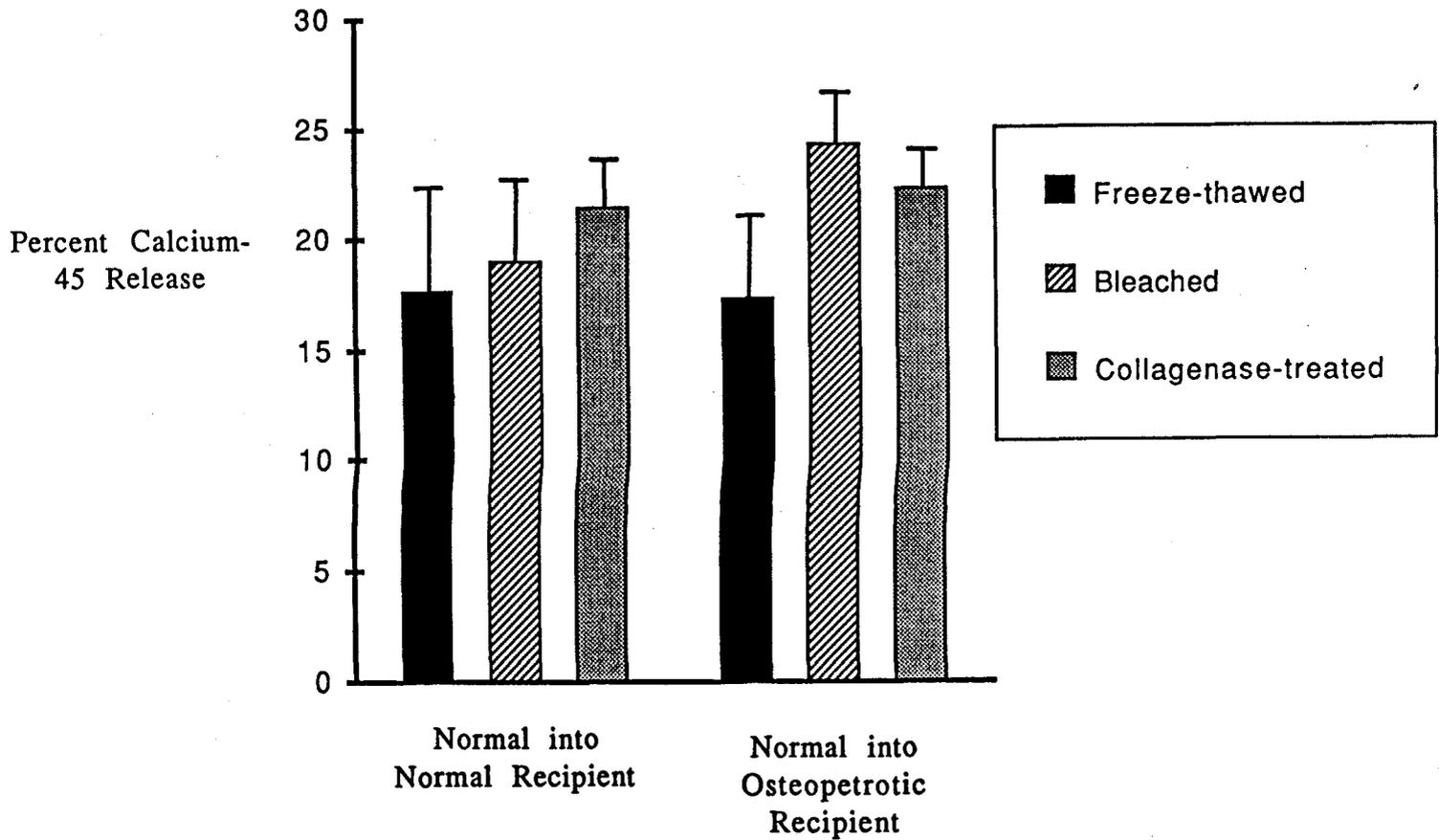


Figure 3.

Mean percent \pm S.D. of total bone surface covered by MNGC's two weeks after implantation of freeze-thawed, bleached, and collagenase-treated bone chips into normal and ia rats.

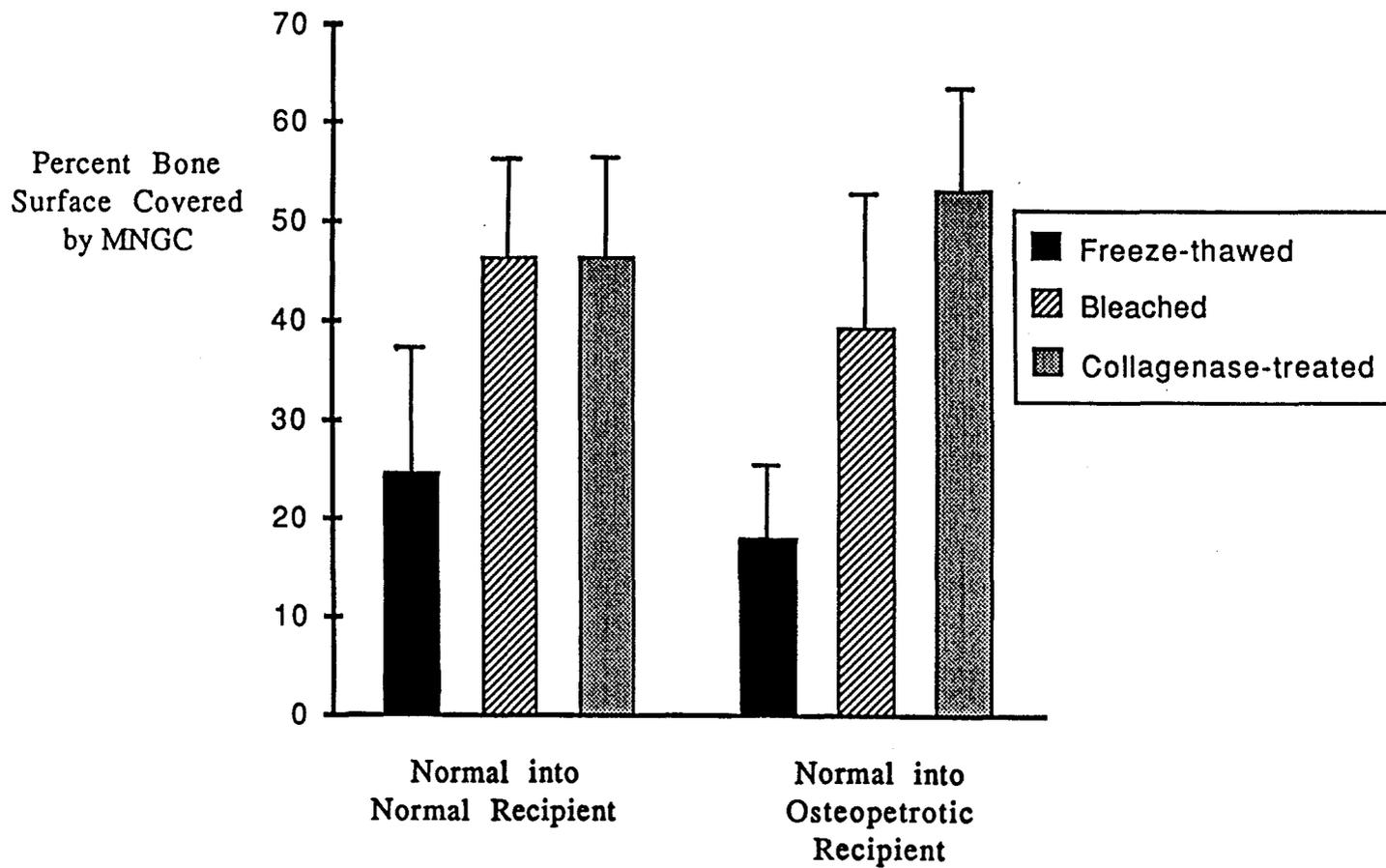


Figure 4.

At 3 days, mononuclear cells adhering to the bone (b) surface.

x 400

- a) Freeze-thawed bone into normal rat
- b) Freeze-thawed bone into ia rat
- c) Collagenase-treated bone into normal rat
- d) Collagenase-treated bone into ia rat

Figure 5.

Electron micrograph of mononuclear cells surrounding a devitalized bone (b) at three days in an ia rat. Note the extensive interdigititation with nearby mononuclear cells.

x 10,000

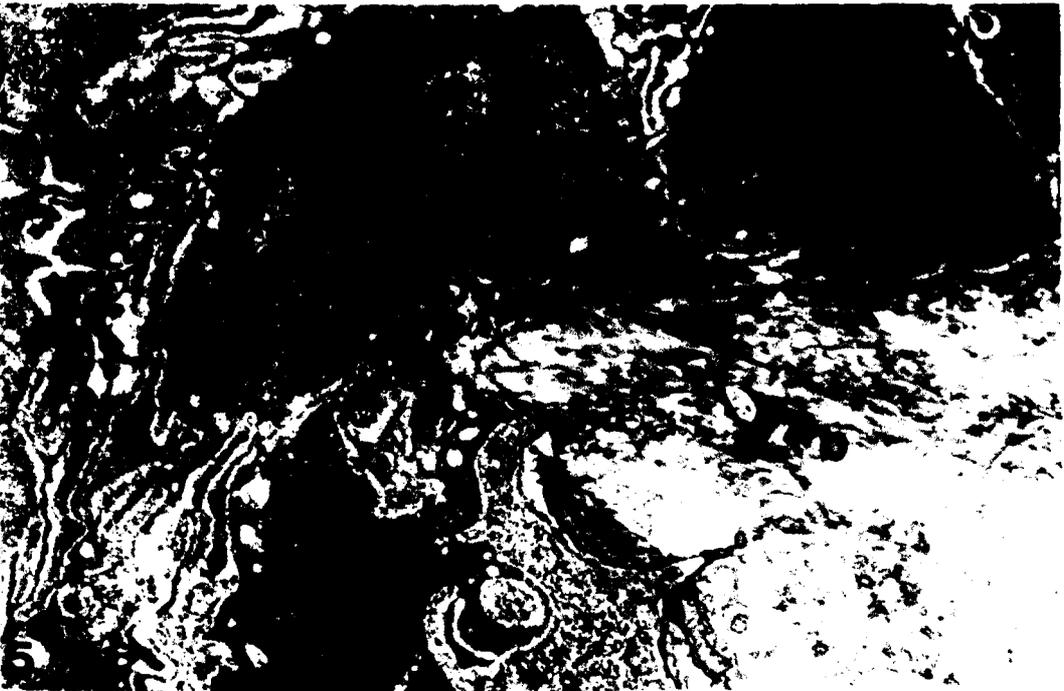


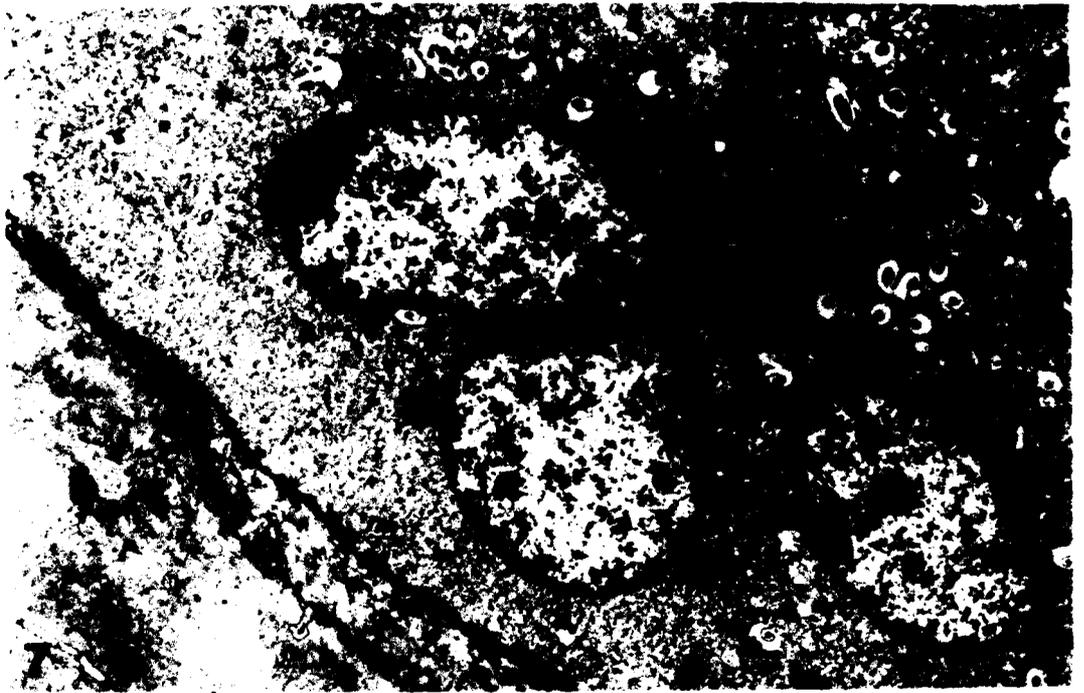
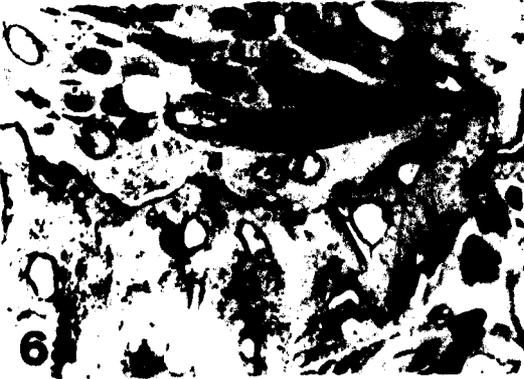
Figure 6.

MNGC's (arrows) adjacent to the bone (b) at 14 days. x 400

- a) Collagenase-treated bone into normal rat
- b) Collagenase-treated bone into ia rat. A vacuolated cytoplasm is present at the bone-cell interface.

Figure 7.

At 14 days, MNGC contacting a bleached bone (b) surface in a normal rat. The MNGC shows no plasma membrane specializations at its surface in contact with the bone. x 6100



CHAPTER IV

CELLULAR RESPONSE TO ECTOPICALLY IMPLANTED
SILK SUTURES AND OSTEOPETROTIC BONE

ABSTRACT

Faulty osteoclasts, characteristic of the ia rat mutation of osteopetrosis, cause a bone-resorbing defect which results in the persistence of immature, highly mineralized bone matrix. We implanted osteopetrotic (ia) bone subcutaneously into normal and ia rats to determine if ia bone could induce functionally active and morphologically identifiable osteoclasts at the implant surface. Results of functional studies, ^{45}Ca assays, showed that normal and ia recipients were capable of equivalent cell-mediated label release over a 2-week implant period, indicating that the ia resorptive defect was not reproduced at this ectopic site. Osteopetrotic freeze-thawed bone demonstrated a 2-fold increase in ^{45}Ca release over that of normal bone. This difference was eliminated by collagenase treatment. Cells attracted to bone and suture implants were subjected to light and electron microscopic examination. Cellular profiles were similar in both normal and ia animals regardless of the implant preparation. At 3 days, both bone and suture were surrounded by mononuclear cells. By 14 days, multinucleated cells were observed at the implant surfaces. Morphological comparison of the implant-induced multinucleated cells and tibial osteoclasts indicated that bone-elicited multinucleated cells did not exhibit ruffled borders characteristic of the active osteoclast and more closely resembled suture-induced macrophage polykaryons. We conclude that ectopically implanted ia bone elicits a

different functional response as compared to normal bone from structurally similar cell populations. Morphologically, bone-elicited multinucleated cells could not be classified as active osteoclasts despite evidence that ^{45}Ca release had occurred. Thus, the label release which occurred was probably due to the action of mononuclear phagocytes and macrophage polykaryons rather than to osteoclastic resorption.

INTRODUCTION

Osteopetrosis is a disease characterized by excessive bone accumulation (Marks and Walker 1976). The primary defect in the incisors-absent (ia) rat mutation of osteopetrosis is reduced bone resorption due to non-functional osteoclasts (Marks 1973). Osteoclasts from ia rats do not form ruffled borders or release normal amounts of lysosomal enzymes. In addition, ia osteoclasts have extensive clear zones at the bone-cell interface, which may prevent enzymatic contact with the bone surface (Marks 1983). As a result, bone resorption is reduced, normal remodeling does not take place, and a generalized skeletal sclerosis occurs (Marks 1984). Biochemically, deficiencies in remodeling are expressed as alterations in matrix composition (Boskey and Marks 1985). Metaphyseal bone isolated from ia rats, exhibits elevated hexosamine levels as compared to normal age-matched controls, an indication of persistent cartilage within the

bone. The ia metaphyseal bone also contains a higher mineral content than its normal counterpart. Similar, though much less significant findings were noted between normal and ia calvarial bone (Boskey and Marks 1985).

In previous studies involving the subcutaneous implantation of calvarial bone chips into normal and osteopetrotic (ia) rats (Walters and Schneider 1985), we examined the premise that the multinucleated giant cells (MNGC's) elicited by the implanted bone matrix were cells equivalent to osteoclasts and, therefore, are appropriate investigational surrogates for the study of osteoclastic lineage and function (Teitelbaum and Kahn 1980; Holtrop et al. 1982). Our results indicated that at this ectopic site, calvarial bone chips were unable to elicit morphologically identifiable osteoclasts despite evidence that bone resorption had occurred.

The present series of experiments was designed to determine if ia trabecular bone isolated from the tibial metaphysis could provide a more suitable substrate for the induction of osteoclast-like cells on the basis of its altered matrix composition and elevated mineral content. We assessed the results of ia versus normal metaphyseal bone implantation by examining the functional responses and morphological characteristics of the cells found adjacent to the bone implants. Because contact with bone mineral may be necessary to induce and activate osteoclasts (Chambers et al. 1984), we evaluated the effects of increased mineral exposure on this implant system by incubating

some of the bone fragments with collagenase prior to implantation (Green et al., 1985). The formation of macrophage polykaryons (MK's) was also induced in the recipient animals by the introduction of silk suture into adjacent subcutaneous sites, permitting comparison of the morphological characteristics of tibial osteoclasts from the metaphyseal area, suture-induced macrophage polykaryons, and bone-elicited multinucleated giant cells from the same recipient animal. This comparison was done to reveal any similarities or differences in the structures of these three categories of multinucleated cells which may be important in defining the relationship between them. In addition, implantation into both normal and ia rats was performed to determine if the usual osteopetrotic resorptive defect was mimicked at this subcutaneous site and if present, was it accompanied by morphological differences between bone-elicited multinucleated giant cells similar to those found between normal and ia osteoclasts. If both the resorptive defect and morphological discrepancies could be duplicated at this site, these findings would help define the nature of the bone-elicited multinucleated giant cells and support their use as appropriate investigational surrogates for the study of osteoclastic lineage and function.

MATERIALS AND METHODS

Animals

Rats were obtained from our colony bred to maintain the osteopetrotic (ia) mutation. Breeding groups were established to produce litters of ia/ia and ia/+ genotypes. The former can be identified on the tenth day after birth by failure of eruption of the incisors (Greep 1941). The latter are phenotypically identical with +/+ rats of this stock (Marks 1973) and were used as normal controls.

Calcium-release assay

Radioactively labeled bone chips were prepared in the following manner. Seven-day-old normal and osteopetrotic (ia) rats were injected with $40\mu\text{Ci } ^{45}\text{Ca}$ (in 0.1ml distilled water). To insure adequate label uptake, the injections were repeated at ten days of age. Three days later, the animals were killed by ether inhalation, the tibiae removed and the adherent soft tissues stripped. The bone marrow was extracted by repeated flushing with distilled water. Each tibia was divided, retaining the proximal metaphysis and a small portion of the adjacent diaphysis, but excluding the epiphysis. The tibiae were devitalized by freeze-thawing x3 in liquid nitrogen and sonication in distilled water for 7 minutes. Tibiae designated for mineral-exposed matrix studies were incubated in 5mg/ml crude collagenase (Lot #113F-6831, 245 U/mg dry wt collagenase, .10 U/mg dry

wt clostripain, <.01 U/mg dry wt trypsin, 85 U/mg dry wt neutral protease, Sigma, St. Louis, Mo.) in phosphate buffered saline for 2 hours at 37°C followed by thorough rinsing. All tibiae were bisected resulting in one pair of bone chips per tibia. The chips were dried at 60°C for 20 minutes and then weighed. After overnight exposure to ultraviolet light, the four types of bone chip pairs (ia freeze-thawed, ia collagenase-treated, normal freeze-thawed, and normal collagenase-treated) were allocated to experimental groups.

Group 1: uniformity of labeling within each bone chip pair

Each member of the bone chip pair was digested in an individual vial with 0.5 ml concentrated formic acid at 100°C for 30 minutes. After cooling, 10ml of aqueous scintillation cocktail was added to each vial and radioactivity assessed by liquid scintillation counting. Comparison of the ⁴⁵Ca content (counts/mg) of the members of each bone chip pair was performed to determine the uniformity of ⁴⁵Ca label within the pair.

Group 2: diffusion chamber implants

Materials to be used in preparation of the diffusion chambers were sterilized by overnight exposure to ultraviolet light. A 0.45um Millipore filter was glued to one side of a 13-mm plexiglass O ring. One half of each bone chip pair was placed in the chamber, after which the chamber was completed by gluing a second Millipore filter to the O

ring. Two to four chambers per animal were implanted into the dorsal subcutaneous tissue of (7) four week old normal and (9) ia rats. The remaining bone chip half was digested and the ^{45}Ca content was determined. Two weeks following implantation the chambers were removed and their contents were examined by light microscopy to determine whether they had remained intact and cells had been excluded. Those chambers which were not intact were excluded from this study. The devitalization process was repeated, the bone chips were digested, and ^{45}Ca content determined. The implant ^{45}Ca content was then compared to that of the control chip (corrected for natural radioactive decay over the 2 week period).

Group 3: direct bone chip implantation

One member each of 3 normal freeze-thawed (+ F-T) and 3 normal collagenase-treated (+ C-T) bone chip pairs was implanted directly into the dorsal subcutaneous tissue of a four week old ia or normal rat. The same procedure was followed using ia freeze-thawed (ia F-T) and ia collagenase-treated (ia C-T) bone chip pairs. A total of 4 rats (2 ia and 2 normal) received implants. The remaining control tibial half was digested and the ^{45}Ca content determined. Two weeks after implantation, the bone chips were recovered, devitalized, digested, and the ^{45}Ca content measured. Comparison of the ^{45}Ca content of the calculated control versus the implant was done as in the diffusion chamber studies.

Morphology

Two each of nonradioactive freeze-thawed and collagenase-treated bone chips, prepared from 13 day old normal and ia rat tibia as previously described, were implanted into the dorsal subcutaneous tissue of 6 ia and 6 normal rats. In addition small pieces of multiple strand silk suture (5-0, ETHICON, INC.) were also implanted. At 3, 7, and 14 days postimplantation, bone chips, suture, and the metaphyseal portions of recipient tibia were recovered from 2 ia and 2 normal rats, fixed in 2.5% glutaraldehyde in cacodylate buffer (pH 7.2) for 2 hours, decalcified in 10% EDTA (pH 7.3) at 4°C for 48 hours, postfixed in 1% OsO₄ for 1 hour, dehydrated in ethanol, and embedded in Epon. The tissue was sectioned at 2 um, stained with toluidine blue, and evaluated by light microscopy. Areas warranting further investigation were thin sectioned and stained with uranyl acetate and lead citrate for viewing with the Hitachi H600 electron microscope.

Morphometric analysis

Sixty-seven individual fields from 14 day old implanted bone chip sections were photographed, magnified to 670x, and analyzed to determine the percentage of bone surface lined by multinucleate giant cells (MNGC's). All measurements were taken with a BQ CAM computer aided morphometry image analysis system (r and m Biometrics Corp.,

Nashville, Tn.). Percentage of bone length lined by MNGC's per field was calculated as follows:

$$\frac{\text{Length of bone covered by MNGC's}}{\text{Total length of bone surface}} \times 100 = \%$$

The statistical comparison of these percentages was performed using the unpaired Student's t-test.

RESULTS

Uniformity of label within bone chip pairs

Tables 1 and 2 record the results of our comparison of label uniformity between corresponding members of each bone chip pair (counts/mg). The mean variation in ^{45}Ca content for each category of bone preparation was calculated from each group's individual percent errors. Mean variations in label of $4.8\% \pm 0.7\%$ for normal and $2.2\% \pm 1.6\%$ for ia freeze-thawed pairs and $3.2\% \pm 1.9\%$ and $5.1\% \pm 2.3\%$ for normal and ia collagenase-treated pairs respectively were determined. This degree of uniformity in label content between members of individual pairs allowed their use as control and implant chips in subsequent experiments.

Diffusion chamber implants

These studies were performed to assess the amount of in vivo non-cell mediated ^{45}Ca release occurring over a 2 week implantation

period for each bone chip preparation. The results (Fig. 1) were tabulated from chambers found by light microscopic examination to be cell-free. There was a mean $4.2\% \pm 1.6\%$ ^{45}Ca loss from + F-T bone chips, and a mean decrease of $7.18\% \pm 4.4\%$ from ia F-T chips, while +C-T and ia C-T had mean label releases of $7.2\% \pm 1.8\%$ and $3.4\% \pm 2.5\%$ respectively over the same implant period.

Direct implantation studies

Figure 2 illustrates the results of the direct implantation studies. Normal recipients responded to + F-T and + C-T bone chips with mean ^{45}Ca losses of $11.0\% \pm 2.7\%$ and $30.4\% \pm 11.4\%$, respectively. Osteopetrotic recipients showed similar ^{45}Ca releases from + F-T ($12.8\% \pm 3.8\%$) and + C-T ($32.1\% \pm 3.7\%$) implants. Implantation of freeze-thawed ia tibial bone resulted in mean label decreases of $23.8\% \pm 4.7\%$ in normal recipients and $25.5\% \pm 3.7\%$ in ia rats while ia collagenase-treated implants showed label losses of $30.8\% \pm 9.4\%$ in normal animals and $41.5\% \pm 10.1\%$ in osteopetrotic recipients. The mean percent ^{45}Ca release reflects the total label loss from the implanted bone chips over the 2 week implant period. This percentage minus the appropriate non-cell mediated ^{45}Ca release from diffusion chamber studies reflects the net amount of cell mediated ^{45}Ca loss. These results show that the amount of ^{45}Ca released from direct subcutaneous implantation exceeded that of the non-cell mediated baseline, indicating that a large portion of the label release from

the direct implants was due to a cell-mediated process rather than a passive diffusion event. Both normal and osteopetrotic recipients were capable of equivalent ^{45}Ca releases, implying that the osteopetrotic resorptive defect was not mimicked at this ectopic site. In addition, both types of recipients showed increased label release from collagenase-treated bone implants over that of freeze-thawed implants. Figure 2 also illustrates that both types of recipient animals responded to freeze-thawed osteopetrotic bone with a two-fold increase in ^{45}Ca release over that of normal freeze-thawed implants. This difference was eliminated by collagenase treatment.

Morphometric analysis

Because normal and ia recipients showed similar functional responses to + freeze-thawed bone implants, the individual percentages of bone length lined by MNGC's for each field were summated and a mean \pm S.E. determined. This procedure was also followed for the ia freeze-thawed and collagenase-treated implants. Normal freeze-thawed implants showed a mean of $38.9\% \pm 2.6\%$ bone length covered by MNGC's, while ia freeze-thawed and collagenase-treated demonstrated means of $45.6\% \pm 2.1\%$ and $45.6\% \pm 2.7\%$ respectively(Fig. 3). There was a tendency for both ia freeze-thawed and collagenase-treated implants to be more extensively covered by MNGC's when compared to normal freeze-thawed implants ($P = .05$). No significant difference between the

percentage of bone length covered by MNGC's was noted between ia freeze-thawed and collagenase-treated bone implants.

Morphology

Osteoclasts, from normal recipient tibiae at 3, 7, and 14 days post-implantation, were identified as multinucleated cells demonstrating a striated border with associated vacuolation of the cytoplasm adjacent to the bone surface (Fig. 4a). In osteopetrotic (ia) osteoclasts, the striated region and vacuolation were reduced or lacking (Fig. 4b). Ultrastructurally, at the bone-cell interface, normal active osteoclasts displayed an area of extensive cytoplasmic infolding called the ruffled border which was surrounded by an organelle-free region, the clear zone (Fig. 5a). Osteopetrotic osteoclasts lacked ruffled borders, but did exhibit extensive clear zones along the bone surface (Fig. 5b).

Implanted suture and bone matrices were recovered at 3, 7, and days. At 3 days, the suture and bone implants were surrounded by a variety of mononuclear cell types (Figs. 6a and b), predominately neutrophils and monocyte-macrophages. At the transmission electron microscope level, both neutrophils and macrophages were observed in tissues surrounding the implants, however, macrophage-like mononuclear cells were most frequently found adjacent to the implant surface. These cells possessed irregularly shaped nuclei with a thin rim of heterochromatin distributed along the nuclear envelope and a prominent

nucleolus. The cytoplasm contained the usual complement of organelles which included numerous lysosome-like bodies and what appeared to be phagosomes. The non-bone apposing surfaces of these cells were often thrown into elaborate cytoplasmic folds that interdigitated with those of adjacent cells (Fig. 7).

By 7 days, the connective tissue surrounding the implants had organized into a fibrous capsule. There was a marked decrease in the number of neutrophils present on and around the implants. Although mononuclear cells similar to those seen at 3 days continued to be the predominate cell type found along both the bone and suture implant surfaces, occasional multinucleated cells were also seen at this time (Fig. 8a and b).

Fourteen days post-implantation, multinucleate giant cells (MNGC's) could be found covering much of the implanted bone and suture. The bone-elicited MNGC's were often seen to occupy shallow depressions on the bony surface. The 14 day MNGC's were larger and contained more nuclei than did the MNGC's observed at 7 days. At the light microscope level in both bone and suture implants, two types of MNGC's could be distinguished on the basis of nuclear arrangement. In the first type of MNGC's, the nuclei were aligned at the periphery of the cell, whereas in the second type of MNGC's, the nuclei were more centrally located and grouped in clusters (Figs. 9a and b). Ultra-structurally, no difference, other than nuclear arrangement, could be noted between these two MNGC profiles. Both types of MNGC's contained

numerous mitochondria, ribosomes and variable amounts of rough endoplasmic reticulum (Fig. 10). Occasionally the MNGC's exhibited relatively organelle-free regions of cytoplasm at the implant surface. These areas contained large numbers of polyribosomes, but did not display the perpendicular arrangement of filamentous material typical of clear zones (Fig. 11). At no time was a ruffled border, characteristic of the actively resorbing osteoclast, observed within either bone-elicited MNGC's or suture-induced macrophage polykaryons (MK's). No morphological distinction could be made between the MNGC's found adjacent to bone or suture, regardless of the bone chip preparation or the type (normal or ia) of implant recipient.

DISCUSSION

This investigation was designed to assess the resorptive capabilities and morphological characteristics of the cells found adjacent to subcutaneously implanted trabecular bone, in general, and osteopetrotic (ia) trabecular bone, in particular. It also compared, on a structural basis, the osteoclast, the suture-induced macrophage polykaryon, and the bone-elicited multinucleated giant cell in an effort to define the relationship between these three multinucleated cells.

Results of the functional studies, ^{45}Ca release assays, show that both osteopetrotic and normal implant recipients were capable of equivalent label release over the two week implant period and that a large

portion of the observed label loss occurred as the result of a cell-mediated process. Quantitation of the amount of ^{45}Ca released from bone is commonly used as an index of bone resorption, therefore, the results of these studies, indicate that the reduced bone resorption typical of the ia rat was not duplicated at this ectopic site by trabecular bone implants. Generally, both ia and normal rats responded to mineral-exposed bone (C-T) implants with increased label releases over that of osteoid-exposed (F-T) implants. These findings are in agreement with our previous studies using calvarial bone as the implant substrate (Walters and Schneider 1985). The functional response of the recipient animals to ia F-T trabecular bone, however, differed from that observed in ia F-T calvarial bone studies in which similar amounts of ^{45}Ca were released from +F-T and ia F-T calvarial bone implants. In the present investigation both ia and normal animals responded to ia F-T bone with almost two-fold increases in label release over that of + F-T bone. The increase in ^{45}Ca release may reflect the significantly higher mineral content of osteopetrotic trabecular bone over that of normal trabecular bone (Boskey and Marks 1985). Morphometric analysis demonstrated that ia F-T and collagenase treated implants had similar percentages of bone length covered by MNGC's profiles while + F-T implants tended to have less total length lined by MNGC's. These data suggest that mineral exposure may be a factor in the induction of MNGC's at the bone surface and that ia F-T and collagenase-treated bone may share similar patterns of increased

mineral exposure. The elevation in ia trabecular bone mineral content may be expressed as a greater amount of surface-exposed mineral when compared to normal trabecular bone which has undergone mineral removal during remodeling. Exposed bone mineral appears to provide a stimulus that is capable of initiating bone resorptive behavior in the normal osteoclast (Chambers et al. 1984; Chambers and Fuller 1985). The activated osteoclast is then believed to resorb the bone located in the mineral-exposed area. Osteopetrotic (ia) osteoclasts, though recruited to the mineral-exposed surface, are faulty and unable to resorb the bone effectively, resulting in greater amounts of surface-exposed mineral in osteopetrotic trabecular bone. Osteopetrotic bone may more closely resemble collagenase-treated bone which also expresses greater than normal surface-exposed mineral (Green et al. 1985; Chambers et al. 1985), providing an explanation for the relatively high levels of ^{45}Ca release from ia F-T bone which approach that of collagenase-treated bone. The discrepancy in mineral content is much less apparent between normal and ia calvarial bone (Boskey and Marks 1985) which may account for the difference in results obtained in our previous study.

Morphologically, the ia and normal cell populations found adjacent to the bone and suture surfaces appeared to be the same. The suture-induced macrophage polykaryons (MK's) and bone-elicited multinucleated cells (MNGC's) displayed similar morphological features at the light and electron microscope levels. At no time did the bone-

elicited MNGC's exhibit ruffled borders, characteristic of active osteoclasts, despite evidence, ⁴⁵Ca release assays, that bone mineral had been removed from the bone implants; nor did the bone-elicited MNGC's demonstrate the extensive clear zones typically observed in the ia osteoclast. Based on their structural similarities to suture-induced MK's and their lack of definitive osteoclastic morphology, bone-elicited MNGC's could not be classified as osteoclasts, but may instead represent macrophage polykaryons like those formed in response to the chronic inflammatory stimulus provided by the implanted silk suture (Van der Rhee et al. 1979). Macrophages and monocytes have been found to respond chemotactically to the products of normal bone resorption and components of bone matrix (Mundy et al. 1978; Malone et al. 1982) and appear capable of bone resorption in vitro (Mundy et al. 1977; Kahn et al. 1978; Teitelbaum et al. 1979). Bone-elicited macrophage polykaryons or other members of the mononuclear phagocyte system do appear to be capable of cell-mediated bone resorption in our implant system as well as others. Devitalized bone powder implanted into calvarial defects (Glowacki et al. 1981) induced the formation of multinucleated cells around the bone fragments. As in our study, ultrastructural assessment of these multinucleated cells failed to reveal the presence of ruffled borders in areas of bone-cell contact despite morphometric evidence that bone resorption had taken place. These findings imply that members of the mononuclear phagocyte system (MPS), like osteoclasts, are capable of bone resorption in vivo, but

that MPS-mediated osteolysis may occur through a different mechanism than that of osteoclastic-mediated resorption. Osteoclasts, but not macrophages or their derivatives, form ruffled borders at the bone surface. Osteoclasts do not exhibit all of the same enzyme or receptor characteristics of monocytes and macrophages, nor do they respond to the same hormonal stimuli (Marks 1983; Horton et al. 1984). Members of the mononuclear phagocyte family placed on a smooth bone surface failed to demonstrate the resorption lacunae typical of the active osteoclast, as detected by scanning electron microscopy (Chambers and Horton 1984). Burger et al. (1984) in their in vitro studies of osteoclastic lineage, found that functional osteoclasts could only be formed from precultured bone marrow mononuclear cells in the presence of live, but not devitalized bone, and that only live bone was resorbed by osteoclasts. Devitalized bone, placed into the cultures, was resorbed, but according to the authors, most probably by mature macrophages which comprised 50% of the bone marrow mononuclear cells. They concluded that this type of bone resorption was different from osteoclast-mediated resorption. These data, therefore, appear to provide evidence in support of a difference in the mechanisms involved in MPS-mediated versus osteoclastic-mediated bone resorption.

In summary, we were able to conclude the following from our present investigation of the MNGC's found adjacent to ectopically implanted bone matrix and suture:

- 1) Ectopically implanted freeze-thawed osteopetrotic bone elicits a different functional response when compared to normal bone from the cells recruited to the implant surface.
- 2) The ia and normal cell populations surrounding the bone and suture implants appear to be morphologically identical, regardless of the bone chip preparation.
- 3) Osteopetrotic (ia) and normal animals are capable of equivalent cell-mediated ^{45}Ca releases over the two week implant period, indicating that the typical reduction in bone resorption seen in this osteopetrotic mutation is not reproduced at this ectopic site.
- 4) The MNGC's found adjacent to the implanted bone surface can not be classified as active osteoclasts, despite evidence of ^{45}Ca release, but more closely resemble the macrophage polykaryons induced by the implanted suture.
- 5) Based on (3) and (4), the cell-mediated ^{45}Ca release which occurred in this study is probably the result of action by members of the mononuclear phagocyte system rather than from true osteoclastic activity.

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Table 1. Uniformity of ^{45}Ca label within freeze-thawed bone chip pairs

<u>Bone chips</u> ¹	<u>Weight(mg)</u>	<u>Counts/mg</u>	<u>Mean</u> ²	<u>Percent error</u> ³
Normal				
1	3.0	154,975	159,842 \pm 6,882	4.3
	3.3	164,708		
2	2.4	170,745	165,983 \pm 6,734	4.1
	3.5	161,221		
3	1.8	190,296	184,915 \pm 7,610	4.1
	2.3	179,534		
4	4.3	177,402	170,867 \pm 9,243	5.4
	2.6	164,331		
5	3.0	202,678	195,319 \pm 10,407	5.3
	3.0	187,960		
6	3.2	117,156	112,642 \pm 6,384	5.7
	3.1	108,128		
Osteopetrotic				
1	3.6	167,736	168,984 \pm 1,764	1.0
	3.7	170,231		
2	3.4	170,390	168,583 \pm 2,555	1.5
	2.8	166,776		
3	4.6	156,822	161,026 \pm 5,945	3.7
	2.1	165,229		
4	2.9	283,712	280,949 \pm 3,908	1.4
	1.9	278,185		
5	3.7	128,859	133,210 \pm 6,153	4.6
	3.3	137,561		
6	2.4	284,333	282,998 \pm 1,888	0.7
	2.2	281,663		

¹Samples taken from the tibia of 13-day-old rats.

²Mean \pm standard deviation in counts/mg.

³Percent error = (standard deviation \times 100)/mean.

Table 2. Uniformity of ^{45}Ca label within collagenase-treated bone chip pairs

<u>Bone chips</u> ¹	<u>Weight(mg)</u>	<u>Counts/mg</u>	<u>Mean</u> ²	<u>Percent error</u> ³
Normal				
1	3.7	116,000	115,252 \pm 1,058	0.9
	1.9	114,504		
2	2.4	178,326	184,431 \pm 8,634	4.7
	3.5	190,536		
3	2.8	204,564	203,747 \pm 1,156	0.6
	2.8	202,929		
4	2.3	188,784	182,752 \pm 8,531	4.7
	3.1	176,719		
5	3.0	181,601	187,740 \pm 8,682	4.6
	2.3	193,879		
6	2.4	191,851	186,883 \pm 7,027	3.8
	2.7	181,914		
Osteopetrotic				
1	5.9	184,557	195,127 \pm 14,948	7.7
	1.8	205,696		
2	4.6	191,875	200,077 \pm 11,599	5.8
	2.8	208,279		
3	4.4	202,761	210,842 \pm 11,428	5.4
	2.9	218,922		
4	4.2	195,189	196,222 \pm 1,461	0.7
	3.5	197,255		
5	2.1	210,661	202,247 \pm 11,899	5.9
	6.1	193,833		
6	4.3	218,407	210,926 \pm 10,580	5.0
	3.0	203,444		

¹Samples taken from the tibia of 13-day-old rats.

²Mean \pm standard deviation in counts/mg.

³Percent error = (standard deviation x 100)/mean.

Figure 1.

Mean percent + S.D. ⁴⁵Ca release over a 2 week period from freeze-thawed and collagenase-treated normal or osteopetrotic bone chips in diffusion chambers implanted into 4 week old normal and ia rats.

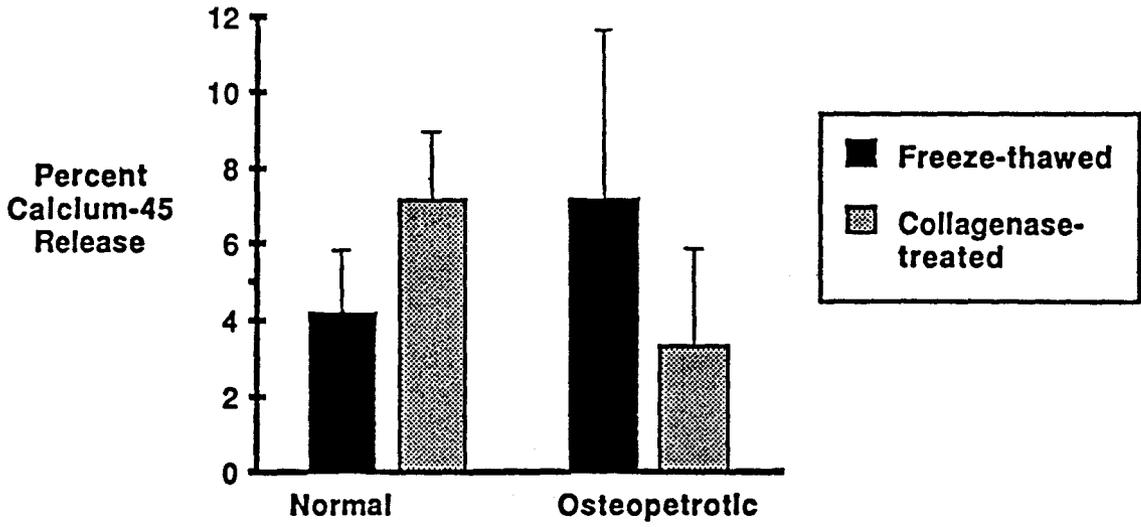


Figure 2.

Mean percent \pm S.D. ^{45}Ca release over a 2 week period from freeze-thawed and collagenase-treated normal or osteopetrotic bone chips implanted into normal and ia rats.

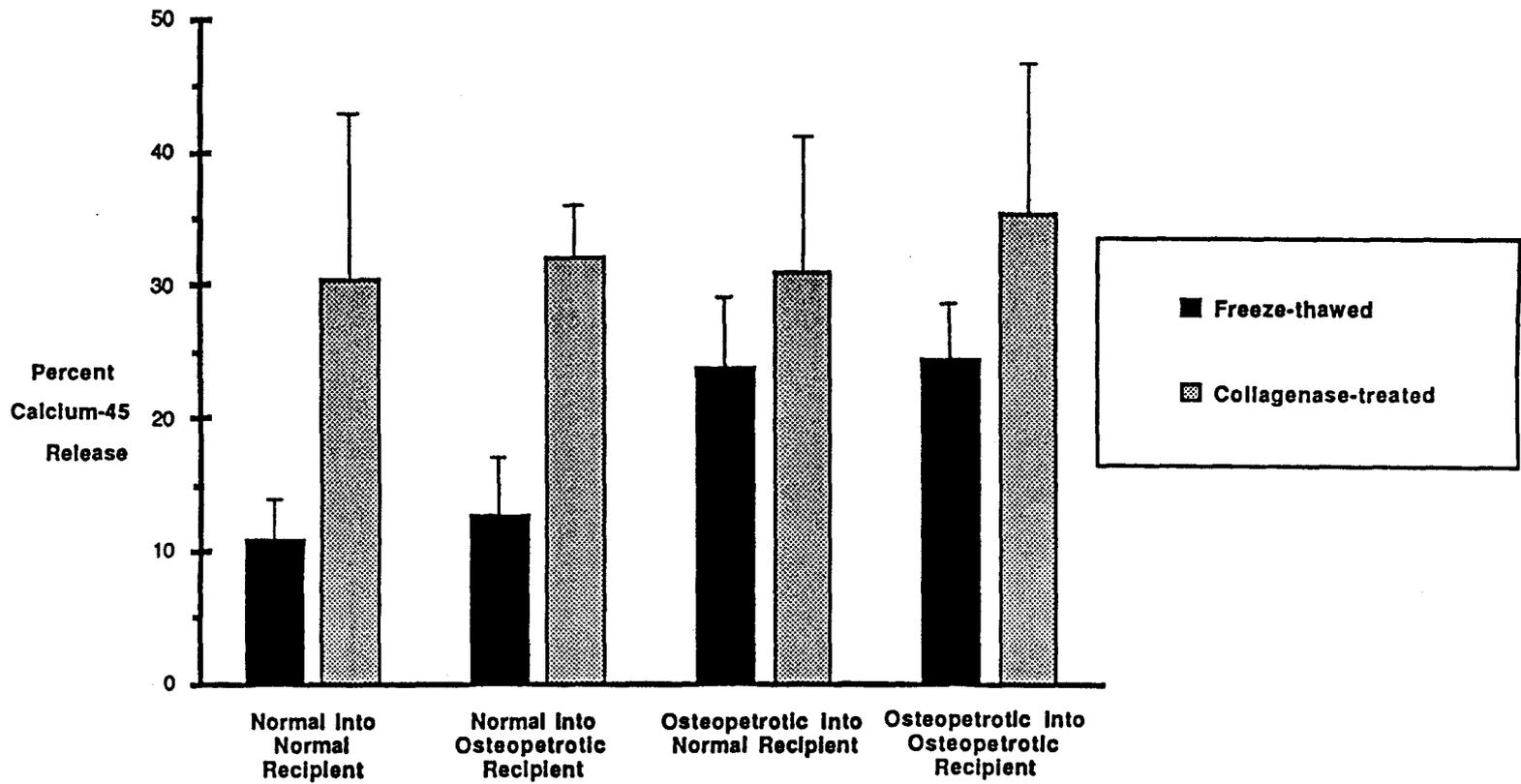


Figure 3.

Mean percent \pm S.E. of total bone length covered by multinucleated giant cells (MNGC's) from normal (+) freeze-thawed, ia freeze-thawed, and collagenase-treated bone chips 2 weeks after implantation into normal and ia rats.

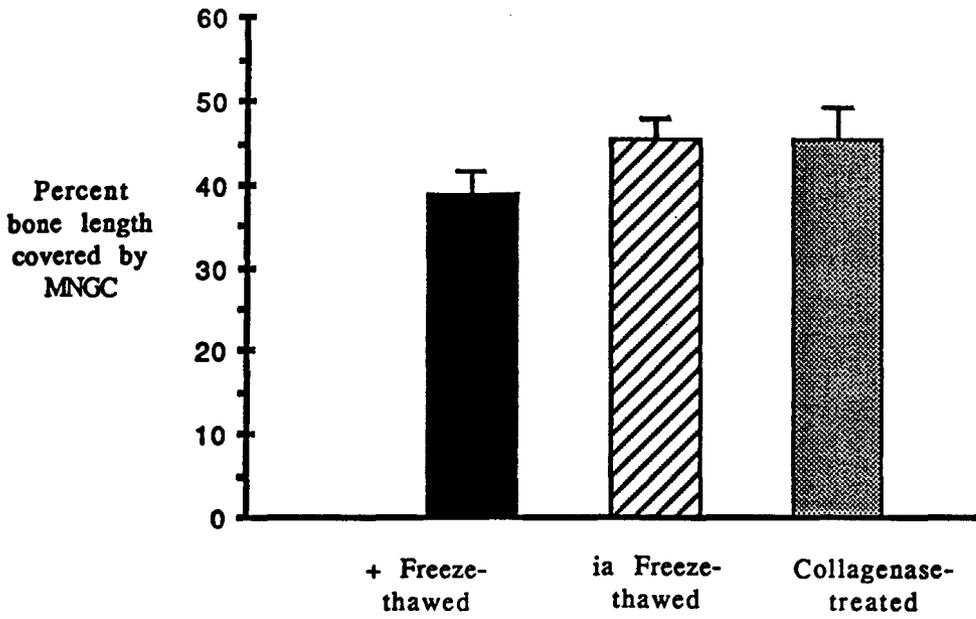


Figure 4.

In situ osteoclasts adjacent to bone from normal and ia recipients 3 days post-implantation. Original magnification x 100
Bar = 10um

- a. Normal osteoclast (arrows) exhibiting a striated border with associated vacuoles at the bone surface.
- b. Osteopetrotic osteoclasts (arrows) lacking specializations at the bone-cell interface.

Figure 5.

Electron micrographs of normal and osteopetrotic osteoclasts from recipient rats 3 days after implantation.

- a. Normal osteoclast displaying a ruffled border (arrows) surrounded by a clear zone (c) at the bone (b) surface. Original magnification x 5000
Bar = 1um
- b. Osteopetrotic osteoclasts with an extensive clear zone (arrows) adjacent to the bone (b) matrix.
Original magnification x 8000 Bar = 1um



Figure 6.

Mononuclear cells adhering to bone and suture implants at 3 days post-implantation. Original magnification x 100 Bar = 10um

a. Suture (s) implanted into an ia rat.

b. Freeze-thawed osteopetrotic bone (b) into a normal rat.

Figure 7.

Electron micrograph of mononuclear cells surrounding a freeze-thawed normal bone (b) at 3 days after implantation into an ia rat. Note the extensive interdigitation with adjacent mononuclear cells. Original magnification x 5000 Bar = 1um

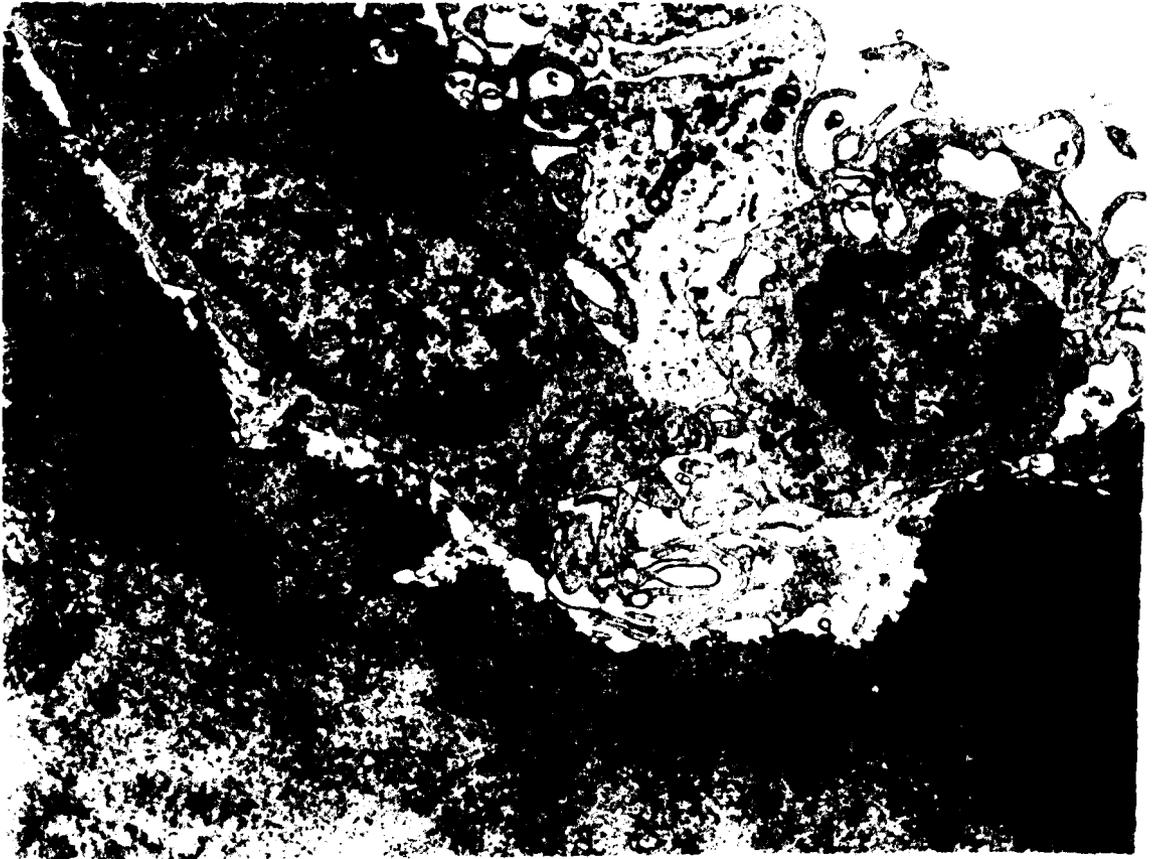
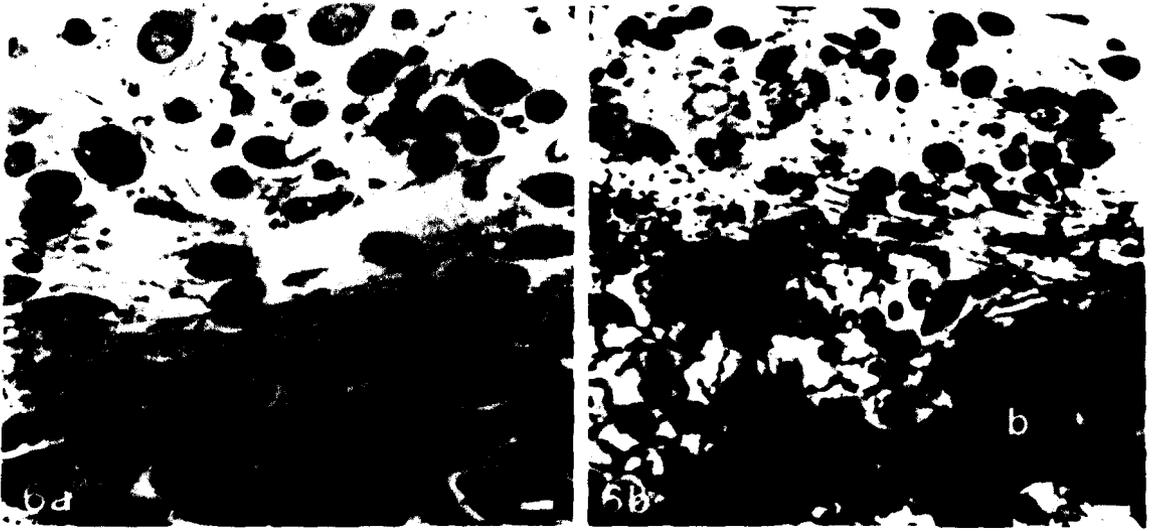


Figure 8.

Multinucleated cells (arrows) at bone and suture surface 7 days post-implantation. Original magnification x 100 Bar = 10um

- a. Suture (s) into normal rat.
- b. Collagenase-treated osteopetrotic bone (b) into ia rat.

Figure 9.

At 14 days, multinucleated giant cells (arrows) covering much of the suture and bone implants. Original magnification x 100

Bar = 10um

- a. Suture (s) into ia rat.
- b. Freeze-thawed normal bone (b) into a normal rat.

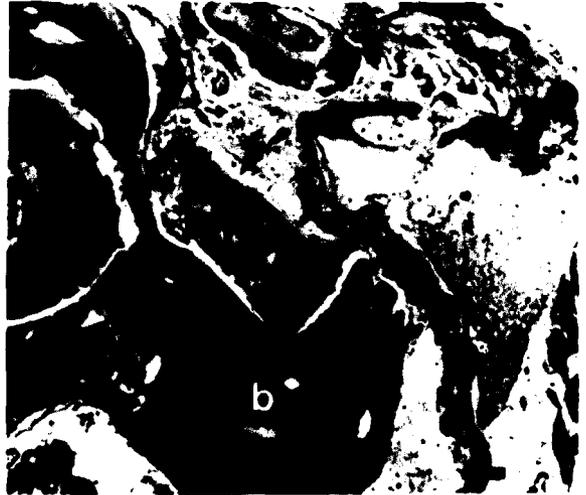
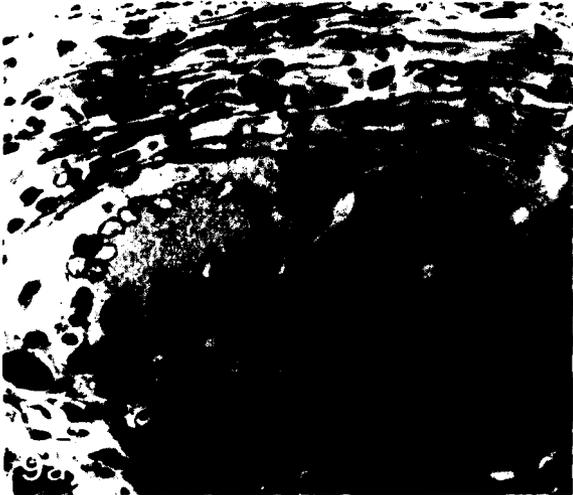
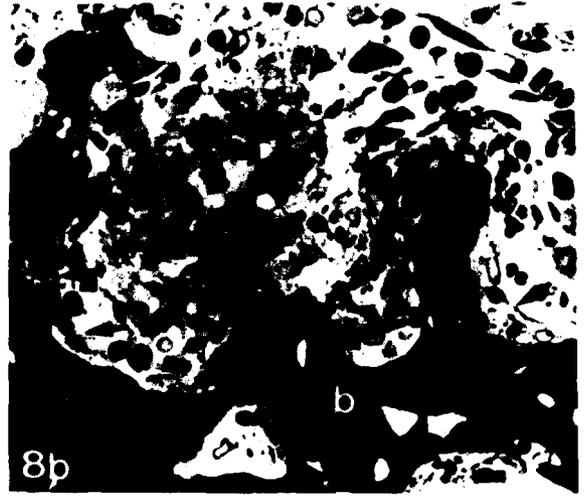
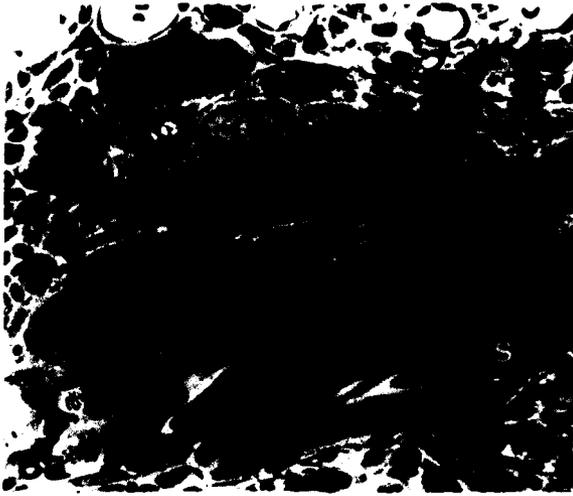
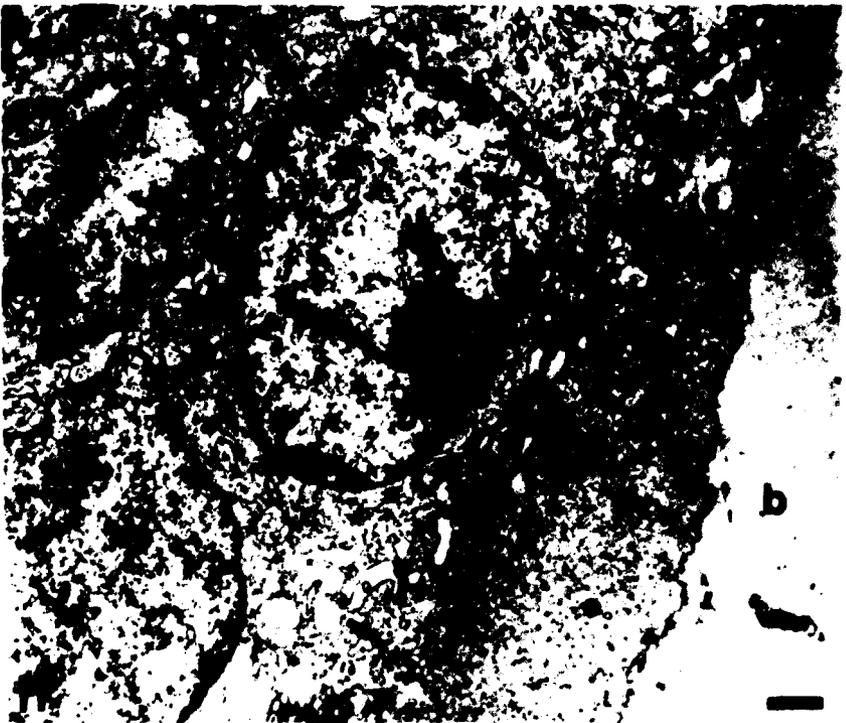
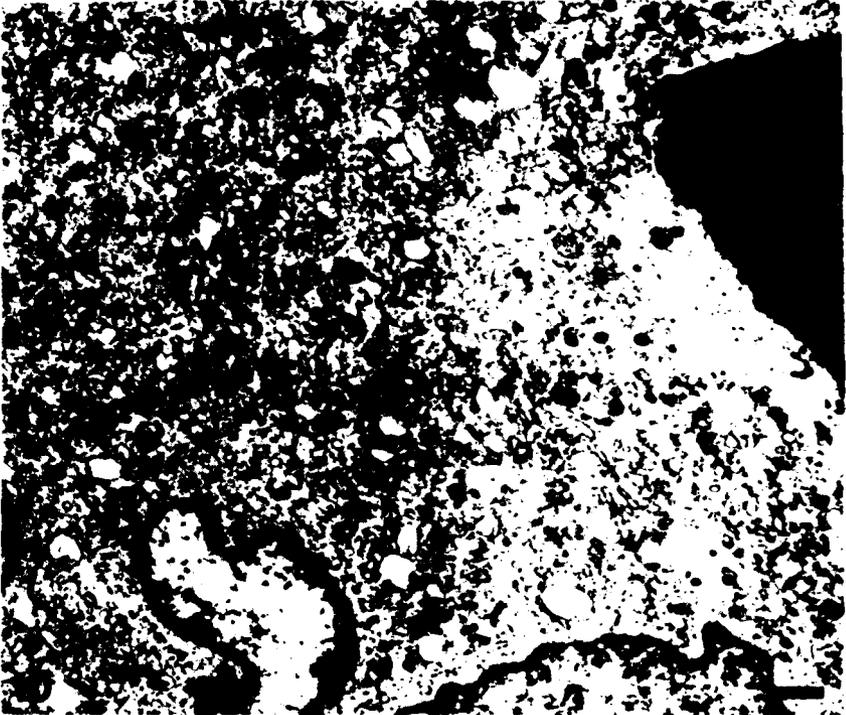


Figure 10.

Macrophage polykaryon adjacent to suture (s) implanted into normal rat 14 days after implantation. No plasma membrane specializations are present at its surface in contact with the suture. Original magnification x 3000 Bar = 1 μ m

Figure 11.

Bone-elicited multinucleated giant cell 14 days post-implantation adjacent to the freeze-thawed normal bone implant in a normal recipient. Note the relatively organelle-free region of cytoplasm (arrows) at the bone surface (b). Original magnification x 4900 Bar = 1 μ m.



CHAPTER V

TARTRATE-RESISTANT ACID PHOSPHATASE
ACTIVITY IN TIBIAL OSTEOCLASTS AND CELLS
ELICITED BY ECTOPIC BONE AND SUTURE IMPLANTS
IN NORMAL AND OSTEOPETROTIC RATS

ABSTRACT

Bone-induced multinucleated cells have been suggested as surrogates for the study of osteoclastic lineage and function. This study evaluates this proposal by comparing acid phosphatase localization in tibial osteoclasts with that of cell populations elicited by subcutaneous implantation of bone and suture into normal and osteopetrotic (ia) rats, emphasizing tartrate-resistant acid phosphatase, an osteoclastic marker. The ia rat mutation of osteopetrosis is characterized by defective osteoclasts which typically express enhanced TRAP activity when compared to normal; ia macrophage populations do not share the same osteoclastic defect and demonstrate normal amounts of acid phosphatase reactivity. The majority of the acid phosphatase activity expressed by implant elicited mononuclear cells was sensitive to tartrate. An increase in the percentage of tartrate-sensitive, but not TRAP-positive, mononuclear cells was observed during the 14-day implantation period, suggesting the mononuclear cells did not undergo osteoclastic differentiation. Both normal and ia osteoclasts contained high concentrations of TRAP reaction product while bone and suture-induced multinucleated cells examined at 14 days post-implantation were only mildly TRAP reactive. We conclude that devitalized bone matrix implanted at this ectopic site is capable of the formation of TRAP-positive multinucleated cells, but when compared on the basis of strength of TRAP activity, the bone-induced multi-

nucleated cells do not resemble active osteoclasts, but are similar to suture-elicited macrophage polykaryons. Therefore, we suggest caution in the use of bone-induced multinucleated cells as surrogates for the study of osteoclasts.

INTRODUCTION

Based on functional similarities, a proposed precursor relationship and ready availability for experimentation, macrophages, monocytes and macrophage polykaryons have been suggested as investigational surrogates for the study of osteoclastic differentiation and function (Teitelbaum and Kahn, 1980). This premise implies that monocytes and macrophages, when confronted with a bone matrix, are capable of fusion into multinucleated cells functionally and morphologically equivalent to the osteoclast. Such a proposal also equates the mechanism involved in macrophage-monocyte osteolysis with that of osteoclastic bone resorption.

One in vivo method used to examine the relationship between the osteoclast and the macrophage polykaryon involves implantation of mineralized bone matrix into a variety of ectopic sites. Ultrastructural assessment of the multinucleated cells generated in response to ectopically implanted bone matrix failed to identify them as osteoclasts despite evidence that cell-mediated bone resorption had

occurred (Holtrop et al., 1982; Walters and Schneider, 1985). In addition, morphological comparison of bone-induced multinucleated cells with suture-elicited macrophage polykaryons showed them to be structurally similar. At no time did either type of implant-induced multinucleated cell exhibit a ruffled border, characteristic of the active osteoclast (Walters and Schneider, 1986). The lack of demonstrable ruffled borders denies the equivalency of bone-induced multinucleated cells with osteoclasts and also implies that the osteolysis which occurred in this implant system may be the result of a different resorptive mechanism than that employed by the osteoclast.

Tartrate-resistant acid phosphatase (TRAP) has been proposed as an osteoclastic marker (Hammarstrom et al., 1971; Minkin, 1982; Chappard et al., 1983) and, as such, has been used to study osteoclastic differentiation in vivo (Baron et al., 1986) and in vitro (Jilka, 1986). In the present study we compared TRAP localization in cells found adjacent to subcutaneously implanted bone and suture with that of the osteoclast to determine if these three categories of multinucleated cells possess similar or distinctive TRAP profiles. Because exposure to bone mineral may be important in the induction and activation of osteoclasts (Chambers et al., 1985), we incubated some of the bone chips with collagenase prior to implantation (Green et al., 1985). Implantation into both normal and osteopetrotic (ia/ia) rats was performed, using normal and osteopetrotic bone. The bone resorbing defect in the ia osteopetrotic mutation is due to non-

functional osteoclasts that are unable to form ruffled borders or release normal amounts of lysosomal enzymes which accumulate in their cytoplasm (Marks, 1973). Osteoclasts from ia rats demonstrate increased acid phosphatase activity when compared to normals (Handelman et al., 1964; Schofield et al., 1974), most of this increase in enzyme activity appears to be tartrate-resistant (Hammarstrom et al., 1983). Reproduction of the intense pattern of TRAP localization within bone-induced multinucleated cells at this ectopic site in ia rats would lend support to the use of such cells for the study of osteoclastic lineage and function.

MATERIALS AND METHODS

Animals

Rats were obtained from our colony bred to maintain the osteopetrotic (ia) mutation. Breeding groups were established to produce litters of ia/ia and ia/+ genotypes. The former can be identified on the tenth day after birth by failure of eruption of the incisors (Greep, 1941). The latter are phenotypically identical with +/+ rats of this stock (Marks, 1973) and were used as normal controls.

Preparation and implantation of implant substrates

Tibial and calvarial bone chips were prepared in the following manner. Thirteen day old normal and osteopetrotic rats were killed by

ether inhalation, the tibiae and calvaria removed and adherent soft tissues and periosteum stripped. The bone marrow was extracted from the tibiae by repeated flushing with distilled water. Each tibia was divided, retaining the proximal metaphysis and a small portion of adjacent diaphysis, but excluding the epiphysis. Both tibiae and calvaria were devitalized by freeze-thawing x3 in liquid nitrogen and sonication in distilled water for 7 minutes. Following devitalization, tibiae and calvaria designated for mineral-exposed studies were incubated in 5 mg/ml crude collagenase (Lot #113F-6831, 245 U/mg dry wt collagenase, .10 U/mg dry wt clostripain, <.01 U/mg dry wt trypsin, 85 U/mg dry wt neutral protease, Sigma, St. Louis, Mo.) in phosphate buffered saline for 2 hours at 37°C. All tibiae were bisected resulting in one pair to bone chips per tibiae. Calvaria were divided into frontal, parietal and occipital sections; these were bisected along the midline suture, resulting in 3 pairs of bone chips per calvarium. The chips were dried at 60°C for 20 minutes after which they were exposed to ultraviolet light overnight. This resulted in 8 different bone chip preparations: 1a trabecular freeze-thawed, 1a calvarial freeze-thawed, 1a trabecular collagenase-treated, 1a calvarial collagenase-treated, normal trabecular freeze-thawed, normal calvarial freeze-thawed, normal trabecular collagenase-treated and normal calvarial collagenase-treated. One each of the 8 types of bone chip preparations was implanted into the dorsal subcutaneous tissue of 6 1a and 6 normal rats. In addition small pieces of sterile multiple

strand silk suture (5-0 black-braided, ETHICON, INC.) were also implanted into the same animals.

Fixation and embedding procedures

Except where indicated, all of the following procedures were conducted at 4°C. Bone chips, suture and the metaphyseal portions of recipient tibia were recovered from 2 ia and 2 normal rats at 3, 7, and 14 days post-implantation. The tissue was fixed for 2 hours in 2.5% cacodylate-buffered gluteraldehyde (pH 7.4) containing 7% sucrose, rinsed 3 times, stored overnight in cacodylate buffer, decalcified in 10% EDTA (pH 7.3) for 48 hours, dehydrated through 95% in acetone, infiltrated overnight in JB-4 solution A with catalyst (Polysciences, Inc., Warrington, PA) and embedded in complete JB-4 medium in BEEM capsules. During embedding, the capsule trays were placed on cracked ice to reduce the high temperature which accompanies JB-4 polymerization at room temperature. The blocks were allowed to polymerize for several days at 4°C. Sections (3µm) were cut dry at room temperature and placed on ice cooled slides which had been alcohol cleaned and gelatin-subbed. The sections were air dried at 4°C for 5-7 days.

Histochemical staining (Cole and Walters, 1986; Appendix B)

Burstone's complete medium for acid phosphatase (Pearse, 1968) was prepared by dissolving 4mg naphthol AS-BI phosphate substrate

(Sigma, St. Louis, MO) in 0.25ml N,N-dimethyl formamide followed by the addition of 25ml of 0.2M acetate buffer (pH 5.0), 35mg Fast Red Violet LB (Sigma) as the coupling agent, and 2 drops (60ul) 10% MgCl₂. The media was then filtered into acid-cleaned Coplin jars. As a control, the substrate was omitted from some Coplin jars. For inhibition studies, 50mM L(+)-tartaric acid (Sigma) was added to Coplin jars containing 25ml of filtered, complete media. Tissue sections were allowed to come to room temperature and were then incubated for 45 minutes at 37°C. Following incubation, the slides were washed for 30 minutes, air-dried at room temperature and counterstained with 1% aqueous Fast Green FCF (Fisher Scientific Co., Chicago, IL, C.I. 42053). Cover slips were mounted with Euparal (Gallard-Schlesinger Chem. Mfg. Corp., Carle Place, N.Y.). The tissue sections were examined for the presence of absence or reaction product. The percentage of acid phosphatase-positive and tartrate-resistant mononuclear cells per 200 cells counted in sections of bone and suture implants at 3, 7 and 14 days post-implantation were calculated. The percentage of bone or suture-induced multinucleated cells exhibiting reaction product in the presence or absence of tartrate 14 days after implantation was also determined.

RESULTS

Acid phosphatase localization demonstrated by this histochemical procedure results in a granular red-maroon reaction product distributed throughout the cell cytoplasm. Occasional diffuse staining of the tibia and implanted bone surface, often near multinucleated cells, was observed. Generally, the addition of tartrate to the incubation medium decreased the concentration, but not the intensity, of the reaction product within the cells. No difference was detected in the histochemical response of ia and normal implant recipients to any of the 8 bone matrix preparations. For this reason, results of mononuclear cell counts obtained from sections of the bone implants were summated.

Osteoclasts

Osteoclasts observed in sections taken from normal and ia rat tibiae were strongly positive for acid phosphatase. The high concentration of reaction product was not diminished by the presence of tartrate during incubation. A significant increase in the amount of TRAP localization in ia osteoclasts as compared to normal osteoclasts could not be consistently demonstrated (Figs. 1a and b).

Three day samples

At 3 days post-implantation, the bone and suture implants were surrounded by mononuclear cells typical of an acute inflammatory reaction. Morphologically, these cell populations appeared similar;

however, a difference in the percentage of acid phosphatase-positive mononuclear cells localized in sections of bone when compared with suture implants was observed in both normal and ia recipients. Table 1 illustrates that at 3 days 12% of the (400) suture-elicited and 29.7% of the (2800) bone-induced mononuclear cells counted were acid phosphatase-positive. Similar counts from adjacent sections of bone and suture implants, incubated in the presence of tartrate, showed less than 1% of the mononuclear cells present to be reactive. These data indicate that tartrate-sensitive acid phosphatase is largely responsible for the 2-fold increase in acid phosphatase activity generated in response to bone implants as compared to suture implants. Suture-elicited mononuclear cells generally contained fewer and more sparsely distributed granules of tartrate-sensitive reaction product (Fig. 2a and b) than did bone-induced mononuclear cells (Figs. 3a and b).

Seven day samples

Although occasional small multinucleated cells were observed, mononuclear cells continued to be the predominate cell type adjacent to the implants. Acid phosphatase was localized in 42.1% of the (400) suture and 48.5% of the (2000) bone mononuclear cells counted (Table 1). Collectively, less than 3% of the implant-generated mononuclear cells exhibited TRAP activity. By 7 days the suture-elicited and bone-induced mononuclear cells demonstrated similar patterns of acid phosphatase localization. Seven-day mononuclear cells contained more

numerous and intensely stained granules of reaction product than their 3-day counterparts (Figs. 4a and b; Figs. 5a and b).

Fourteen day samples

By 14 days the implants were surrounded by an organized connective tissue capsule. Table 1 shows that 64.8% of the (450) mononuclear cells adjacent to suture at 14 days contained acid phosphatase reaction product; of these cells 3.0% were found to be TRAP-positive. Fifty-four percent of the (2600) bone mononuclears counted exhibited acid phosphatase reactivity while less than 1% of similar cells from adjacent sections retaining activity in the presence of tartrate. No difference in the pattern of enzyme reactivity between ia and normal mononuclear cells was noted.

At 14 days multinucleated cells covered a large portion of the bone and suture implant surface. These multinucleated cells were larger and contained more nuclei than 7-day multinucleated cells. Table 2 illustrates that 80.5% of (132) bone-induced multinucleated cells in normal recipients were acid phosphatase-positive whereas only 56.1% of (114) suture-elicited macrophage polykaryons were reactive. Similarly, 77.1% of (126) bone-induced multinucleated cells in osteopetrotic rats exhibited acid phosphatase activity as compared to 69.5% of (131) suture-elicited macrophage polykaryons. Although ia recipients when compared to normal recipients demonstrated a higher percentage of acid phosphatase reactive suture-induced macrophage polykaryons, bone implanted into both ia and normal rats generally gener-

ated a stronger acid phosphatase response from adjacent multinucleated cells than did implanted suture. This enhancement of acid phosphatase activity appeared to be due in part to increased levels of TRAP within the multinucleated cells found in both types of recipient animals. In normal rats 52.2% of (125) bone-induced multinucleated cells were TRAP-positive as compared to 22.3% of (130) suture-elicited macrophage polykaryons. TRAP reaction product was demonstrated in 68.4% of (130) bone-induced multinucleated cells and 61.8% of (163) suture-elicited macrophage polykaryons counted in samples taken from ia rats.

Results of these cell counts suggest that bone matrix implanted at this ectopic site is capable of inducing the formation of TRAP positive multinucleated cells. However, the concentration of TRAP reaction product in suture and bone implant multinucleated cells differed markedly from that observed in the osteoclast. Acid phosphatase positive bone and suture multinucleated cells were only mildly reactive, displaying a sparse distribution of reaction product which was often diminished or eliminated by the addition of tartrate to the incubation medium (Figs. 6a and b; Figs. 7a and b). At no time did suture or bone-induced multinucleated cells exhibit the highly concentrated TRAP profile consistently demonstrated by normal and ia osteoclasts. Therefore, when compared on the basis of strength of TRAP activity, bone-induced multinucleated cells did not resemble osteoclasts, but were similar to suture-elicited macrophage polykaryons.

DISCUSSION

Tartrate-resistant acid phosphatase (TRAP) has been localized within osteoclasts (Hammarstrom et al., 1971; Chappard et al., 1983) while macrophages and monocytes typically exhibit tartrate-sensitive acid phosphatase activity (Seifert, 1984; Cole and Walters, 1986; Appendix B). In the present study, this dicotomy in the acid phosphatase isoenzyme expressed by osteoclasts and mononuclear phagocytes was used to examine the relationship between the osteoclast, the bone-induced multinucleated cell and the macrophage polykaryon. We compared the acid phosphatase profiles of cells found adjacent to bone and suture implanted into normal and osteopetrotic (ia) rats with tibial osteoclasts from the same animals.

Both normal and ia osteoclasts were heavily stained with TRAP reaction product; however, the presence of excessive amounts of acid phosphatase in ia osteoclasts (Hammarstrom et al., 1983) could not be consistently demonstrated. Diffuse staining of the bone adjacent to osteoclasts was occasionally observed. Handelman et al (1964) described similar bands of concentrated acid phosphatase activity at the junction of osteoclasts and bone. They postulated that these junctional bands represent sites into which lysosomal enzymes are secreted during the resorptive process. Ultrastructurally, acid phosphatase has been localized within the extracellular channels of the ruffled border, the bone-cell interspace and matrix underlying the active

osteoclast (Lucht, 1971; Doty and Schofield, 1972). Miller (1985) demonstrated the appearance of extracellular acid phosphatase activity, which was confined to the developing ruffled border and adjacent bone matrix, in quail medullary bone osteoclasts stimulated by parathyroid hormone (PTH). Collectively, these studies indicate acid phosphatase, particularly the tartrate-resistant form, may play an important role in the resorptive function of the osteoclast.

Histochemical evaluation of cell populations found surrounding implanted bone and suture indicated that the majority of acid phosphatase activity expressed by mononuclear cells was tartrate-sensitive. Only occasional tartrate-resistant cells were observed. Monocyte and macrophages are known to be major constituents of the mononuclear cell populations elicited by bone and suture implants (Holtrop et al., 1982; Walters and Schneider, in press), therefore, a preponderance of tartrate-sensitive acid phosphatase activity in the mononuclear cells adjoining such implants is predictable. Nilsen and Magnusson (1981) described a similar pattern of tartrate-sensitive acid phosphatase localization in guinea pig macrophages surrounding intramuscular implants of dentin.

An increase in the percentage of tartrate-sensitive acid phosphatase-positive mononuclear cells found adjacent to the implants was observed during the 14-day implantation period. Initially, bone implants elicited larger numbers of acid phosphatase reactive cells than did suture, however, at seven days post-implantation this

discrepancy in acid phosphatase induction was no longer apparent. A similar enhancement of TRAP localization in implant mononuclear cell populations was not observed, suggesting that macrophages and monocytes activated under these implant conditions did not undergo osteoclastic differentiation. These results differ from those of in vitro in which the acquisition of TRAP activity has been demonstrated. Snipes et al. (1986) reported that monocytes incubated with 1,25 dihydroxyvitamin D₃ and monocyte-derived macrophages cultured for three to seven days expressed TRAP activity while freshly isolated monocytes and vitamin D-stimulated U937 monocytic cells exhibited only tartrate-sensitive acid phosphatase. These findings reflect the heterogeneity of the monocyte-macrophage populations often used for in vitro investigations and the diversity of their responses in culture to osteotropic agents such as 1,25 dihydroxyvitamin D₃.

No significant difference in the acid phosphatase profiles between normal and ia mononuclear cells surrounding the implants was noted. These observations concur with those of Schneider et al. (1981) and Seifert (1984) in their individual investigations of the biology of macrophage populations in osteopetrosis. The authors demonstrated that, unlike the osteoclast populations, no significant differences in structure, acid phosphatase content or phagocytic activity were present in ia macrophages when compared with those from normal littermates. These results suggest that macrophages may be a cell type distinct from the osteoclast and that less mature monocytic

cells may represent better choices for the study of osteoclast-mononuclear phagocyte linkage. Investigations evaluating the characteristics of less differentiated mononuclear cell populations have also employed TRAP as an index of osteoclastic differentiation. The formation of TRAP-positive multinucleated cells, possessing some of the morphological characteristics and hormonal responsiveness of the osteoclast, was observed in long-term cultures of feline and primate bone marrow-derived mononuclear cells (Ibbotson et al., 1984; Roodman et al., 1985). Data from these and similar studies using mouse marrow cells (Burger et al., 1982; Burger et al., 1984) demonstrated that the osteoclast-like multinucleated cells were formed by fusion of non-adherent monocytic progenitor cells. These results suggest that osteoclasts and mononuclear phagocytes may be related through an early common stem cell, but, subsequently, take separate differentiation pathways.

In studies of human monocytes cultured in the presence of γ interferon, the formation of macrophage polykaryons was accompanied by increasing levels of TRAP activity, leading to speculation that γ interferon may be capable of the transformation of monocytes into osteoclasts (Weinberg et al., 1984). Morphologically, the interferon-stimulated polykaryons resembled multinucleated cells observed during inflammatory reactions in vivo (Weinberg et al., 1984). No direct comparison of the TRAP profiles of these cultured multinucleated cells with those of similarly stimulated osteoclasts was conducted. Our

present study allows the comparison of the acid phosphatase activity present in inflammation-elicited multinucleated cells with that present in osteoclasts from the same animal. Results indicated that bone implants induced the formation of larger numbers of acid phosphatase-positive multinucleated cells than did suture implants. In addition, a greater proportion of bone multinucleated cells, particularly in ia recipients, retained this activity in the presence of tartrate; however, the concentration of reaction product in both bone and suture-induced TRAP-positive cells differed markedly from that of the osteoclast. Implant-induced cells could only be described as mildly reactive, exhibiting sparsely scattered granules of TRAP reaction product throughout their cytoplasm, while both normal and ia osteoclasts demonstrated intense TRAP activity. Therefore, although bone implants were capable of inducing the formation of TRAP-positive cells, the bone multinucleated cells shared a similar concentration and pattern of TRAP distribution with suture-elicited macrophage polykaryons, suggesting that these two categories of multinucleated cells may be distinct from or not equivalent to the osteoclast. Alternatively, the maturation of the bone-induced multinucleated cells into histochemically identifiable osteoclasts may have been prevented at this ectopic site by the absence of a specific inductive matrix component, humoral agent or cellular population required for completion of the differentiation process. Burger et al. (1984), studying the origin of the osteoclast in mouse long bone primordia,

reported that osteoclasts developed only in the presence of live bone. This finding indicates that environmental factors found within living bone may be necessary for osteoclastic differentiation. Thus, the devitalized bone matrix used in the present study may not provide the appropriate substrate for the elaboration of typical osteoclastic TRAP profiles in bone-induced multinucleated cells.

In summary, this investigation was conducted to compare the TRAP characteristics of bone-induced multinucleated cells with those of the osteoclast and suture-elicited macrophage polykaryon to define more clearly the relationship between these three categories of multinucleated cells. In this study, bone implants were capable of inducing the formation of TRAP-positive multinucleated cells, however, the distribution of TRAP reaction product within these cells did not resemble that of the osteoclast, but was similar to that found within suture-elicited macrophage polykaryons. These results imply that the bone-induced multinucleated cells, formed under these implant conditions, may not be equivalent to the osteoclast, but may, in fact, be macrophage polykaryons elicited during an inflammatory response to the implanted bone matrix. Based on the results of this investigation and others which have also demonstrated morphological differences between bone-induced multinucleated cells and osteoclasts (Walters and Schneider, 1985; Walters and Schneider, in press), we suggest caution in the use of bone-induced multinucleated cells as surrogates for the study of osteoclastic lineage and function.

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Table 1. Percent Acid Phosphatase-Positive Mononuclear Cells

<u>Implant</u> ¹	3-day		7-day		14-day	
	<u>AcP</u> ²	<u>TR</u> ³	<u>AcP</u>	<u>TR</u>	<u>AcP</u>	<u>TR</u>
Suture	12.0	<1.0	42.1	<1.0	64.8	<3.0
Bone	29.7	<1.0	48.5	<1.0	54.0	<1.0

¹Samples taken from normal and ia rats

²Percent acid phosphatase-positive mononuclear cells in sections incubated without tartrate

³Percent tartrate-resistant mononuclear cells

Table 2. Percent Acid Phosphatase-Positive Multinucleated Cells

<u>Implants</u> ¹	Normal		<u>ia</u>	
	<u>AcP</u> ²	<u>TR</u> ³	<u>AcP</u>	<u>TR</u>
Suture	56.1	22.3	69.5	61.3
Bone	80.5	52.2	77.1	68.4

¹Samples taken from normal and ia rats 14 days post-implantation

²Percent acid phosphatase-positive multinucleated cells in sections incubated without tartrate

³Percent tartrate-resistant multinucleated cells

Figure 1.

Osteoclasts (arrows) from normal and ia tibiae, containing high concentrations of granular TRAP reaction product, located adjacent to the bone surface (b).

a. Normal osteoclasts. x 700

b. Osteopetrotic osteoclasts. x 720.



Figure 2.

Adjacent sections of mononuclear cells surrounding suture (s) implanted into a normal rat 3 days post-implantation. x 513

- a. Acid phosphatase-positive cells (arrows) exhibiting a few granules of reaction product.
- b. In the presence of tartrate, fewer cells show enzyme activity.

Figure 3.

At 3 days mononuclear cells located near normal freeze-thawed trabecular bone (b) implanted into a normal recipient. x 475

- a. Reactive mononuclear cells (arrows) in tissue surrounding the implanted bone matrix.
- b. Complete tartrate inhibition of mononuclear cells similar to those present in Fig. 3a located in an adjacent tissue section.

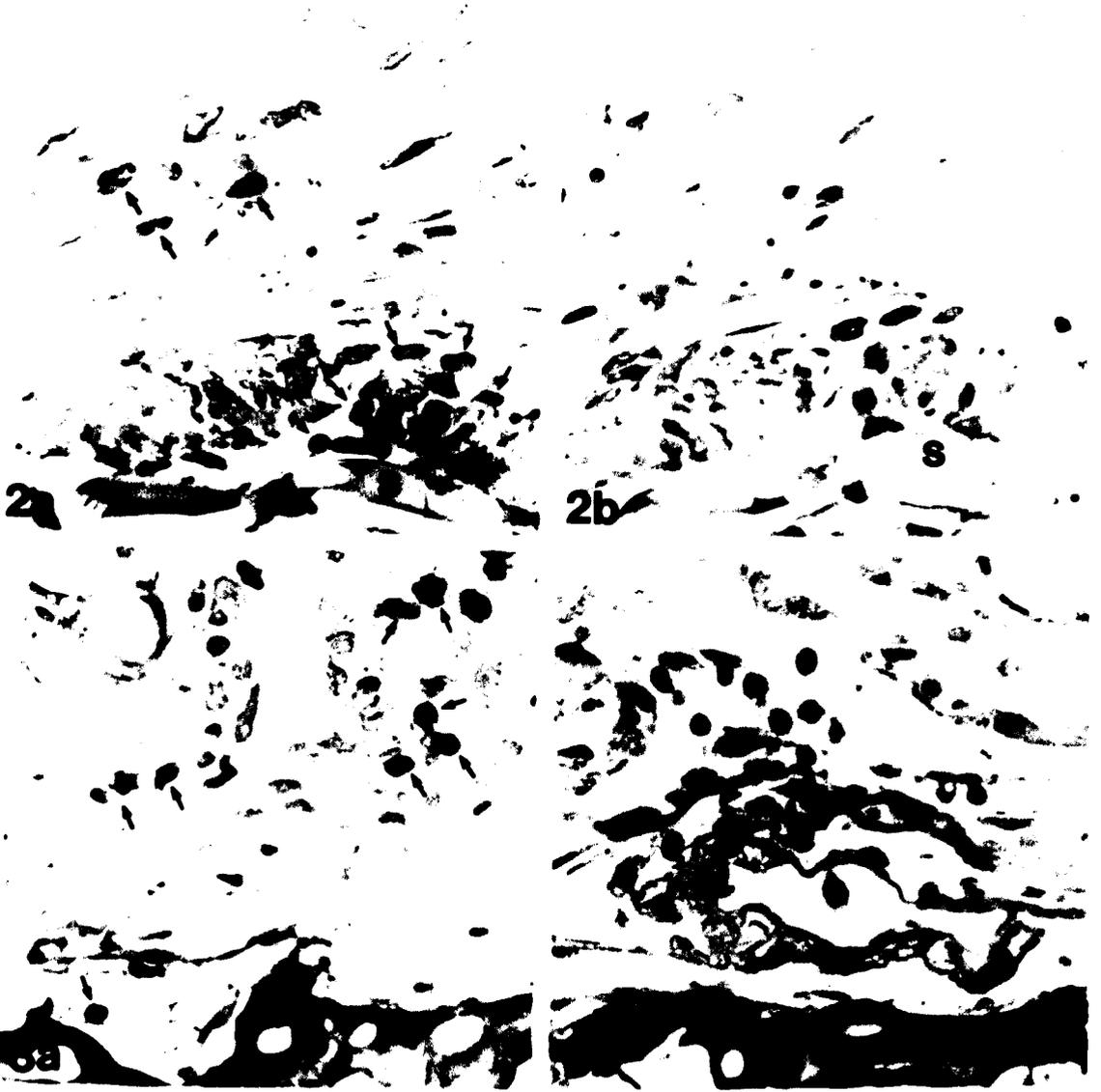


Figure 4.

Acid phosphatase activity of cell populations elicited by suture (s) implanted into an ia rat 7 days after implantation.

x 475

- a. Mononuclear cells demonstrating reaction product (arrows). Note small non-reactive multinucleated cells immediately adjacent to strands of suture.
- b. With tartrate, the acid phosphatase activity within mononuclear cells is completely inhibited in this section. Multinucleated cells remain non-reactive.

Figure 5.

At 7 days histochemical response of cells located near normal freeze-thawed trabecular bone (b) implanted into an ia rat.

x 388

- a. Acid phosphatase reaction product localized within mononuclear cells in tissue adjoining bone matrix. Multinucleated cells (arrows) at the bone surface do not exhibit activity.
- b. Tartrate inhibition of an adjacent section through the same bone implant.

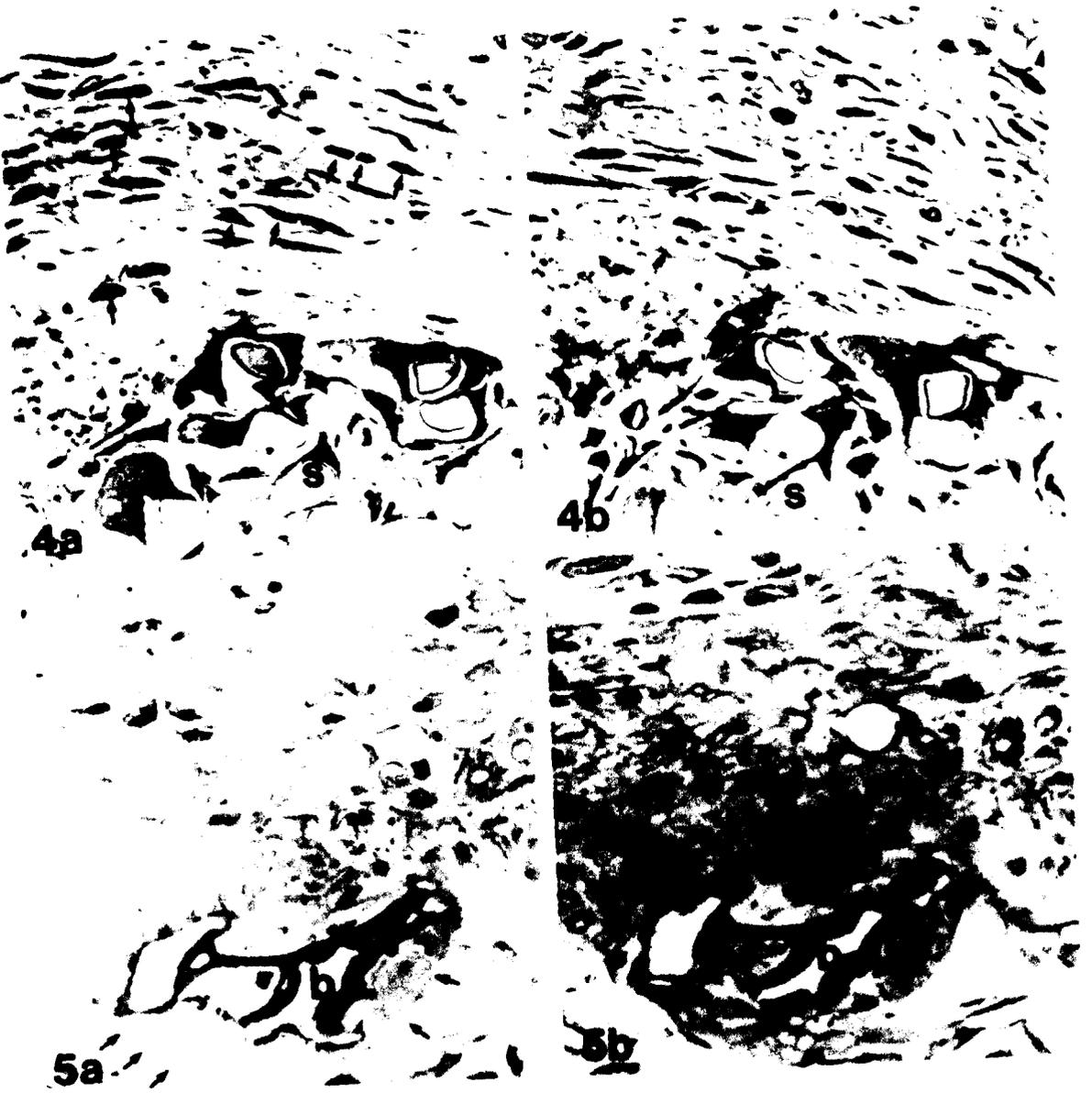


Figure 6.

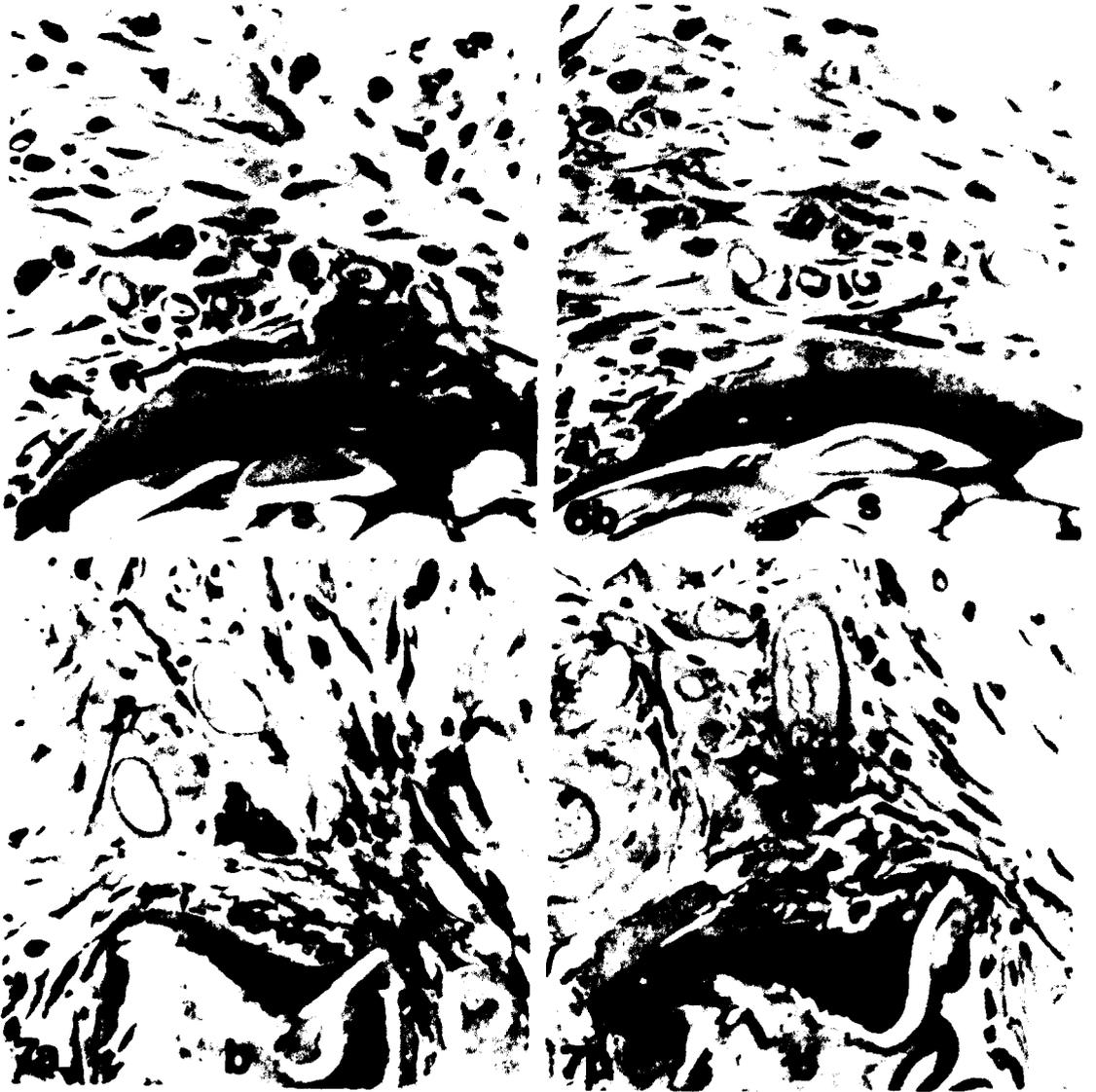
Acid phosphatase staining characteristics of mononuclear and multinucleated cells present around suture (s) implanted into an 1a rat 14 days post-implantation. x 500

- a. Numerous strongly reactive mononuclear cells lie within the connective tissue capsule. A mildly reactive multinucleated cell (arrows) is also present adjacent to the suture.
- b. An adjacent tissue section incubated with tartrate. Mononuclear cells do not demonstrate reaction product. The multinucleated cell retains mild activity.

Figure 7.

At 14 days normal collagenase-treated trabecular bone (b) implanted into a normal rat. x 500

- a. Acid phosphatase localization within mononuclear and multinucleated (arrows) cells at or near the bone surface.
- b. With inclusion of tartrate, reaction product within mononuclear cells is almost completely eliminated while the multinucleated cell retains mild reactivity.



CHAPTER VI

DISCUSSION

Osteoclasts play a principal role in the degradation of both the organic and inorganic components of bone; however, the exact mechanism by which osteoclasts resorb bone is unknown. Because they comprise only a small percentage of the cells present in bone, isolation of homogeneous populations of osteoclasts in sufficient numbers for in vitro experimentation has been difficult, limiting the use of such techniques in defining the nature of osteoclastic bone resorption. Discovery that mononuclear phagocytes, putative osteoclast precursors, are capable of contact-mediated bone resorption in culture has led to speculation that monocytes, macrophages, and macrophage polykaryons could serve as investigational surrogates for the study of osteoclastic lineage and function (Teitelbaum and Kahn, 1980). This proposal suggests that, in the presence of bone, monocytes and macrophages undergo fusion and subsequent differentiation into multinucleated cells which are equivalent to osteoclasts, equating the mechanisms involved in monocyte-macrophage osteolysis with those of osteoclastic bone resorption. Numerous in vitro investigations, co-culturing various mononuclear phagocyte populations with devitalized bone matrix often in the presence of osteotropic agents such as

vitamin D₃, have been conducted (Mundy et al., 1977; McArthur et al., 1980; Bar-Shavit et al., 1983). These studies often do not consider the heterogeneity of the mononuclear phagocytes used for experimentation or the diversity of their responses in culture to osteotropic agents. In addition, they rarely include direct comparisons of cultured mononuclear phagocytes with similarly stimulated osteoclasts, making correlation to in vivo situations difficult.

The present series of investigations evaluated the premise that macrophages and monocytes may serve as osteoclast surrogates in an in vivo setting. A functional, morphological and histochemical assessment of the cell populations found adjacent to devitalized bone implants was conducted to determine if the formation of multinucleated cells equivalent to osteoclasts could be induced at a subcutaneous site. Implantation was performed into both normal and osteopetrotic (ia) rats to determine if the usual resorptive defect seen in this mutant (Marks, 1973) could be duplicated at this ectopic site, and if so, was this defect reflected by morphological differences between normal and ia bone-induced multinucleated cells similar to those found between normal and ia osteoclasts. Duplication of such differences would lend validity to the use of bone-induced multinucleated cells as osteoclastic surrogates. In the morphological and histochemical studies, silk sutures were also implanted, allowing comparison of cells found adjacent to both bone and suture implants with tibial osteoclasts from the same recipient animal. The effects of substrate

composition on the recruitment, differentiation and function of cells to the implant substrate was also performed through the use of primarily mineral-exposed (collagenase-treated) and primarily osteoid-exposed (freeze-thawed) normal and osteopetrotic bone matrix.

The functional assessment (^{45}Ca assays) of the resorptive capabilities of the implant-elicited cell populations permitted quantitation of passive and cell-mediated label releases. Results showed that significant cell-mediated ^{45}Ca loss had occurred in both normal and ia rats during the implantation period, suggesting a large part of the bone resorption which takes place in this implant system is an active cell-related process rather than a passive diffusion event. Primarily mineral-exposed implant matrix exhibited more label release than did primarily osteoid-exposed matrix. Osteopetrotic and normal animals demonstrated similar patterns of ^{45}Ca release, indicating that the typical ia resorptive defect was not mimicked at this ectopic site. This finding implies that the cells involved in bone resorption under these implant conditions may not be osteoclasts which are defective in the ia animal or, alternatively, that this ectopic site may provide cues, missing in the ia bone environment, which are necessary for the normal differentiation of osteoclasts. Morphological assessment of the cells recruited to the implant surface was conducted to determine which of these alternatives might be occurring.

Light and electron microscopic evaluation showed that the cellular events occurring around bone and suture implants were similar

in both normal and ia animals regardless of the bone matrix preparation implanted. At 3 and 7 days post-implantation, monocytes and macrophages were the predominant mononuclear cells surrounding the implants, while multinucleated cells extensively covered much of the bone and suture surfaces at later stages in the implantation period. No morphological differences were observed between multinucleated cells generated in response to primarily mineral-exposed versus primarily osteoid-exposed implants; however, the results of morphometric analysis indicated that mineral-exposed bone implants tended to have more total length covered by multinucleated cells than did osteoid-exposed implants, suggesting that mineral-exposure may play a role in the induction of multinucleated cells. Chambers et al. (1984) have suggested that contact with bone mineral may be important in the initiation of resorptive behavior by osteoclasts; however, in the present study, increased mineral exposure at the implant surface did not promote the phenotypic expression of osteoclast-like morphology in cells adjacent to the bone matrix. Ultrastructural assessment of bone-induced mononuclear and multinuclear cells failed to reveal the presence of ruffled borders, hallmarks of actively resorbing osteoclasts, at the bone-cell interface. Osteoclastic bone resorption is believed to occur in a well defined extracellular compartment, the ruffled border. Recent studies by Baron et al. (1985) suggest that the region between the ruffled border and the bone surface is actively acidified by the osteoclast. This acidification may assist in the

solubilization of bone mineral and provide pH optima for the actions of lysosomal enzymes (acid hydrolases), secreted by the osteoclast into the pericellular space, which then degrade the organic components of bone. The clear zone, an organelle-free, actin-rich region of the osteoclast, is believed to form a tight seal around the ruffled border to maintain the specialized resorptive environment created by the osteoclast. The observation that bone resorptive cells in this implant system did not exhibit structures similar to ruffled borders implies that the cells are either inactive osteoclasts, which is not supported by the ^{45}Ca release data, or they are cells other than osteoclasts. Possibly mononuclear phagocytes and polykaryons were responsible for releasing the radioactive label from the implanted bone matrix. Bone-induced multinucleated cells structurally resembled suture-elicited macrophage polykaryons, providing evidence in support of this hypothesis.

Histochemical localization of tartrate-resistant acid phosphatase (TRAP), a proposed osteoclastic marker (Minkin, 1982), in cells surrounding bone and suture implants also revealed significant differences between implant-induced cell populations and tibial osteoclasts, furnishing additional evidence that cell populations recruited to this ectopic site may not form cells equivalent to osteoclasts. The majority of acid phosphatase-reactive mononuclear cells adjacent to both bone and suture implants exhibited tartrate-sensitive activity. Only occasional TRAP-positive mononuclear cells were observed. The per-

centage of tartrate-sensitive mononuclear cells increased over the implant period. A corresponding increase in TRAP-positive cells was not seen, suggesting that the mononuclear cells elicited under these implant conditions did not undergo osteoclastic differentiation, but remained macrophages as histochemically defined. Generally, bone matrix implanted into both normal and ia rats generated a stronger acid phosphatase response from adjacent multinucleate cells than did suture. In addition, a larger number of bone-induced multinucleated cells retained this activity in the presence of tartrate; however, the concentration of TRAP reaction product in both bone and suture-elicited multinucleated cells differed significantly from that found in osteoclasts. Both normal and osteopetrotic osteoclasts demonstrated high concentrations of TRAP reaction product, while bone and suture-elicited multinucleated cells could only be classified as mildly TRAP reactive. These results suggest that bone matrix is capable of inducing the formation of TRAP-positive multinucleated cells, but when compared on the basis of strength of TRAP reactivity, bone-induced multinucleated cells more closely resembled suture-elicited macrophage polykaryons than they did osteoclasts.

The overall results of the present series of investigations have shown that cell-mediated bone resorption, as assessed in ^{45}Ca release assays, occurs in this implant system. However, multinucleated cells exhibiting the morphological and histochemical characteristics of osteoclasts could not be demonstrated at the bone implant surface in

either normal or ia rats. Instead, bone-induced multinucleated cells shared similar structural and enzymatic features with suture-elicited macrophage polykaryons, implying that bone-induced multinucleated cells, formed under these implant conditions, may not be equivalent to osteoclasts, but may, in fact, be macrophage polykaryons generated during an inflammatory response to the implanted devitalized bone matrix. The results of these studies, therefore, suggest that under the conditions imposed by this implant system, mononuclear phagocytes do not appear capable of forming osteoclasts as morphologically and histochemically defined, despite evidence that cell-mediated bone resorption has occurred.

One explanation for the findings of this study may be that the devitalized bone matrix used as the implant substrate in these experiments did not provide the factors necessary for the differentiation of osteoclasts from mononuclear phagocytes at this ectopic site. Burger et al. (1984) have suggested that devitalized bone may not be an appropriate substrate for the formation of osteoclasts. The authors, using cultured embryonic bone rudiments, reported that osteoclasts developed in the presence of live, but not devitalized, bone, indicating that factors within the vital bone environment may be necessary for the differentiation of osteoclasts. They propose that the presence of osteogenic cells may be required for osteoclast formation from mononuclear progenitors. Evidence in support of this hypothesis has been provided by Osdoby (1986) who demonstrated that chick monocytes

or bone marrow cells cultured with osteoblasts formed multinucleated cells which expressed osteoclastic antigens while similar cells cultured alone or with fibroblasts did not. He concluded that osteoblastic expression, possibly contact-mediated, may be involved in osteoclastic differentiation. Studies by Rodan and Martin (1981) and McSheehy and Chambers (1986) have also shown that osteoblasts may play a role in the hormonal control of bone resorption by mediating the effects of osteotropic agents such as vitamin D₃ and parathyroid hormone on osteoclasts. The results of these investigations suggest that the presence of environmental factors or cells such as osteoblasts within vital bone, may be important for the formation, activation and function of osteoclasts. Thus, the absence of these factors or cells from the implantation site in the current studies may have limited the ability of the mononuclear cells recruited to the bone matrix to differentiate into osteoclasts or prevented the expression of active osteoclastic morphological and histochemical profiles by multinucleated cells covering the bone surface. An evaluation of the importance of vital bone and osteogenic cells in the formation of osteoclasts at this ectopic site might be accomplished through the use of demineralized bone matrix as an implant substrate. Subcutaneous implantation of demineralized bone powder into experimental animals induces de novo bone formation (Reddi and Huggins, 1972; Sampath and Reddi, 1984). By days 10-12 post-implantation, osteoblasts can be identified on the newly formed bone surface (Reddi,

1985). Co-implantation of demineralized bone powder and mineralized bone chips, therefore, may provide a means by which osteogenic cells and vital bone could be introduced at the implantation site in our model system, permitting evaluation of their roles in osteoclastic differentiation of cells recruited to mineralized implants at this ectopic site.

Another plausible explanation for the failure of mononuclear phagocytes to form morphologically and histochemically identifiable osteoclasts under these implant conditions may be related to the maturity of the monocyte and macrophage cell populations elicited at the implant site. The mononuclear phagocytes present at the site of inflammatory reactions, like those generated by the bone and suture implants in the present study, represent cells which have undergone extensive structural, enzymatic and receptor modification from their unactivated counterparts in response to the inflammatory stimulus (Mariano and Spector, 1974; van der Rhee et al., 1979; Treves, 1984). There is mounting evidence that such mature or activated monocytes and macrophages may not be capable of forming osteoclasts. Burger et al. (1982) have demonstrated that osteoclasts developed in fetal mouse long bone co-cultured with embryonic liver or weakly adherent radio-sensitive bone marrow cells, but not with strongly adherent cells, resident or elicited peritoneal macrophages. Ibbotson et al. (1984) reported similar findings in long-term cultures of feline marrow-derived cells. Injection of cell suspensions of mature macrophages

into ia rats does not effect the cure of osteopetrosis whereas injections of pluripotent stem cells were capable of reversing the ia resorptive defect and generating normal osteoclasts within ia long bones. These investigations suggest that immature or less differentiated mononuclear phagocytes may be able to give rise to osteoclasts while mature monocytes and macrophages cannot, but instead, fuse to form macrophage polykaryons, cells which are not equivalent to osteoclasts.

Macrophages and monocytes are associated with the breakdown and remodeling of connective tissues in general. They are involved in the involution of organs, such as the post-partum uterus and the mammary gland after the cessation of lactation, and participate in the events of wound debridement and repair (Vaes, 1985). Mononuclear phagocytes exert their effects either by direct contact with cell or tissue targets or, alternatively through a variety of soluble mediators (Davies et al., 1980; Vaes, 1985). Werb et al. (1980), studying the degradation of insoluble smooth muscle extracellular matrix in vitro, postulated that macrophage-mediated degradation of connective tissue matrices could occur at three sites: (1) extracellularly by secretion of neutral proteases, (2) intracellularly through the actions of lysosomal hydrolases and (3) at the cell surface and adjacent pericellular space by cell-surface bound enzymes. Evidence in support of this hypothesis has been provided by numerous investigations. In vitro studies have shown that resident and elicited macrophages

secrete neutral proteases such as plasminogen activator, elastase, and collagenase which are capable of degrading the glycoprotein, elastin and collagen found in most connective tissues (Jones and Werb, 1980; Werb et al., 1980; Vaes, 1985). Lysosomal enzymes such as cathepsins B and D, which are capable of collagen and proteoglycan digestion, have also been demonstrated within macrophages (Vaes, 1985). Roberts and Dean (1986) reported that a cell-surface associated enzyme, probably a neutral protease, may participate in the degradation of bovine nasal cartilage in vitro. Wright and Silverstein (1984) have shown that phagocytosing macrophages exclude proteins from the zone of contact with opsonized targets implying that clear zone-like regions, previously described in macrophages (Rifkin et al., 1979) may function to isolate the area of phagocytosis from the surrounding extracellular space.

Although macrophage-mediated tissue degradation and osteoclastic bone resorption do share many similar features, some important differences do exist. Osteoclasts appear to preferentially employ the actions of lysosomal acid hydrolases to affect bone resorption rather than those of neutral proteases such as collagenase which have been implicated in mononuclear phagocytic tissue degradation (Vaes, 1985; Blair et al., 1986). The addition of calcitonin to cultures of peripheral blood monocytes and elicited peritoneal macrophages has no effect on macrophage-mediated bone dissolution (McArthur et al., 1980); however, low concentrations of calcitonin induce dramatic

changes in osteoclasts (Holtrop et al., 1974; Chambers and Magnus, 1982). Monocytes, macrophages and macrophage polykaryons incubated on slices of human cortical bone or seeded onto whale dentine failed to demonstrate morphological signs of resorptive activity, as detected by scanning electron microscopy, whereas osteoclasts, cultured under similar conditions, carved out classical resorption lacunae on the underlying matrix (Chambers et al., 1984; Ali et al., 1984). Another important structural difference between macrophage-mediated and osteoclastic bone resorption appears relevant to the present study. Ruffled borders, previously described as sites of active osteoclastic bone resorption, have not been observed at the macrophage-bone interface, indicating that macrophages and osteoclasts may not employ all of the same structural machinery to affect bone degradation. Chambers (1985) suggests that mononuclear phagocytes may release calcium and hydroxyproline through the digestion of phagocytosed whole bone particles rather than by true extracellular resorption of bone surfaces. Therefore, the mechanisms by which mononuclear phagocytes degrade bone matrix may not be equivalent to those of osteoclast-mediated bone resorption.

The implant system employed in this investigation does not appear to be an appropriate model for studying osteoclastic differentiation from mononuclear phagocytes or for examining the process of normal bone resorption. However, it may represent an in vivo model for the study of inflammation-related skeletal tissue destruction, a

topic of clinical importance. Case reports of bone foreign body granulomas and destructive arthritis, elicited by silicone prostheses used as joint replacements, describe inflammatory reactions which extend into bone and articular cartilage, causing extensive bone and joint damage (Rosenthal et al., 1983; Manes, 1985). Cartilage destruction and subchondral bone loss are associated with chronic inflammatory diseases, such as rheumatoid arthritis and periodontitis (Mergenhagen et al., 1975; Bromley et al., 1985). Macrophage-like cells, containing abundant lysosomes, mitochondria and vacuoles, are seen in the invading soft tissue pannus in rheumatoid joints and are the predominant cell type present at the cartilage surface undergoing degradation. These cells often send cytoplasmic extensions deep into the eroding cartilage matrix (Shiozawa et al., 1983; Bromley and Woolley, 1984). Macrophage-like cells are also believed to secrete the cartilage degrading enzymes such as collagenase and cathepsin D which have been localized at the rheumatoid cartilage-pannus junction (Kobayashi and Ziff, 1975; Woolley et al., 1977). Mononuclear and multinuclear phagocytic cells can also be identified in areas of active periodontal disease, characterized by inflammation of the gingiva and loss of supporting bone and connective tissue and at site of pressure induced necrosis in experimental models of tooth movement (Rifkin and Heijl, 1979; Cohen et al., 1985; Nixon and King, 1985). Many of the salient features commonly observed in these osteolytic diseases are mimicked by the implant system described in the present

investigation (generation of an inflammatory reaction, significant cell-mediated bone loss, and the presence of mononuclear phagocytes at the implant surface and in surrounding connective tissues). An evaluation of tissues taken from subjects with these diseases might be conducted to determine if cell populations in the tissue samples share similar morphological and histochemical profiles with implant-induced cells. The demonstration of such similarities would provide further validation for the use of this implant system as a model for the study of inflammation-related osteolysis.

In conclusion, the premise that macrophages and monocytes are capable of undergoing fusion and differentiation to form morphologically and histochemically identifiable osteoclasts is not supported by the results of the in vivo implantation system described in this dissertation. The bone resorption which occurred during the implantation period, as indicated by ^{45}Ca assays, most probably occurred through the osteolytic actions of members of the mononuclear phagocyte system. This implantation system does not, therefore, appear to represent a model for the study of osteoclastic lineage and function, but may reflect an appropriate technique for the examination of inflammation-related bone loss.

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APPENDIX A

The material in this appendix supplements Chapter 3. It reports the results of label uniformity, diffusion chamber, and direct implantation studies using freeze-thawed and collagenase-treated osteopetrotic calvarial bone matrix as the implant substrate. Bleached osteopetrotic bone was not evaluated. These data indicate that label uniformity and label releases from the osteopetrotic bone implants were similar to those from normal bone implants in both normal and ia rats.

Uniformity of ^{45}Ca label within osteopetrotic freeze-thawed bone chip pairs

<u>Bone chips</u> ¹	<u>Weight(mg)</u>	<u>Counts/mg</u>	<u>Mean</u> ²	<u>Percent error</u> ³
Frontal				
left	1.9	127,877	125,931 ± 2,753	2.2
right	1.9	123,984		
left	2.7	249,577	256,042 ± 9,142	3.6
right	2.1	262,506		
left	2.2	217,040	218,632 ± 2,251	1.0
right	2.1	220,224		
Parietal				
left	4.0	120,105	119,685 ± 595	0.5
right	3.7	119,264		
left	3.4	231,136	227,913 ± 4,558	2.0
right	4.0	224,690		
left	--	--		
right	--	--		
Occipital				
left	3.8	119,868	122,888 ± 4,271	3.5
right	3.6	125,908		
left	3.0	239,383	234,572 ± 6,804	2.9
right	3.1	229,760		
left	2.3	196,090	197,885 ± 2,539	1.3
right	2.0	199,680		

¹Samples taken from 13-day-old rats.

²Mean ± standard deviation in counts/mg.

³Percent error = (standard deviation x 100)/mean.

Uniformity of ^{45}Ca label within osteopetrotic collagenase-treated bone chip pairs

<u>Bone chips</u> ¹	<u>Weight(mg)</u>	<u>Counts/mg</u>	<u>Mean</u> ²	<u>Percent error</u> ³
Frontal				
left	1.9	195,993	200,365 ± 6,183	3.1
right	1.8	204,737		
left	2.3	191,460	196,133 ± 6,608	3.4
right	1.8	200,805		
left	1.6	205,106	209,816 ± 6,660	3.2
right	1.4	214,525		
Parietal				
left	2.9	168,867	171,351 ± 3,513	2.1
right	3.3	173,835		
left	3.0	167,477	164,620 ± 4,041	2.5
right	3.0	161,762		
left	1.7	169,231	170,013 ± 1,105	0.6
right	2.6	170,794		
Occipital				
left	2.4	194,275	198,494 ± 5,966	3.0
right	2.4	202,712		
left	2.3	185,005	193,490 ± 11,999	6.2
right	2.5	201,975		
left	2.1	186,157	190,433 ± 6,047	3.2
right	2.0	194,709		

¹Samples taken from three 13-day-old rats.

²Mean ± standard deviation in counts/mg.

³Percent error = (standard deviation x 100)/mean.

Figure 1.

Mean percent \pm S.D. ^{45}Ca release over a two week period from freeze-thawed ($5.7\% \pm 2.1\%$) and collagenase-treated ($6.8\% \pm 3.2\%$) osteopetrotic calvarial bone chips in diffusion chambers implanted into normal rats.

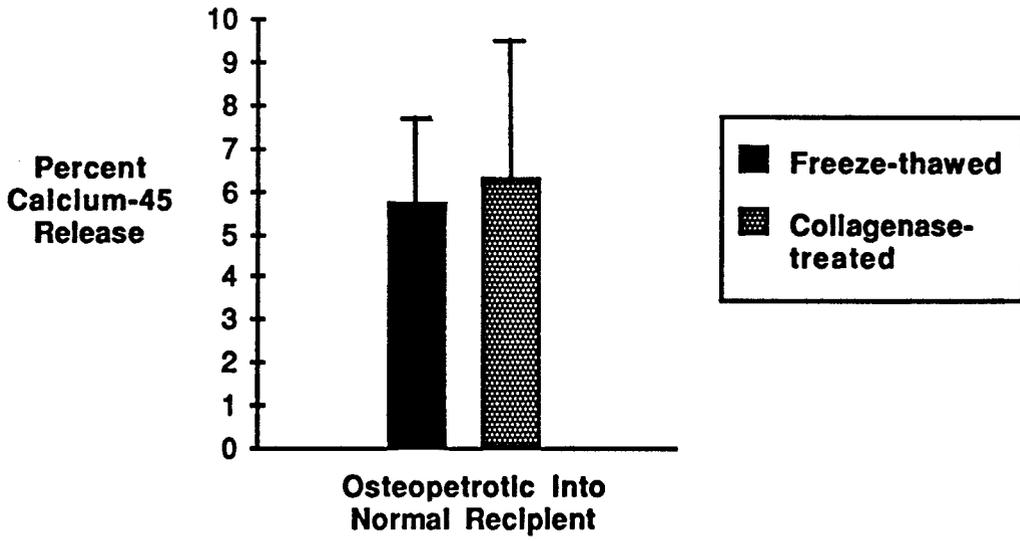
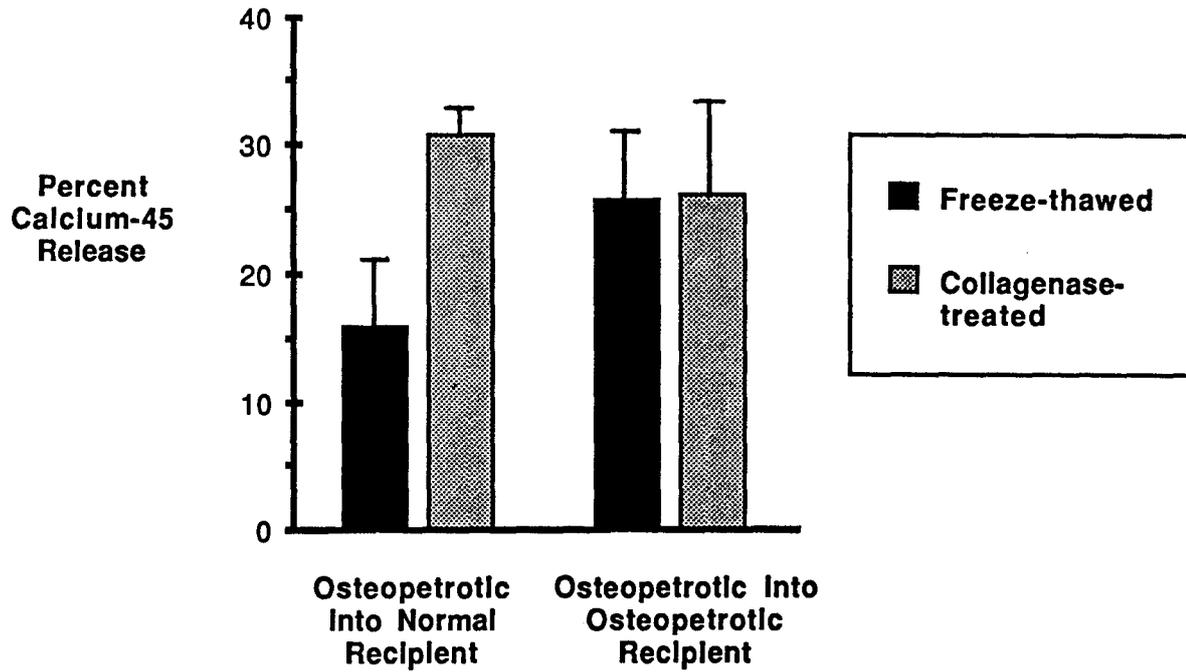


Figure 2.

Mean percent \pm S.D. ^{45}Ca release over a two week period from freeze-thawed and collagenase-treated osteopetrotic bone chips implanted directly into normal and ia rats. Normal recipients demonstrated mean label releases of $15.9\% \pm 5.3\%$ and $30.9\% \pm 2.8\%$ from freeze-thawed and collagenase-treated bone chips respectively. Osteopetrotic rats had mean ^{45}Ca releases of $25.9\% \pm 5.3\%$ from freeze-thawed and $26.3\% \pm 6.7\%$ from collagenase-treated bone implants.



APPENDIX B

This Appendix represents data obtained through the combined efforts of the author and Ada A. Cole, a fellow graduate student. Because this document will be included in both dissertations, the Graduate School has ruled that this work can be included within the dissertation of each student, but only in an Appendix, separate from the main body of each dissertation.

TARTRATE-RESISTANT ACID PHOSPHATASE IN BONE AND CARTILAGE
FOLLOWING DECALCIFICATION AND COLD-EMBEDDING IN PLASTIC

ABSTRACT

Tartrate-resistant acid phosphatase (TRAP) has been proposed as a cytochemical marker for osteoclasts. We have developed an improved technique for the localization of TRAP in rat and mouse bone and cartilage. This procedure employs JB-4 plastic as the embedding medium, permits decalcification, and results in improved morphology compared with frozen sections. Peritoneal lavage cells were used to determine the appropriate isomer and concentration of tartrate necessary for inhibition of tartrate-sensitive acid phosphatase. Following incubation in medium containing 50 mM L(+)-tartaric acid, osteoclasts and chondroclasts were heavily stained with reaction product. On the basis of their relative sensitivity to tartrate inhibition, three populations of mononuclear cells could also be distinguished. These three populations may represent: (1) heavily stained osteoclast/chondroclast precursors, (2) sparsely stained osteoblast-like cells lining the bone surface, and (3) unstained cells of monocyte/macrophage lineage. Our results are consistent with the use of TRAP as a histochemical marker for study of the osteoclast.

INTRODUCTION

On the basis of sensitivity to tartrate inhibition, two types of acid phosphatase can be identified in bone. Both forms have been biochemically characterized with respect to substrate specificity, pH optima, and have been shown to be functionally responsive to osteotropic hormones in vitro (Anderson and Toverud, 1982; Ibbotson et al., 1984; Minkin, 1982; Wergedal, 1970). The tartrate-sensitive acid phosphatase has been cytochemically localized in osteoblasts, osteocytes, and osteoclasts, while the tartrate-resistant acid phosphatase (TRAP) is present in osteoclasts (Hammarstrom et al., 1983; Minkin, 1982). Osteoclasts have been identified primarily by morphological criteria as large multinucleated cells which contact calcified bone matrix and exhibit ruffled borders surrounded by clear zones (Gothlin and Ericsson, 1976). Tartrate-resistant acid phosphatase as a histochemical and biochemical marker for the osteoclast would provide an additional experimental tool for studying osteoclastic differentiation and function. Baron et al. (1986) employed TRAP as such a marker for the cytochemical identification of proposed osteoclast precursors at both the light and electron microscopic levels.

Various investigators have used different techniques to localize TRAP within tissues making comparison of the results of the studies difficult. Unfixed, frozen sections have been used to localize bone TRAP in vivo (Hammarstrom, et al, 1983; Minkin, 1982). These methods

demonstrate enzyme product, but lack of fixation results in a loss of morphological detail. Chappard et al. (1983) describe a TRAP procedure which includes cold-embedding in a mixture of glycol and methyl methacrylates. This technique requires sectioning of undecalcified bone and the purification of methacrylates. We have incorporated glutaraldehyde fixation into a histochemical procedure based on the cold-embedding method of Namba et al. (1983) which utilizes commercially available JB-4 (Polysciences, Inc.) as the embedding medium eliminating the need for purification of methacrylate. In addition, our procedure permits the use of EDTA decalcified bone and cartilage for ease in sectioning. The description of this technique includes an evaluation of two isomers of tartaric acid, L(+)- and D(-)-, as effective inhibitors of tartrate-sensitive acid phosphatases. This evaluation was accomplished through the use of peritoneal lavage cells, the majority of which are macrophages known to contain tartrate-sensitive acid phosphatase (Schneider et al., 1981; Seifert, 1984). Rodent peritoneal macrophages appear to be capable of bone resorption in vitro and have been repeatedly investigated as a possible cell source of osteoclast precursors (McArthur et al., 1980; Teitelbaum et al., 1979). The use of peritoneal lavage cells, therefore, provides not only a means to evaluate the effective inhibition of tartrate-sensitive acid phosphatase, but also a means to compare the acid phosphatase characteristics of these proposed osteoclast precursors with those of *in situ* osteoclasts. Our procedure is an

attempt to provide a convenient and reproducible method for TRAP localization within skeletal tissues.

MATERIALS AND METHODS

Animals

All animals used for this study were obtained from breeding colonies housed in a centrally located, fully accredited animal care facility.

Fixation and Embedding

Except where indicated, all of the following procedures were conducted at 4°C (Namba et al., 1983). Proximal tibiae from 2 week old rats and proximal femurs including growth plates from 1 week old mice were removed and dissected free of adherent soft tissue. Tissues were fixed for 2 or 4 hours in either 3.7% formalin in phosphate buffer (pH 7.4) (Lillie, 1965) with 7% sucrose or in 2.5% cacodylate-buffered glutaraldehyde (pH 7.4) containing 7% sucrose, rinsed 3 times, and stored overnight (17-19 hr) in the appropriate buffer. Both bones and growth plates were decalcified for 48 hours in 10% EDTA in Tris buffer, pH 7.4 (Pearse, 1968). Undecalcified tissue was used as a control. The formalin-fixed tissue was dehydrated in 50, 75 and 95% (2 changes) acetone for 15 min each; the glutaraldehyde-fixed tissue was dehydrated in either 50, 75, and 95% (2 changes) ethanol or ace-

tone for 15 min each. The tissue was infiltrated overnight in JB-4 solution A with catalyst (Polysciences, Inc., Warrington, PA) and embedded in complete JB-4 medium in BEEM or gelatin capsules. During embedding, the capsule trays were placed on cracked ice to reduce the high temperature which accompanies JB-4 polymerization at room temperature. The blocks were allowed to polymerize overnight at 4°C. Sections (3 μ m) were cut dry at 25°C on a Dupont Sorvall JB-4 microtome with glass knives and placed on ice-cooled slides which had been alcohol-cleaned and gelatin-subbed. The sections were allowed to air-dry at 4°C for 5-7 days.

Preparation of Peritoneal lavage cells

Cells obtained by peritoneal lavages from adult mice and 4 week old rats were used as a control to determine the appropriate isomer and concentration of tartrate for inhibition of tartrate-sensitive acid phosphatase. The abdominal cavity was injected with 10 ml of 0.9% saline and massaged for 2 min. The peritoneal fluid was removed and centrifuged. The cell pellets were resuspended in 0.5 ml of 0.9% saline. The cells were smeared onto gelatin-coated glass slides, air-dried at 4°C, fixed in 3.7% formalin fumes for 5 min, and stored at 4°C.

Histochemical Staining

Burstone's complete medium for acid phosphatase (Pearse, 1968)

was prepared by dissolving 4 mg naphthol AS-BI phosphate substrate (Sigma, St. Louis, MO) in 0.25 ml of N,N-dimethyl formamide followed by the addition of 25 ml of 0.2M acetate buffer (pH 5.0), 35 mg of either Fast Red Violet LB or Fast Garnet GBC diazonium salt (Sigma) as the coupling agent, and 2 drops (60ul) of 10% $MgCl_2$. The media was then filtered into acid-cleaned Coplin jars. As a control, the substrate was omitted. In addition, sodium tartrate (Mallinckrodt, Paris, KY), L(+)-tartaric acid, disodium salt (Sigma), or D(-)-tartaric acid (Sigma) at concentrations of 1, 32.5, 50, or 100 mM were added to individual Coplin jars containing 25 ml of filtered, complete media in media prewarmed to 37°C. Both tissue sections and peritoneal lavage preparations were allowed to come to room temperature and incubated for 30 min to 3 hr. Following incubation, the slides were washed for 30 min in running water, allowed to air-dry at 25°C, counterstained with 1% aqueous Fast Green FCF (Fisher Scientific Co., Chicago, IL, C.I. 42053) for approximately 1 min. Cover slips were mounted with Euparal (Gallard-Schlesinger Chem. Mfg. Corp., Carle Place, N.Y.), and preparations were examined for the presence or absence of reaction product. For each isomer and concentration of tartrate, the percentage of stained peritoneal lavage cells per 1000 cells was calculated.

RESULTS

The red-maroon acid phosphatase reaction product was granular and confined to the cell cytoplasm in all tissues, except for some diffuse staining of the bone matrix adjacent to osteoclasts. No difference in reaction product or intensity was observed between 2 or 4 hour fixation or between phosphate-buffered formalin or cacodylate buffered glutaraldehyde. The 4 hour glutaraldehyde fixation demonstrated superior morphological preservation and is the preferred fixative. Using cacodylate-buffered glutaraldehyde also eliminates possible artifactual staining due to the presence of phosphate in the buffer. Decalcification of bone and growth plate for 48 hours at 4°C did not decrease the reaction product. When Fast Garnet GBC was used as the capture agent, the tissue non-specifically stained yellowish-orange, and a heavy, red precipitate often covered the sections. With Fast Red Violet LB, there was no non-specific staining of the tissue and very little precipitate adhered to sections incubated up to 90 min. Rat bone, incubated for 45 min, gave a strong reaction product; in mouse bone and growth plate a 90 min incubation was required to give the same intensity. No difference in reaction product was noted in sections from tissue blocks stored up to four months; however, after six months of storage, decreased reaction product was noted.

An attempt was made to define the inhibitory concentration of each isomeric form of tartrate by determining the concentration at

which reaction product was absent from peritoneal lavage cells. At 50 mM L(+)-tartaric acid, no reaction product was seen in peritoneal lavage cells (Table I) or in a population of bone marrow mononuclear cells located adjacent to blood vessels. Reaction product localized within tissue sections incubated with 50 mM L(+)-tartaric acid reflected the presence of TRAP in those tissues. Multinucleated cells immediately adjacent to bone or calcified cartilage matrix contained a heavy concentrated reaction product uniformly distributed throughout the cytoplasm (Fig. 1). The majority of mononuclear cells along the bone surface contained a few granules of reaction product which was not inhibited even at concentrations as high as 100 mM L(+)-tartaric acid (Fig. 2). In addition, small populations of mononuclear cells within the bone marrow and perichondrium (Fig. 3) exhibited a staining pattern similar to that of the multinucleated cells (Fig. 1).

DISCUSSION

This study describes a technique for TRAP localization in bone and cartilage based on a modification of the acid phosphatase technique of Namba et al. (1983). The recommended technique is:

- 1) fixation in 2.5% cacodylate-buffered glutaraldehyde,
- 2) decalcification in 10% buffered EDTA, 3) dehydration in acetone,
- 4) embedding in JB-4 medium, and 5) incubation in medium containing Naphthol AS-BI phosphate as substrate, Fast Red Violet as capture

agent and L(+)-tartaric acid (50 mM) to inhibit tartrate-sensitive acid phosphatase. This method results in an easily distinguished granular TRAP reaction product within the cells which remained stable over a period of months. The enzyme preservation in this technique may be due to cold polymerization in JB-4 (Namba et al. 1983), decalcification with cold buffered EDTA (Pearse, 1968) or the inclusion of sucrose in the solutions used in tissue processing. The addition of sucrose has been shown to prevent osmotic damage to cells during processing (Pearse, 1968) and is recommended for enzyme preservation (Holt, 1959).

In frozen sections of bone, Hammarstrom et al. (1983) described an intense TRAP reaction in osteoclasts and a weak TRAP reaction in osteoblasts following incubation with 100 mM sodium tartrate. Chappard et al. (1983), using glycol and methyl methacrylate embedded sections and 1 mM L(+)-tartaric acid, localized TRAP in osteoclasts but not in osteoblasts. Baron et al. (1986) employing an alveolar bone model to study osteoclastic lineage, identified TRAP positive osteoclasts and mononuclear cells with 10 mM sodium tartrate inhibition. The authors describe the TRAP containing mononuclear cells as members of the mononuclear phagocyte system and probable osteoclast precursors. Our study using plastic embedded sections and 50 mM L(+)-tartaric acid also demonstrated the intense localization of TRAP in osteoclasts. Our results differ from those of Chappard et al. (1983) in that mononuclear cells lining bone also contained granules

of reaction product although in decreased amounts as compared to that found in the osteoclasts. We were able to distinguish populations of mononuclear cells present in the bone marrow and perichondrium exhibiting the same concentration of reaction product as found in osteoclasts. These cells may represent osteoclast/chondroclast precursors. A third type of mononuclear cell can be identified based on complete inhibition by tartrate. These tartrate-sensitive cells, like the peritoneal lavage macrophages, may represent members of the monocyte-macrophage lineage. Walters and Schneider (1986) examined the TRAP-staining characteristics of the cell populations recruited to subcutaneously implanted bone and suture. The tissue from the implant studies was processed through gluteraldehyde fixation, EDTA decalcification and JB-4 embedding procedures identical to those used in processing the bone and cartilage sections of the present study. They found that the macrophage populations surrounding the implants displayed similar acid phosphatase inhibition with 50 mM L(+)-tartaric acid. Peritoneal lavage cells, tissue macrophages and some bone marrow mononuclear cells, therefore, exhibit similar patterns of tartrate inhibition with 50 mM L(+)-tartaric acid while the osteoclast does not. With our technique, then, we are able to distinguish the osteoclast from some members of the mononuclear phagocyte system. Our findings, therefore, differ from those of Baron et al. (1986) who were unable to distinguish between members of the mononuclear phagocytic system and osteoclasts. This difference could possibly be explained

by their use of 10 mM tartrate rather than our higher 50 mM concentration. On the basis of these observations, our studies are consistent with the use of TRAP as a histochemical marker for the study of osteoclastic differentiation and lineage.

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TABLE I. PERITONEAL LAVAGE CELLS STAINED WITH TARTRATE-RESISTANT ACID PHOSPHATASE

<u>INCUBATION MEDIA</u>	<u>% STAINED CELLS*</u>
Control	0.00
Complete Media	87.60
with 1.0 mM Sodium Tartrate	74.77
32.5 mM	1.20
50.0 mM	0.20
with 1.0 mM L(+)-tartaric acid	48.70
32.5 mM	1.00
50.0 mM	0.00
with 1.0 mM D(-)-tartaric acid	80.96
32.5 mM	80.22
50.0 mM	82.70

*Determined from samples of 1000 cells

Figure 1.

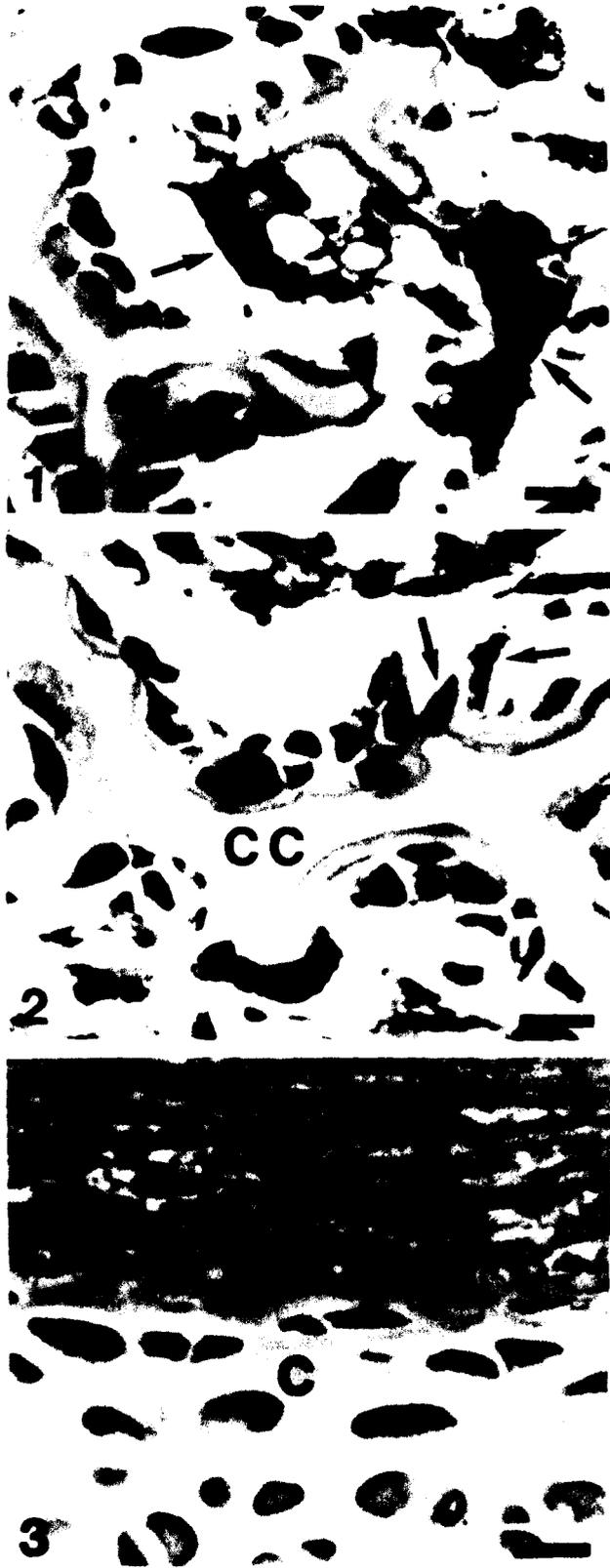
Section of 2 week old rat tibia, counterstained with Fast Green, demonstrating osteoclasts (arrows) stained intensely with granular TRAP reaction product. Original magnification x160
Bar = 11.3um

Figure 2.

Trabecular bone (rat) with calcified cartilage core (CC) lined by mononuclear cells containing a few intensely stained TRAP granules (arrows). Note the heavily stained osteoclastic profile at the bottom of the field. Counterstained with Fast Green. Original magnification x160 Bar = 10.4um

Figure 3.

Heavily stained mononuclear cells (arrows) located in mouse perichondrium (P) adjacent to cartilage matrix (C). Chondrocytes are negative for reaction product. Counterstained with Fast Green. Original magnification x160 Bar = 13.8um



APPROVAL SHEET

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The final copies have been examined by the director of the dissertation and the signature which appears below verifies the fact that any necessary changes have been incorporated and the dissertation is now given final approval by the committee with reference to content and form.

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

11/14/86

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