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## Lectin-Mediated Effects on Bone Resorption in Vitro: A Morphological and Functional Study

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LECTIN-MEDIATED EFFECTS ON BONE RESORPTION IN VITRO:  
A MORPHOLOGICAL AND FUNCTIONAL STUDY

by

Steven N. Popoff

A Dissertation Submitted to the Graduate School  
of Loyola University of Chicago  
in Partial Fulfillment of the  
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## VITA

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The author is married to Kathleen Popoff.

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## CHAPTER I

### INTRODUCTION

Lectins have been used in a variety of biological assays to study the surface structure and function of cells and tissues. Lectins are proteins that are highly specific in their saccharide binding requirements as demonstrated by hapten inhibition studies where the interactions between lectins and cell surface glycoconjugates can be inhibited by simple sugars (Sharon and Lis, 1972). In addition, many lectins exhibit differential binding affinities for various mammalian cells due to cell-specific differences in the expression of membrane glycoconjugates. These properties promote the use of lectins as probes to study the structure and activity of cell surface components in both normal and pathologically-altered tissues. In biological assays, lectins have been used to mimic the functional effects of documented, physiological mediators of cellular activity, such as hormones, lymphokines and vitamins. Therefore, lectins have been used as in vitro model systems to evaluate the mechanisms of cellular activation and/or inhibition.

Although the biological effects of many lectins have been investigated on a variety of cellular systems both in vivo and in vitro, few studies have used lectins to evaluate the structure and

function of the skeletal system. The studies involving the skeletal system (Wang et al., 1982; Horton et al., 1982; Ryder et al., 1982) indicate that some lectins (i.e. PHA-P and con A) can induce changes in the structure and function of bone cells, particularly osteoclasts. The osteoclast is a large multinucleate cell that is widely accepted as the primary effector cell responsible for normal bone resorption (Marks, 1983). Although the definitive origin of the osteoclast remains uncertain, there is abundant evidence to support the hypothesis that osteoclasts are derived from mononuclear cells of hemopoietic origin, possibly of the monocytic lineage (Walker, 1975; Marks and Walker, 1981; Schneider and Byrnes, 1983).

Bone-organ cultures of long bones or calvariae from neonatal rodents are model systems used to study the morphological and functional effects of biologically active mediators on osteoclastic bone resorbing activity. This type of culture system has the advantage of correlating changes in morphology with altered functional activity as compared to other culture systems in which the cells being studied are not on their normal substrate. Reynolds and Dingle (1970) reported several advantages in using organ cultures of rodent calvariae in comparison to organ cultures of long bones. The distribution and numbers of osteoclasts on the inner surface of the flat bones of the growing rodent skull are well-documented (Barnicott, 1947; Abe et al., 1983; Jones and Boyde, 1976 Jones and Boyde, 1977).

The first part of this dissertation is designed to investigate

the biological effects of various lectins on bone resorption using a calvarial culture system. The remaining studies are designed to evaluate possible mechanisms responsible for lectin-induced changes in the ability of osteoclasts to resorb bone. Concanavalin A (con A) is the most widely used lectin in biological assays and many of its properties have been well-documented and thoroughly investigated. Other lectins that are frequently used to study the structure and function of cells and tissues include wheat germ agglutinin (WGA), soybean agglutinin (SBA) and peanut agglutinin (PNA). The saccharide binding specificities of these four lectins are distinctly different from one another and the sugar residues to which these lectins bind are common to mammalian membrane-associated glycoconjugates.

The four lectins, con A, WGA, SBA and PNA, were used in conjunction with a  $^{45}\text{Ca}$  pre-labeled calvarial-organ culture system to assess their effects on bone resorption. However, the primary emphasis throughout this dissertation focuses on the lectin, con A. In the first study, we treated osteoclasts and osteoblasts on the endocranial surface of 10-day old rat calvariae with con A and hemocyanin to determine whether or not specific con A binding sites are present on these bone cell membranes as visualized by scanning electron microscopy (SEM). In addition, we conducted a series of  $^{45}\text{Ca}$  bone release assays using pre-labeled calvariae to evaluate the effects of various concentrations of con A on the bone resorbing capacity of osteoclasts. After determining the con A-mediated

effects, we were interested in the effects of the other 3 lectins, WGA, SBA and PNA, on  $^{45}\text{Ca}$  release in the calvarial culture system. In the second study, we conducted another series of  $^{45}\text{Ca}$  bone release assays using a lectin concentration of 50  $\mu\text{g}/\text{ml}$  for each of the lectins to be tested. The 50  $\mu\text{g}/\text{ml}$  lectin concentration was chosen on the basis of the results obtained from the first study.

The third and fourth studies are designed to gain some insight into the mechanisms that may be responsible for lectin induced changes in bone resorption and osteoclastic function. Because of the difficulty in obtaining pure populations of osteoclasts, most studies on bone resorption are performed on intact animals or bone-organ cultures. These systems do not permit detailed analysis of the cellular mechanisms of bone resorption or of the means whereby resorbing cells attach to the bone surface (Teitelbaum et al., 1979). Since monocytes and macrophages are capable of attaching to and subsequently degrading a bone matrix, the macrophage-devitalized bone culture system serves as a useful model to study the mechanisms of bone resorption in tissue culture. (Kahn et al., 1978; Teitelbaum et al., 1979; Bar-Shavit et al., 1983a).

In the third study, we used a macrophage-devitalized bone culture system to evaluate the effects of 2 lectins, con A and SBA, on the morphology of macrophage attachment to a devitalized bone surface and the subsequent functional ability of these lectin-treated cells to mobilize bone mineral. SEM was used to assess the qualitative and

quantitative differences in the attachment of macrophages to bone in the presence and absence of lectin. Furthermore,  $^{45}\text{Ca}$  bone release assays were conducted to evaluate the functional significance of the morphological findings.

The induction of many of the functional effects by cell-specific mediators of cellular activity (ligands) requires the internalization of ligand-receptor complexes subsequent to the binding of a particular ligand to specific membrane receptors. The internalization of a cell-bound ligand usually includes an initial redistribution followed by endocytosis of the ligand-receptor complexes. These metabolically-dependent events have been studied in a variety of substrate-attached mononuclear cell cultures. Some studies have employed the use of biologically active lectins conjugated to ultrastructural and cytochemical marker molecules to follow the movement of the lectin-receptor complexes both on the cell surface and in the cytoplasm. In the fourth study, we investigated the processing of con A-receptor complexes by osteoclasts in culture. Con A conjugated to fluorescein isothiocyanate (con A-FITC) was used to evaluate the redistribution of cell-bound con A via epifluorescence microscopy. In addition, an ultrastructural study using con A-ferritin was conducted to determine whether these lectin-receptor complexes are internalized. The endocytosis of lectin-receptor complexes may represent a general mechanism that is used by osteoclasts to process other endogenous physiological agents that bind

directly to the osteoclast membrane thereby regulating osteoclastic activity.



## CHAPTER II

### REVIEW OF RELATED LITERATURE

#### Lectins - General Review

Lectins, the term proposed by Boyd in 1970, are proteins possessing the ability to agglutinate red blood cells as well as a variety of other cell types. They are isolated from a wide variety of sources, ranging from the seeds of plants to the horseshoe crab. Besides their agglutinating ability, lectins exhibit a host of other interesting biological properties (Lis & Sharon, 1973). Some lectins are specific in their reaction with human blood groups and are used in the typing of human blood and to study the chemical structure of substances defining blood groups. Certain lectins are mitogenic; they can stimulate the transformation of small resting lymphocytes into large blast-like cells which undergo mitotic division. This polyclonal stimulation provides an important tool for examination of the biochemical events involved in the conversion of a resting cell into an active, dividing cell. Lectins have also been used in cytogenetics to study the relationships between chromosome abnormality and human diseases.

Lectins bind to specific saccharides on the cell surface, providing a useful tool to study cell surface architecture. Lectins

specifically precipitate polysaccharides and glycoproteins, a property useful in the isolation and purification of carbohydrate-containing polymers (Sharon & Lis, 1972). By virtue of their specific interactions with polysaccharides and glycoproteins, they have served as a useful model to study the antigen-antibody reaction. There has been a surge of interest in lectins among investigators engaged in cancer research. Some lectins, such as con A, WGA and SBA, preferentially agglutinate transformed mammalian cells grown in tissue culture, indicating that the cell surface of a transformed cell differs significantly from the surface of its untransformed counterpart (Inbar & Sachs, 1969a; Inbar & Sachs, 1969b; Burger & Goldberg, 1967; Sela et al., 1970). The use of lectins to inhibit the growth of malignant cells in vivo and in vitro (Burger & Noonan, 1970; Shoham et al., 1970), and the use of lectin-binding synthetic polymers to immunize mice against various tumors (Shier, 1971), are examples of the potential clinical usefulness of lectins in oncology. Recently, lectin histochemistry has been used in developmental biology to evaluate changes in cell surface glycoconjugates during the development of various tissues and organ systems (Wu et al., 1983; Lee and Damjanov, 1984; Farnum and Wilsman, 1984; Wu et al., 1984).

Green et al. (1981) and Fleisher et al. (1981) have shown that con A-activated peripheral blood mononuclear cells produce a soluble immune suppressor supernatant (SISS) which contains certain factors that are capable of suppressing immune responses in vitro. Barrett et

al. (1983) reported that WGA could inhibit lymphocyte blastogenesis, induced by a variety of mitogenic lectins. Thus, WGA-mediated inhibition of mitogenesis was found to mimic the suppressive effect of SISS on lymphocyte blastogenesis. They also demonstrated that the inhibition of mitogenesis induced by either WGA or SISS could be blocked by the addition of N-acetylgalactosamine, the specific sugar inhibitor of WGA binding. Furthermore, WGA and SISS compete with one another for the same cell surface receptor site. These findings support the hypothesis that inhibitory or suppressive signals for lymphocyte activation can be generated by the binding of soluble saccharide-specific factors that interact with cell surface glycoconjugates (Green et al., 1981; Fleisher et al., 1981). A study by Leu et al. (1980) compared a lectin, fucose binding protein (FBP), with a lymphokine, migration inhibitory factor (MIF), for its binding interactions with peritoneal macrophages using a migration inhibition assay. Their results demonstrated that both FBP and MIF inhibited the migration of macrophages in similar dose-response profiles. In addition, L-fucose, the specific sugar inhibitor of FBP, blocked the inhibitory effect of both FBP and MIF on macrophage migration. Therefore, the interaction of exogenous lectins or endogenous lymphokines with saccharide-associated cell surface receptors may represent a common mechanism by which soluble factors regulate immunological responses. All of these studies provide evidence to substantiate the use of certain lectins as in vitro models for

saccharide-specific, biologically relevant mediators of cellular activity. This conclusion lends some additional credibility to the possibility that lectin-induced changes in cellular morphology and function, may mimic the effects of well-documented, physiological agents that regulate the structure and function of cells in vivo.

The study of lectins was initiated by Stillmark in 1888, when he described the agglutination of erythrocytes by plant extracts. He found that castor beans contained a toxic protein, which he named ricin, capable of agglutinating both human and animal red blood cells. Ehrlich used ricin to study and describe a fundamental principle of immunology. In 1891, he reported that mice could be immunized against a lethal dose of ricin by repeated subcutaneous injections of minute amounts of the toxin, and that the serum of these immunized mice was capable of neutralizing the toxin. In 1908, Landsteiner and Raubitschek demonstrated that various seed extracts exhibited differential agglutinating activities when tested with erythrocytes from different animals, and they compared this specificity with that of animal serum antibodies. Although it was originally presumed that plant hemagglutinins were non-specific with respect to blood groups, these initial observations by Landsteiner and Raubitschek (1908) led to the discovery that certain lectins are specific for one or more human blood group antigens (Boyd & Reguera, 1949).

Most of the initial studies using lectins were conducted using crude extracts of plant origin. Although the first purified lectin,

concanavalin A, was obtained in crystalline form from the jack bean by Sumner as early as 1919, it was not until 1936 that Sumner and Howell showed that purified con A agglutinated horse red blood cells at concentrations as low as  $0.1 \mu\text{g/ml}$ , thus linking the agglutinating activity of the jack bean extract with con A. Since then, con A has become the most widely used lectin in biological assays. Other lectins which have been widely used to study the structure and function of various cellular systems include WGA, SBA and, more recently, PNA. These three lectins have also been isolated in purified form and their chemical composition determined.

Lectins are highly specific in their cell surface saccharide binding requirements. This conclusion is based on hapten inhibition studies which demonstrate that the interactions of lectins with cells can be specifically inhibited by simple sugars. Some lectins discriminate between  $\alpha$ - and  $\beta$ -anomers of a particular sugar residue, while others bind to both the  $\alpha$ - and  $\beta$ -anomeric structures with equal affinity. Some lectins require that the binding sugar be located in a terminal non-reducing position in the oligosaccharide chain, while others can bind to sugars within the oligosaccharide chain. The affinity between a lectin and its binding site can exhibit a great deal of variation due to small changes in the sequence of sugars within an oligosaccharide. Because of the binding specificity that each lectin has for a particular carbohydrate structure, even oligosaccharides with identical sugar compositions can be separated

and distinguished from one another.

Most lectins that have been isolated and analyzed to date are composed of multiple subunits, which are held together by non-covalent bonds. These subunits are polypeptide chains that usually contain covalently bound carbohydrate side chains. Con A, WGA, SBA and PNA are multimeric lectins; con A and PNA are each composed of 4 identical subunits, whereas WGA and SBA are each composed of 2 identical subunits. SBA and PNA are glycoproteins, and the sugars which have been isolated from these lectins are common to animal cell membranes. In contrast, con A and WGA are devoid of covalently bound carbohydrate side chains. It is the multimeric structure of lectins that allows them to agglutinate cells and form precipitates with glycoconjugates in a manner similar to antigen-antibody interactions. Although most lectins can bind to a variety of different cell types, agglutination of cells occurs only in some cell types. On the other hand, some lectins do not bind to particular cell types, which is probably due to the absence or masking of the suitable receptor oligosaccharides on the cell surface.

The sugar specificities of con A, WGA, SBA and PNA are distinctly different from one another. All of the sugar residues to which these lectins bind are common to many mammalian membrane-associated glycoconjugates. Con A has a broad biological applicability primarily because it recognizes 2 commonly occurring cell surface saccharides,  $\alpha$ -D-mannose and  $\alpha$ -D-glucose. Although it was originally postulated

that the specificity of con A is directed toward a single sugar residue, studies by So et al. (1968) demonstrated that  $\alpha$ -linked mannose disaccharides and trisaccharides are stronger inhibitors of the con A-dextran interaction by 5 and 20 times respectively, than is  $\alpha$ -methylmannoside, the haptenic sugar inhibitor of con A binding. These results suggest that the con A binding site is more complex in both its primary structure and three-dimensional configuration than originally proposed. WGA can bind oligosaccharides containing terminal N-acetylglucosamine (GluNAc) residues, with preferential binding to dimers and trimers of this saccharide. SBA preferentially binds to oligosaccharides with terminal  $\alpha$ - or  $\beta$ -linked N-acetylgalactosamine (GalNAc) residues, and to a lesser extent to D-galactose residues. PNA binding is directed toward D-galactose residues, although this lectin binds to the disaccharide, galactosyl  $\beta$ -1,3 GalNAc, with greatest affinity. This disaccharide is called the T-antigen, and its presence on the surface of human erythrocytes is responsible for a phenomenon known as T-polyagglutination, a problem encountered in routine serology. T-polyagglutination, the agglutination of red blood cells irrespective of blood type, is caused as a result of in vitro contamination of blood specimens by the degradative action of bacterial neuraminidase which exposes the T determinant (Goldstein & Hayes, 1978). The discovery of PNA has greatly simplified the determination of T-polyagglutination in blood specimens.

### Processing of Concanavalin A

Since con A is the most widely used lectin in biological assays, it has been used extensively as a probe to study cell surface interactions. The various biological activities of con A all appear to involve initial binding of con A to the cell surface. The manner in which cells process this cell-bound lectin has been the focus of a great number of investigations. Although various cell types have been used in these studies, most of the observations have been made using lymphocytes, since it is well documented that con A is mitogenic for lymphocytes. Taylor et al. (1971) demonstrated cap formation of surface immunoglobulin (Ig) determinants on mouse lymphocytes induced by the interaction of FITC-labeled anti-Ig antibodies with the Ig molecules of the cell membrane. Following capping, the surface Ig determinants were pinocytosed. Since this initial discovery, antibody-induced patch and cap formation has been described in various cell types. In 1972, Yahara and Edelman reported that con A prevents anti-Ig patch and cap formation, presumably by restricting the mobility of cell surface Ig determinants. Karnovsky and Unanue (1973) reported that the redistribution of con A receptors, induced by the binding of con A, did not alter the distribution of surface Ig molecules and vice versa. These findings indicate that the inhibition of patching and capping by con A cannot be attributed to the linkage of mobile receptors to immobile con A receptors by con A, nor to



extensive cross-linkage of various specific receptors by con A. Gunther et al. (1973) observed that divalent succinylated con A cannot inhibit receptor mobility although it directly competes with its non-derivatized counterpart for the same binding sites. These observations suggest that the binding valency of the lectin is an important factor in the inhibition of receptor mobility and the cross-linkage of receptor molecules is a necessary condition for inhibition of receptor mobility.

In contrast to its inhibitory effects on patch and cap formation, con A has been shown to induce patching and capping of its own binding sites under certain conditions of incubation. Unanue et al. (1972) used FITC-con A to demonstrate that patching and capping of con A binding sites occurred when lymphocytes were labeled with con A at 4°C, then washed to remove unattached con A molecules, followed by incubation at 37°C. Yahara and Edelman (1973) showed that lymphocytes incubated at 37°C in concentrations of con A above 10 µg/ml did not exhibit patch or cap formation, whereas at concentrations below 10 µg/ml capping of con A binding sites occurred in a small percentage of cells. In contrast to the findings observed at 37°C, when lymphocytes were pre-incubated with con A at 4°C, then washed to remove excess con A and incubated at 37°C, patch and cap formation was observed in a large percentage of lymphocytes regardless of the concentration of con A. These observations were confirmed by Bhalla et al. (1979) who demonstrated that if washing is omitted after

pre-incubation at 4°C or if con A is added again after washing, the percentage of cells forming caps at 37°C is drastically reduced. These results indicate that depending on the conditions of incubation employed, con A exhibits 2 antagonistic properties when bound to lymphocytes. Con A exhibits the ability to induce cap formation of its own binding sites and it can also inhibit the redistribution of ligand-receptor complexes including that of its own.

Since it has been postulated that some of the biological effects of con A initiated at the cell surface may be a result of its multiple binding valency, it is necessary to compare the effects of different forms of this molecule, each having the same binding specificity but different valence. Wang and Edelman (1978) reported the preparation and characterization of monovalent, divalent and tetravalent con A molecules that are stable at physiological pH. They demonstrated that all three derivatives were potent mitogens for mouse splenic lymphocytes suggesting that multipoint attachment and cell surface receptor bridging are not necessary for lymphocyte activation. In contrast, at concentrations above the mitogenic optimum, only tetravalent con A was capable of inhibiting both cap formation and cell proliferation. These results indicate that the inhibition of binding site mobility and DNA synthesis does require extensive cross-linkage of con A binding sites. The observations made in this study can explain the inconsistent observations of Greaves et al. (1972) and Sallstrom et al. (1972), who reported that a large

percentage of lymphocytes incubated at 37°C in high concentrations of con A exhibited patch and cap formation. It has been shown that the fluorescein-labeled con A molecules used by these investigators were irreversibly denatured, and therefore, these molecules were either monovalent or divalent derivatives. The observations that con A can bind to various molecules that do not contain any saccharide residues in their structure led to the identification of a hydrophobic binding site within the con A molecule that is distinct from and independent of its saccharide binding site (Edelman & Wang, 1978). After con A binds to the cell surface via its saccharide binding site it is capable of interacting with hydrophobic ligands; this secondary interaction may induce surface alterations necessary for some of the biological effects mediated by this lectin.

Although cross-linkage of receptor sites can partially account for the ability of con A to inhibit the redistribution of ligand-receptor complexes including that of its own, this event does not account for the difference in the number of cap forming cells following pre-incubation at 4°C as compared to cells incubated at 37°C. It is well known that antimitotic agents, such as colchicine and vinblastine, or incubation in cold (4°C) will cause the disruption of microtubules. Numerous studies have demonstrated that microtubule disassembly is directly correlated with enhanced con A cap formation on lymphocytes and other cell types (Edelman et al., 1973; Unanue & Karnovsky, 1974; Oliver, 1976; Bhalla et al., 1979; Oliver et al.,

1980). These observations suggest a role for microtubules in limiting the lateral movement of con A-binding site complexes within the membrane. Edelman et al. (1973) have proposed an hypothesis that relates con A-mediated cell surface events to colchicine-sensitive binding proteins (CBP) in the cytoplasm. These proteins may either be the components of microtubules or be closely related to microtubular proteins. They suggest that con A binding sites may occur in 2 states; free (not attached to CBP) or attached (anchored to CBP). Thus, the treatment of cells with an agent that disrupts microtubules allows greater mobility because the equilibrium between these two receptor states shifts greatly in favor of the free receptors. This hypothesis provides an explanation for the increase in con A-induced cap formation observed when cells are pre-labeled at 4°C or treated with a microtubular poison.

Microfilaments have also been implicated in lectin-mediated cell surface activation. Studies have demonstrated that the aggregation of con A-binding site complexes into caps can be prevented by metabolic inhibitors and by agents, such as cytochalasin B, that impair microfilament function (Ryan et al., 1974; dePetris, 1975; Schreiner & Unanue, 1976). Condeelis (1979) isolated con A cap-associated membrane fragments at various stages of formation and demonstrated a submembranous network of microfilaments in association with these capped membrane fragments. These studies support the hypothesis that con A patch and cap formation depends on active contractile movements

of microfilaments. Although con A caps are underlain by a dense network of microfilaments in lymphocytes and other cell types, Albertini et al. (1977) demonstrated that the accumulation of microfilaments in the region of the eventual site of con A cap formation is not induced by con A. Instead, they showed that the accumulation of microfilaments follows the disruption of microtubules. Their results indicate that the aggregation of microfilaments and con A cap formation are independent events. Once the cap is formed, interactions between con A-binding site complexes and microfilaments appear to stabilize the localization of both the surface-bound lectin and the submembranous network of microfilaments. The subsequent internalization of con A-binding site complexes is a metabolically dependent event (Smith & Hollers, 1970), and it is believed that the interaction between surface-bound lectin and underlying microfilaments is necessary for the endocytosis of these complexes. Once con A has been internalized there are several possible mechanisms by which the lectin can mediate some of its biological effects.

#### Biological Effects of Lectins on the Skeletal System

The great number of studies examining con A binding and subsequent processing by lymphocytes have provided hypotheses that attempt to explain the mechanisms by which lectins interact with cell surfaces, thereby altering the structure and activity of membrane components. Aside from the mechanisms which account for lectin-cell

surface interactions, the biological effects of many lectins on a variety of cellular systems have been investigated both in vivo and in vitro. However, only a few studies have used lectins as probes to investigate their effects on the structure and function of the skeletal system. Wang et al. (1982) studied the effects of PHA-P, a mitogen known to selectively stimulate cells of hematogenous or lymphoid monocytic origin, on bone of the growing rat. They demonstrated that the in vivo administration of PHA-P for 15 days caused a reduction in the number of osteoblasts, whereas the number of osteoclasts, the number of  $^3\text{H}$ -labeled osteoclastic nuclei and the average number of nuclei per osteoclast were all significantly increased. They suggest that the increase in the number and size of osteoclasts following in vivo administration of PHA-P is caused by PHA-P induced osteoclast progenitor cell proliferation and the enhanced fusion and differentiation of these progenitor cells into osteoclasts. Horton et al. (1982) in their  $^{45}\text{Ca}$  bone release assays, demonstrated a decrease in resorption of long bones cultured in the presence of high concentrations of con A (50 & 100  $\mu\text{g}/\text{ml}$ ). Subsequent TEM of osteoclasts incubated in 50  $\mu\text{g}/\text{ml}$  con A revealed ultrastructural changes associated with inhibition of osteoclastic activity (Ryder et al., 1982).

The studies involving the skeletal system indicate that some lectins can induce changes in the structure and function of bone cells, particularly osteoclasts. Thus, lectins may prove to be useful

probes to study changes in osteoclast morphology in relation to changes in their bone resorbing activity. Organ culture of calvaria provides an ideal system for studying the effects of lectins on the structure and function of resident osteoclasts on their normal substrate. The endosteal surface represents an easily accessible site to study cell surface receptors on osteoclasts as well as changes in osteoclast morphology as visualized by both SEM and TEM. SEM can be used to study changes in cell surface morphology, whereas TEM can be used to investigate both surface and cytoplasmic changes within the osteoclast. Calvaria pre-labeled with  $^{45}\text{Ca}$  can be used to study the effects of lectins on bone resorption in vitro.

### Biology of the Osteoclast

The osteoclast is a large multinucleate cell that is widely accepted as the primary effector cell responsible for bone resorption (Marks, 1983). Ultrastructural changes associated with hormonal regulation of osteoclastic activity have been well documented both in vivo (Holtrop et al., 1979) and in vitro (King et al., 1978; Wezeman et al., 1979). Recently, various morphological changes of inhibited osteoclasts cultured in the presence of calcitonin and prostacyclin have been described using phase contrast microscopy (Chambers & Dunn, 1982; Chambers & Magnus, 1982; Chambers & Moore, 1983). Similarly, morphological characteristics of presumably active or inhibited osteoclasts, as visualized by SEM, have been described both in situ

(Jones & Boyde, 1977) and in vitro (Allen et al., 1981). Despite all of the observed morphological changes associated with various known mediators of osteoclastic activity, little is known about the mechanisms by which these hormones exert their effects.

Recently, numerous investigators have probed the osteoclast membrane to determine whether or not specific receptors for parathyroid hormone (PTH) and calcitonin (CT), the two primary hormones regulating osteoclastic function, are present. In an in vivo study utilizing  $^{125}\text{I}$ -calcitonin, localization of calcitonin receptors on the osteoclast membrane has been demonstrated (Warshawsky et al., 1980). Calcitonin receptors have also been demonstrated on osteoclasts by immunohistochemical staining (Rao et al., 1981). Chambers and Magnus (1982) confirmed the localization of CT receptors on osteoclasts by demonstrating that calcitonin causes complete inhibition of cytoplasmic motility in isolated osteoclasts in vitro. In contrast, light microscopic autoradiographs of chick embryonic calvaria after exposure to  $^{125}\text{I}$ -parathyroid hormone (PTH; 1-34) have revealed PTH receptors on osteoblasts, but not osteoclasts (Silve et al., 1982). The lack of PTH receptors on osteoclasts is also supported by the recent findings of Chambers and Dunn (1982); the addition of PTH to cultures of isolated osteoclasts caused no change in their behavior or morphology. Similarly, PTH added to isolated osteoclasts that were previously inhibited by CT did not oppose the morphological inhibitory changes induced by calcitonin (Chambers &



Moore, 1983). These results support the hypothesis that some hormones may act directly on osteoclasts (i.e. CT), while others may regulate osteoclastic activity indirectly (i.e. PTH) through the intermediary of another cell type (Chambers & Dunn, 1983).

Besides PTH and CT, several studies have identified other hormones and biologically active molecules that can mediate bone resorption in vitro. The mechanism by which mitogenic lectins stimulate lymphocyte production of osteoclast activating factor (OAF) has been well documented (Horton et al., 1972; Horton et al., 1974; Trummel et al., 1975; Yoneda et al., 1979). Horton et al. (1974) showed that OAF production by lymphocytes depends on an interaction between macrophages and lymphocytes. The release of OAF by lymphocytes stimulates osteoclastic activity resulting in an increased resorption of bone. Gowen et al. (1983) demonstrated that the monokine, interleukin 1, stimulates bone resorption in vitro. Klein and Raisz (1970) demonstrated that the effects of several prostaglandins on bone resorption were similar to those of PTH in stimulating the resorption of fetal long bones in vitro. In 1972, Raisz et al. showed that 1 $\alpha$ ,25 dihydroxyvitamin D<sub>3</sub> (1 $\alpha$ ,25(OH)<sub>2</sub>D<sub>3</sub>) is a potent stimulator for the release of previously incorporated <sup>45</sup>Ca from fetal long bones in culture. Nimberg et al. (1981) recently purified and characterized a glycoprotein from cancer ascites fluid which stimulates the resorption of bone explants in vitro. These findings suggest that the localized osteolysis seen in patients with a wide

variety of neoplasms may be mediated by bone-resorbing humoral factors. Chambers and Dunn (1983) showed that the addition of  $\text{PGE}_2$  or  $1\alpha,25(\text{OH})_2\text{D}_3$  to cultures of isolated osteoclasts caused no change in their behavior or morphology. Since numerous investigators have observed that prostaglandins and  $1\alpha,25(\text{OH})_2\text{D}_3$  have a variety of effects on osteoblasts, a role for osteoblasts in the hormonal control of bone resorption has been postulated (Rodan & Martin, 1981).

The hypothesis that the osteoclast precursor is extraskeletal in origin has been recently accepted (Marks, 1983). Although the definitive origin of the osteoclast remains uncertain, there is abundant evidence to support the hypothesis that osteoclasts are derived from mononuclear cells of hemopoietic origin, possibly of the monocytic lineage (Walker, 1975; Marks and Walker, 1981; Schneider and Byrnes, 1983). This hypothesis has created an extensive investigation to determine if these 2 distinct cell types share a common lineage. Studies have focused on both structural and functional similarities between these two cell types.

Some investigators have studied the cell surface of both osteoclasts and macrophage mononuclear cells to determine whether or not certain receptor sites known to exist on macrophages can also be demonstrated on osteoclasts and vice versa. The findings of one study supporting the presence of common membrane determinants in these two cell types, indicated that elicited peritoneal mononuclear phagocytes possess functional receptors for both PTH and CT (Minkin et al.,

1977). In contrast, although Fc and C3 receptors are normally present on macrophages, no one has been able to demonstrate these receptors on osteoclasts either in situ (Shapiro et al., 1979; Hogg et al., 1979) or in vitro (Chambers, 1979). Other studies have focused on functional similarities between the osteoclast and the macrophage mononuclear cell. Martini et al. (1982) used an in vitro assay to quantitate the attachment of osteoclasts and monocytes to developing bone. Their results demonstrated that twice as many osteoclasts and monocytes adhere to bone as compared to fibroblasts or mesenchymal cells. These observations suggest that osteoclasts and monocytes have a higher affinity for bone than cells that are unrelated to a bone substrate. Zallone et al. (1983) demonstrated that osteoclasts and monocytes exhibit similarities in their intracellular organization of cytoskeletal elements and adhesive properties in vitro.

#### Macrophage Mediated Bone Degradation

In addition to the many studies which have attempted to establish a common lineage between the osteoclast and the macrophage mononuclear cell, it has been well-documented that cells of the mononuclear phagocyte system are capable of resorbing devitalized bone in vitro (Mundy et al., 1977; Kahn et al., 1978; Teitelbaum et al., 1979). Kahn et al. (1978) demonstrated that peripheral blood monocytes are capable of resorbing devitalized bone in culture and that an important component of this process involves the attachment of the monocytes to

the bone matrix. Teitelbaum et al. (1979) developed an experimental system in which bone resorption by mononuclear cells can be precisely quantitated via the release of either  $^3\text{H}$ -proline or  $^{45}\text{Ca}$  from pre-labeled bone. Their results also indicated that optimal mobilization of the radioactive label by mononuclear cells requires cell-matrix contact. In contrast to these findings, Mundy et al. (1977) reported that physical contact between peripheral blood monocytes and bone in tissue culture was not a necessary condition for the release of bone mineral and matrix.

Glowacki et al. (1981) recently developed a model system in which they implanted devitalized bone fragments into calvarial defects in rats. These implants initiated a rapid cell-mediated resorptive sequence. A histological and ultrastructural study of these implants by Holtrop et al. (1982) demonstrated that the cells surrounding the bone fragments belong to the mononuclear phagocyte system. However, none of these cells, even the multinucleate giant cells, had ruffled borders, a morphological hallmark of osteoclasts, and therefore, could not be classified as osteoclasts. Other studies have also demonstrated that macrophages attached to bone acquire a number of the morphological characteristics of osteoclasts in culture, with the exception of ruffled borders (Kahn et al., 1978; Rifkin et al., 1979). Severson (1983) took mononuclear cells isolated from the spleen or bone marrow of young rats and injected them into diffusion chambers containing devitalized bone fragments. She reported that these

mononuclear cells differentiated into osteoclast-like cells as visualized by light microscopy (LM) and SEM. All of these observations support the hypothesis that bone resorption in vivo involves the participation of macrophages in conjunction with the action of osteoclasts (Heersche, 1978).

Since the degradation of mineralized bone matrix in vivo is primarily a function of multinucleated osteoclasts which are derived from mononuclear precursors, investigators have studied whether or not the attainment of the multinucleated state has any effect on the ability to attach to bone and subsequently degrade this matrix. The fusion of macrophages in vitro has been induced by treating macrophage cultures with conditioned media obtained from con A-stimulated lymphocytes (Sone et al., 1981) and from spleen cell cultures treated with PHA, con A or  $1\alpha,25(\text{OH})_2\text{D}_3$  (Abe et al., 1983). In addition,  $1\alpha,25(\text{OH})_2\text{D}_3$  added directly to macrophage cultures will also induce fusion. Bar-Shavit et al. (1983a) showed that  $1\alpha,25(\text{OH})_2\text{D}_3$  elicits monocytic differentiation and multinucleation in human promyelocytic leukemia HL-60 cells, and that this transformation is accompanied by a marked increase in the ability of these cells to bind and resorb bone. Fallon et al. (1983) produced macrophage polykaryons by exposing elicited macrophages to human serum. These macrophage polykaryons and their mononuclear precursors were assessed for their ability to degrade  $^{45}\text{Ca}$ -labeled, devitalized bone in vitro. The results demonstrate that macrophage polykaryons exhibit a greater resorptive

potential suggesting that the formation of multinucleated cells from mononuclear precursors is a physiologically significant event.

CHAPTER III

ULTRASTRUCTURAL LOCALIZATION OF CONCAVALIN-A  
BINDING SITES ON BONE CELLS: EFFECTS OF  
CON A ON OSTEOCLASTIC BONE RESORPTION

## ABSTRACT

When osteoclasts on the endocranial surface of 10-day old rat calvaria are treated with concanavalin-A and hemocyanin, con A binding sites can be visualized by scanning electron microscopy (SEM). The hemocyanin label was uniformly distributed on the dorsal (unapposed) surface of all osteoclasts observed by SEM; it could be visualized on smooth surfaces, microvillous projections and filopodia. Osteoblasts were also labeled with the hemocyanin marker, demonstrating con A binding sites.  $^{45}\text{Ca}$  bone release assays of prelabeled calvaria incubated in varying concentrations of con A were conducted to determine whether or not con A has any functional effects on osteoclasts. At low concentrations of con A (0.1  $\mu\text{g}/\text{ml}$ ), there was an increase in bone resorption at 24, 48 and 72 hours of incubation. In contrast, calvaria incubated in high concentrations of con A (50  $\mu\text{g}/\text{ml}$  and 100  $\mu\text{g}/\text{ml}$ ) showed a marked decrease in bone resorption significantly different from that of controls (incubated without the addition of con A) at each of the three 24 hour time periods. These differences in the release of  $^{45}\text{Ca}$  may indicate direct, dose-dependent effects of con A on osteoclastic bone resorption.



## INTRODUCTION

The osteoclast is a large multinucleate cell which is widely accepted as the primary effector cell responsible for bone resorption. Although the ultrastructural changes associated with various hormones which regulate osteoclastic activity have been well documented (Wezeman et al., 1979; Holtrop et al., 1979), little is known about the mechanisms by which these hormones exert their effect. Recently, investigators have probed the osteoclastic membrane to determine whether or not specific receptors for parathyroid hormone (PTH) and calcitonin (CT), the two primary hormones regulating osteoclastic function, are present. The results suggest that some hormones may act directly on osteoclasts (i.e. CT), while others may regulate osteoclastic activity indirectly (i.e. PTH) through the intermediary of another cell type (Warshawsky et al., 1980; Rao et al., 1981; Silve et al., 1982; Chambers and Dunn, 1982).

The hypothesis that the osteoclast precursor is extraskeletal in origin has been recently accepted (Marks, 1983). Although the definitive origin of the osteoclast remains uncertain, there is abundant evidence to support the hypothesis that osteoclasts are derived from mononuclear phagocytes (Marks, 1983). Some of the studies to determine whether the osteoclast and the macrophage share a common lineage have focused on the similarities and differences of cell surface receptors on these two distinct cell types. Recently,

attempts to demonstrate Fc and C3 receptor sites on osteoclasts, which are characteristically present on macrophages, have been unsuccessful (Shapiro et al., 1979; Chambers, 1979; Hogg et al., 1980).

In addition to studies dealing with highly specific receptors, lectins have been used as a more general probe to study both cell surface characteristics and functions. Horton et al. (1982) in their  $^{45}\text{Ca}$  bone release assays, demonstrated a decrease in resorption of long bones cultured in the presence of high concentrations of con A (50  $\mu\text{g}/\text{ml}$  and 100  $\mu\text{g}/\text{ml}$ ). Subsequent transmission electron microscopy (TEM) of osteoclasts incubated in 50  $\mu\text{g}/\text{ml}$  con A revealed ultrastructural changes associated with inhibition of osteoclastic activity (Ryder et al., 1982). We therefore treated osteoclasts on the inner surface of 10-day old rat calvaria with con A and hemocyanin to determine whether or not specific con A binding sites were present on the osteoclast membrane. Furthermore, we incubated  $^{45}\text{Ca}$  prelabeled calvaria in varying concentrations of con A to evaluate the functional significance of our morphological findings which verified the existence of specific con A binding sites on osteoclasts.

## MATERIALS &amp; METHODS

Cell Surface Labeling

Removal of Calvaria - Twelve 10-day old Norway Hooded rats were anesthetized with ether and sacrificed prior to removal of their calvaria. The calvaria were incubated in Hanks' Balanced Salt Solution (HBSS) for 30 minutes. The soft tissue was dissected off the endocranial surface and the calvaria were rinsed twice in phosphate buffered saline (PBS). Eight calvaria were used for concanavalin-A-hemocyanin labeling and four were prepared for a conventional SEM image.

Concanavalin-A-Hemocyanin Labeling - The labeling procedure employed was similar to that used by Schneider et al. (1979) for lymphocytes. Eight calvaria were pre-fixed in 1.0% glutaraldehyde in 0.1M Na cacodylate buffer. The samples were incubated in 0.1M ammonium chloride in cacodylate for 8 hours at 4°C, which blocks any aldehydes on the cell surface that may cause non-specific hemocyanin labeling. The calvaria were then rinsed in cacodylate buffer and incubated in 100 µg/ml concanavalin-A (con A) (Polysciences, Warrington, Pa.) for 10 minutes at 37°C. This is the natural tetravalent form of con A. In all of the following studies it was used under the appropriate pH and ionic conditions to remain in its tetravalent form. After the con A treatment, one half of the calvaria were incubated in 0.05M α-methyl mannoside (Polysciences, Warrington,

Pa.), a haptenic inhibitor of con A binding, and used as controls to demonstrate the specificity of hemocyanin labeling. Both con A treated and control calvaria were then rinsed and incubated in 1.0 mg/ml hemocyanin (Polysciences, Warrington, Pa.) for 10 minutes at 37°C, followed by another rinse in cacodylate buffer. The hemocyanin was first passed through a 0.45 µm Millipore filter to avoid aggregates which can obscure cell surface morphology.

Conventional SEM Tissue Preparation - After the labeling procedure, all twelve calvaria were incubated in 2.5% glutaraldehyde in 0.1M cacodylate for 1 hour and post-fixed in 1.0% osmium tetroxide in 0.1M cacodylate for 1 hour at 4°C. Following fixation the calvaria were rinsed in cacodylate and dehydrated through a graded series of ethanol. They were then critical point dried via CO<sub>2</sub> using a Denton DCP-1 (Denton Vacuum, Cherry Hill, N.J.). After drying, the specimens were attached to aluminum stubs and coated (200Å thickness) with Gold/Palladium using a Hummer II sputter-coating apparatus (Technics, Alexandria, Va.). The specimens were scanned using an ETEC Autoscan (Hayward, Ca.) at an accelerating voltage of 20kV and a 45° tilt.

#### <sup>45</sup>Ca Bone Release Assay

The calvaria of twelve 7-day old Norway Hooded rats were pre-labeled by injecting the animals with 40 µCi of <sup>45</sup>Ca per rat (specific activity 10-40 mCi/mg calcium, Amersham, Arlington Heights, Il.). Three days later, the animals were anesthetized with ether and

sacrificed. The calvaria were removed under sterile conditions and placed into HBSS. The dura mater and periosteum were dissected off the endocranial surface and the calvaria were rinsed in HBSS. Each calvarium was split longitudinally down the sagittal suture, and corresponding halves were placed into adjacent wells of a 24-well Costar tissue culture cluster. Each well contained 2 ml of BGJb media supplemented with 5% Fetal Bovine Serum, 100 units/ml Penicillin-Streptomycin, and 0.292 mg/ml Glutamine. The calvarial halves were incubated at 37°C for 24 hours in 5% CO<sub>2</sub> and air to remove the labile <sup>45</sup>Ca. After 24 hours, the media was discarded and the wells were rinsed with HBSS to remove any residual <sup>45</sup>Ca.

At this point, the calvarial samples were divided into 4 groups, each containing 3 matching calvarial halves. One half of each particular calvarium was incubated in 2 ml of a specific concentration of con A in supplemented BGJb media while the other half was incubated in 2 ml of supplemented BGJb media without the addition of any con A and used as an internal control. The concentrations of con A used in this study were 0.1 µg/ml, 2.0 µg/ml, 50 µg/ml and 100 µg/ml. At 24, 48, and 72 hours, 0.2 ml samples of culture media were pipetted off each well and added to 10 ml Biocount liquid scintillation counting cocktail (Research Products International, Mount Prospect, Il.). Duplicate samples were taken from each well and the remaining media was discarded at 24 and 48 hours. Each well was rinsed with HBSS and replenished with 2 ml of fresh media. The cultures were terminated at

72 hours. All samples were counted in a Beckman LS 7000 Liquid Scintillation System.

## RESULTS

Osteoclasts sequentially treated with concanavalin-A and hemocyanin, were labeled with the hemocyanin marker molecule as visualized by scanning electron microscopy (SEM). Con A is a tetravalent lectin which has a high affinity for  $\alpha$ -D-glucosyl and  $\alpha$ -D-mannosyl residues associated with membrane macromolecules, such as glycoproteins, glycolipids and polysaccharides. In the labeling procedure employed in this study, the cell-bound lectin was visualized by hemocyanin, a cylindrical-shaped marker molecule. Hemocyanin is a glycoprotein molecule possessing saccharide side chains which can interact with free sugar binding sites on the con A molecule, indicating the site of con A binding. The dorsal (unapposed) surface of all osteoclasts observed by SEM was uniformly labeled (figs. 1 and 2). The hemocyanin label could be visualized on smooth surfaces, microvillous projections and filopodia (figs. 2, 3 and 4). Osteoblasts were also labeled with hemocyanin demonstrating con A binding sites (figs. 5 and 6). The possibility of non-specific hemocyanin labeling was eliminated by treating calvaria with  $\alpha$ -methyl mannoside, a specific sugar inhibitor of con A binding. None of the resident bone cells on the endocranial surface of the calvaria treated with  $\alpha$ -methyl mannoside and subsequently with hemocyanin were labeled with the hemocyanin marker (figs. 7, 8, 9 and 10). These cells were indistinguishable from those observed on the calvaria which were

conventionally prepared for SEM. Therefore, the hemocyanin label was specific for con A binding sites on the outer surface of the cell membrane of both osteoclasts and osteoblasts.

The morphological findings presented indicate that osteoclasts have binding sites for con A on their surface. The  $^{45}\text{Ca}$  bone release assays were conducted to determine whether or not con A, at various concentrations, had any functional effect on osteoclasts. The ability of osteoclasts to resorb bone was determined by the amount of  $^{45}\text{Ca}$  released to the tissue culture media during three 24 hour time periods after the release of labile  $^{45}\text{Ca}$ . Values for the level of radioactivity (cpm) were obtained by counting duplicate samples from each well at each time period. The mean value (cpm) for each calvarial half incubated in a specified concentration of con A was divided by the mean value of the corresponding half incubated in control media (no con A added) to give a treated/control (T/C) ratio for each calvarium. The mean T/C ratio for each of the four experimental groups was calculated and compared to the mean control/control (C/C) ratio for that same experimental group to determine whether the observed changes in bone resorption were significantly different. Significance was evaluated by a two-tailed Student t-test for the difference between two means. The counts per minute of any given duplicate sample never differed by more than 3%.

In samples incubated in 100  $\mu\text{g}/\text{ml}$  con A, the concentration utilized for the morphological studies, there was a marked decrease in



bone resorption apparent at 24 hours ( $T/C = 0.76 \pm 0.05$ ). This trend continued at 48 hours and 72 hours (Table 1). In all cases the differences were significant. At 50  $\mu\text{g/ml}$  con A, there was also a significant reduction in bone resorption, although it was not as pronounced as it was at 100  $\mu\text{g/ml}$ . The decrease in resorption seen at 24 hours ( $T/C = 0.78 \pm 0.06$ ) continued at 48 hours ( $T/C = 0.66 \pm 0.05$ ) and 72 hours ( $T/C = 0.55 \pm 0.05$ ). Presently studies are being conducted to determine whether the decrease in resorption at these concentrations of con A is due to con A induced inhibition of osteoclastic activity, or whether these high concentrations are merely cytotoxic to osteoclasts (see Appendix A).

At mitogenic concentrations of con A (2.0  $\mu\text{g/ml}$ ), the concentration that results in peak proliferation of rat lymphocytes in vitro (Schneider, unpublished), the  $^{45}\text{Ca}$  released from treated calvaria did not differ significantly from that of control calvaria at 24, 48 and 72 hours. However, at a concentration of 0.1  $\mu\text{g/ml}$  con A there was an increase in bone resorption. This increase, which was seen as a trend at 24 hours ( $T/C = 1.32 \pm 0.24$ ), became significant at 48 hours ( $T/C = 1.44 \pm 0.21$ ). Although there was a slight decrease in the mean T/C ratio at 72 hours ( $T/C = 1.36 \pm 0.19$ ), the increase in resorption remained significant (Table 1). Studies must be conducted to determine whether the increase in bone resorption seen at low concentrations of con A is the result of a direct or an indirect stimulatory effect of con A on the osteoclast.

## DISCUSSION

Numerous investigators have recently probed the osteoclast membrane attempting to demonstrate receptor sites for various hormones and biologically active molecules which regulate osteoclastic activity. In an in vitro study utilizing  $^{125}\text{I}$ -calcitonin, localization of calcitonin receptors on the osteoclast membrane has been demonstrated (Warshawsky, et al., 1980). Calcitonin receptors have also been demonstrated on osteoclasts by immunohistochemical staining (Rao et al., 1981). However, light microscope autoradiographs of chick embryo calvaria after exposure to  $^{125}\text{I}$ -parathyroid hormone (PTH; 1-34), another primary hormone affecting bone resorption, have revealed PTH receptors on osteoblasts, but not osteoclasts (Silve et al., 1982). The lack of PTH receptors on osteoclasts is also supported by the recent findings of Chambers and Dunn (1982); the addition of PTH to cultures of isolated osteoclasts caused no change in their behavior or morphology.

The hypothesis is that mononuclear phagocytes are the precursors of osteoclasts has created an intense investigation to determine if these two distinct cell types share a common lineage. Several studies have focused on membrane characteristics to determine whether or not certain receptor sites known to exist on macrophages, can also be demonstrated on osteoclasts. The findings of one study which supported this hypothesis, indicated that elicited peritoneal

mononuclear phagocytes possess functional receptors for both PTH and CT (Minkin et al., 1977). Although Fc and C3 receptors are present on macrophages, no one has been able to demonstrate these receptors on osteoclasts either in situ (Shapiro et al., 1979; Hogg et al., 1980) or in vitro (Chambers, 1979).

Lectins, proteins that selectively bind carbohydrates but do not have enzymatic activity, were initially used to study the surface structure and function of lymphocytes. The addition of certain lectins to cultured lymphocytes results in changes in their surface morphology and function, e.g. lectins have mitogenic activity. Recently, lectins have also been used to study the cell membrane structure and function of a variety of other specific cell types, including phagocytic cells. Haimovitz et al. (1982) demonstrated that the in vitro maturation of monocytes to macrophages was accompanied by a decrease in the ability to bind fluorescein-labeled peanut agglutinin. Horton et al. (1982) demonstrated a dose-dependent decrease in resorption of  $^{45}\text{Ca}$  pre-labeled long bones incubated in high concentrations of con A (50  $\mu\text{g}/\text{ml}$  and 100  $\mu\text{g}/\text{ml}$ ). Subsequently TEM studies of osteoclasts incubated in 50  $\mu\text{g}/\text{ml}$  con A, revealed specific ultrastructural changes associated with inhibition of osteoclastic activity (Ryder et al., 1982). These results suggested the possibility of specific con A binding sites on osteoclasts.

Barnicot (1947) reported the peripheral distribution of osteoclasts on the inner surface of the flat bones of the skull of

10-day old mice. This endocranial surface of calvaria represented an easily accessible site to study membrane receptors on osteoclasts. SEM has been successfully used to identify resident bone cells on the endocranial surface of isolated calvaria (Jones and Boyde, 1976; Jones and Boyde, 1977). Therefore, in this study calvaria were scanned for osteoclasts immediately adjacent to the sagittal and frontoparietal sutures. Fields of osteoblasts occupied centrally located regions of the flat skull bones. The existence of con A binding sites on osteoclasts and osteoblasts was revealed using the methods described by Schneider et al (1979) and Brown and Revel (1976).

The  $^{45}\text{Ca}$  bone release assays were conducted to evaluate the effects of various concentrations of con A on the bone resorbing capacity of osteoclasts. The increased release of  $^{45}\text{Ca}$  from the calvarial halves incubated in 0.1  $\mu\text{g/ml}$  con A at each of the three 24 hour time periods, may be the result of a direct con A-mediated stimulation of osteoclastic activity, or it may be mediated indirectly, following interaction of con A with another cell type. Con A, by virtue of its binding to specific sugar residues, may directly alter the functional state of the osteoclast resulting in increased bone resorption at low concentrations. Alternatively, con A may act indirectly through an intermediary cell. The mechanism by which lectins stimulate lymphocyte production of osteoclast activating factor (OAF) has been well documented (Horton et al., 1972; Horton et al., 1974). The release of OAF by lymphocytes stimulates osteoclastic

activity resulting in an increased resorption of bone.

The activity of osteoclasts incubated in a mitogenic concentration of con A did not significantly differ from that of osteoclasts incubated in control media. This result argues against the possible role that OAF may play in the indirect stimulation of bone resorption. The significant reduction in bone resorption observed at 50 and 100  $\mu\text{g/ml}$  con A, is probably the result of a dose-dependent con A-mediated inhibition of osteoclastic activity as indicated by the TEM studies of osteoclasts incubated in 50  $\mu\text{g/ml}$  for 48 hours (Ryder et al., 1982). Presently, experiments are in progress to verify the reversal of this inhibition of osteoclasts by removing con A from the culture media after 24 hours of incubation (see Appendix A).

Chambers and Dunn (1982) and Chambers and Magnus (1982) have described various inhibitory morphological changes observed in cultured osteoclasts treated with calcitonin and prostacyclin as visualized by phase contrast microscopy. Similarly, morphological characteristics of presumably active and inhibited osteoclasts, as visualized by SEM, have been described, both in situ (Jones and Boyde, 1977) and in vitro (Allen et al., 1981). If indeed the decrease in the bone resorbing activity of osteoclasts observed at high concentrations of con A is due to con A-mediated inhibition, and the stimulation of osteoclasts at low concentrations is a direct effect of con A, this culture system will be useful in describing specific

morphological characteristics of osteoclasts by SEM.

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Fig. 1. Osteoclast treated with con A and hemocyanin demonstrating a rough surface, the result of hemocyanin uniformly distributed over the dorsal (unapposed) surface. Bar equals 5.0  $\mu\text{m}$ .

Fig. 2. High magnification of an osteoclast treated with con A-hemocyanin. The hemocyanin molecules appear as small elevations, giving the microvilli and flat areas of the cell surface a highly irregular outline. Bar equals 0.5  $\mu\text{m}$ .

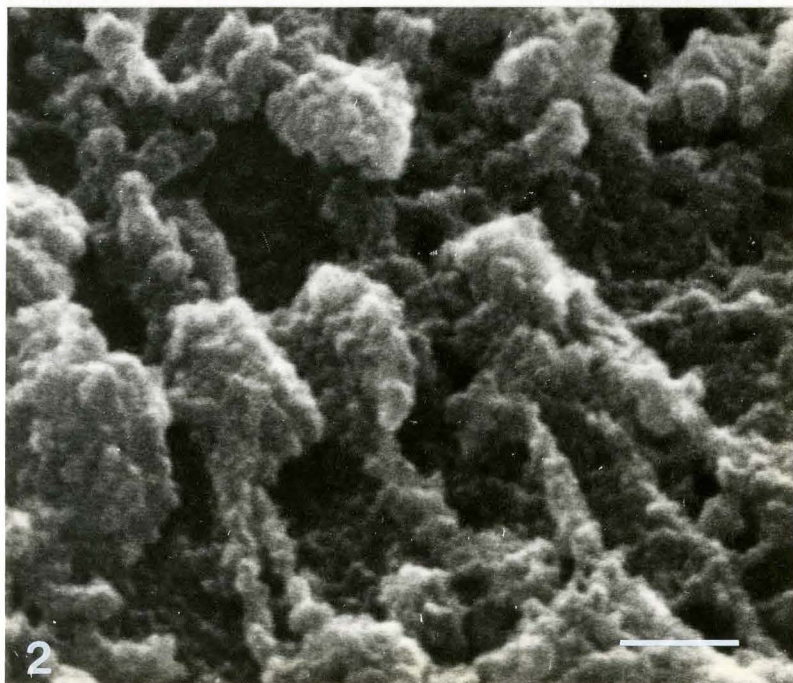
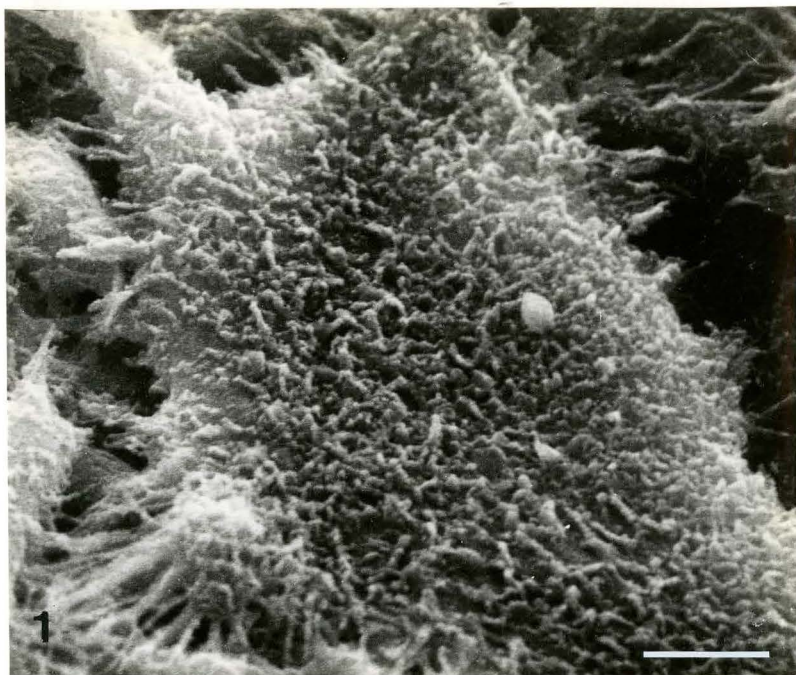


Fig. 3. High magnification of labeled osteoclast illustrating densely packed hemocyanin molecules on the flat areas of the plasma membrane. Bar equals 0.5  $\mu$ m.

Fig. 4. Microvilli at the lateral border of a labeled osteoclast shows numerous marker molecules. Bar equals 0.5  $\mu$ m.

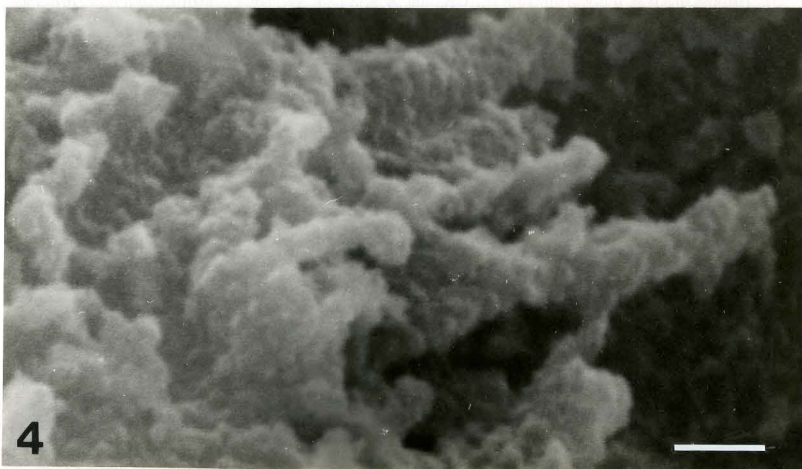


Fig. 5. Field of osteoblasts from a sample treated with con A and hemocyanin. The rough surface is due to hemocyanin molecules. Bar equals 5.0  $\mu\text{m}$ .

Fig. 6. High magnification of a labeled osteoclast, as in figure 5. Note the presence of uniformly distributed hemocyanin molecules. Bar equals 0.25  $\mu\text{m}$ .

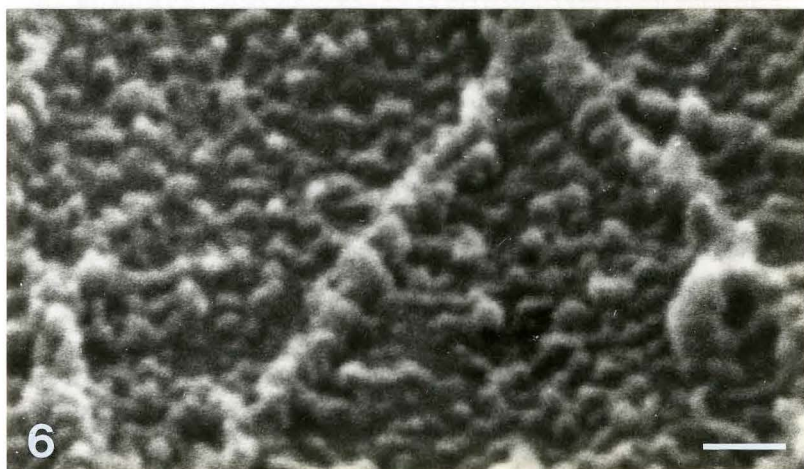
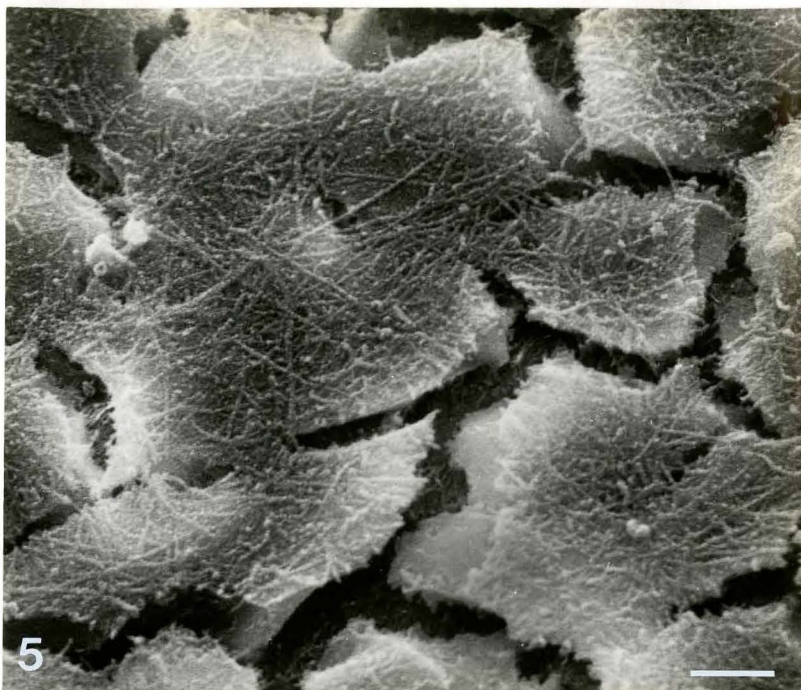


Fig. 7. Control osteoclast treated with con A,  $\alpha$ -methyl mannoside, and hemocyanin. No label is present on the cell surface. Bar equals 5.0  $\mu$ m.

Fig. 8. High magnification of a control osteoclast, as in figure 7. The smooth outline of microvillous projections is apparent. Bar equals 0.5  $\mu$ m.

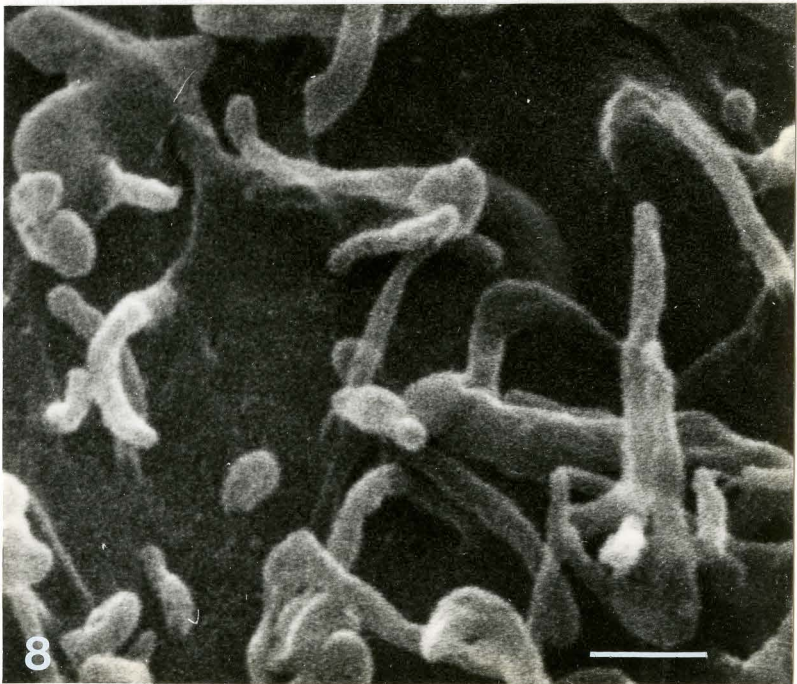
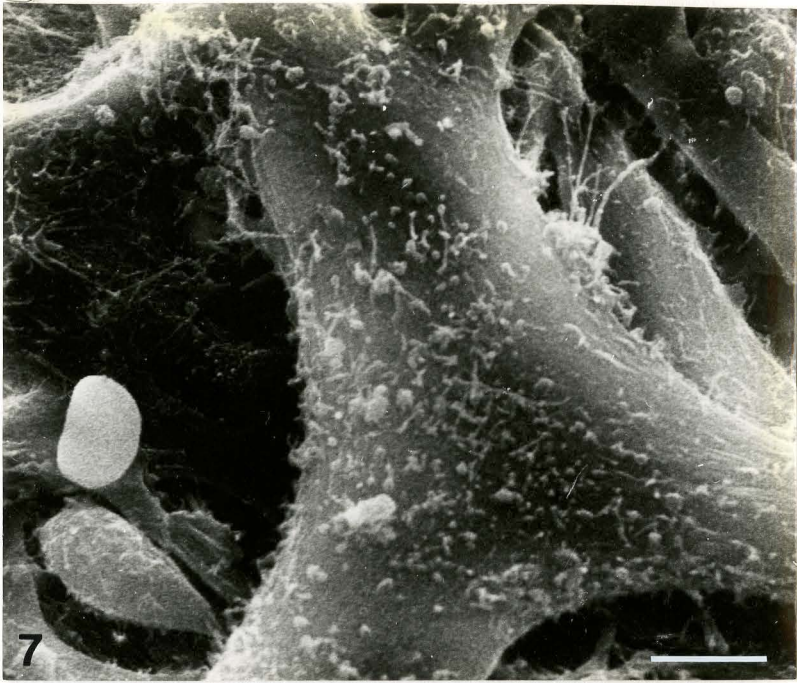




Fig. 9. A field of osteoblasts from a control sample, treated with con A,  $\alpha$ -methyl mannoside, and hemocyanin. Bar equals 5.0  $\mu$ m.

Fig. 10. High magnification of the surface of a control osteoblast. Bar equals 0.25  $\mu$ m.



Table 1

<sup>45</sup>Ca Bone Release Assay

Concentration of con A	24 hour T/C Ratio <sup>a</sup> p <sup>b</sup>	48 hour T/C Ratio p	72 hour T/C Ratio p
0.1 µg/ml	1.32 ± .24 <0.1	1.44 ± .21 <.05	1.36 ± .19 <.05
2.0 µg/ml	1.07 ± .12 N.S.	1.05 ± .10 N.S.	1.03 ± .11 N.S.
50 µg/ml	0.78 ± .06 <.01	0.66 ± .05 <.005	0.55 ± .05 <.002
100 µg/ml	0.76 ± .05 <.005	0.55 ± .01 <.001	0.42 ± .04 <.001

<sup>a</sup> T/C ratio: Mean treated to control ratio of three pairs of corresponding calvarial halves and the standard deviation.

<sup>b</sup> p = probability that the mean T/C ratio was the same as the mean C/C ratio as determined by a two-tailed student t-test.

<sup>c</sup> N.S. = not significant

CHAPTER IV

LECTIN-MEDIATED EFFECTS ON BONE

RESORPTION IN VITRO

## ABSTRACT

Lectins are proteins that are highly specific in their saccharide binding requirements. Although lectins have been used extensively as in vitro biological probes to study the structure and function of cells and tissues, few studies have used lectins to evaluate the skeletal system. Organ cultures of  $^{45}\text{Ca}$  pre-labeled bone have been used as in vitro models to assess the functional effects of various exogenous and endogenous mediators of osteoclastic bone resorbing activity. In a previous study, we demonstrated that concanavalin A (con A) has a dose-dependent biphasic effect on the bone resorbing capacity of osteoclasts using a  $^{45}\text{Ca}$  bone-organ culture system; bone resorption was stimulated at low concentrations and inhibited at high concentrations. In this study we compared the effects of the following 4 lectins, con A, wheat germ agglutinin (WGA), soybean agglutinin (SBA) and peanut agglutinin (PNA), each with distinctly different saccharide binding specificities, on bone resorption. Organ cultures of  $^{45}\text{Ca}$  pre-labeled calvariae were established to quantitate bone resorption and each lectin was tested at a concentration of 50  $\mu\text{g}/\text{ml}$ . Both con A and WGA inhibit bone resorption, although the WGA-mediated effect is not as pronounced as that of con A. In contrast, neither SBA nor PNA have any effect on bone resorption in comparison to the control assay. These observations indicate that con A- and WGA-mediated inhibition of bone resorption is not a

generalized phenomenon that occurs subsequent to the binding of any lectin at the same concentration, suggesting that the specificity of lectin binding to cell surface receptors is an important factor in the regulation of cellular function. Furthermore, lectins such as con A and WGA can be used as in vitro models to study the morphological and biochemical events associated with changes in osteoclastic activity.

## INTRODUCTION

Lectins are proteins that exhibit a variety of biological effects, some of which mimic the effects of endogenous, physiological agents that regulate cellular function in vivo. Therefore, lectins have been used as in vitro models to study the biochemical events and morphological changes associated with alterations of cellular activity. Lectins offer a number of attractive features as biological probes. They are highly specific in their cell surface saccharide binding requirements. Hapten inhibition studies have demonstrated that the interactions between lectins and cell surface saccharides can be inhibited by the addition of simple sugars. Most of the lectins that have been isolated and analyzed to date are composed of multiple subunits which are held together by non-covalent bonds. These subunits are polypeptide chains that frequently contain covalently bound carbohydrate side chains. There are a multitude of lectins that are commercially available in high purity and they can be conjugated to a variety of ultrastructural and cytochemical marker molecules.

Concanavalin A (con A) is the most widely used lectin in biological assays and many of its properties have been well-documented and thoroughly investigated. Other lectins that are frequently used to study the structure and function of cells and tissues include wheat germ agglutinin (WGA), soybean agglutinin (SBA) and peanut agglutinin (PNA). The sugar specificities of con A, WGA, SBA and PNA are

distinctly different from one another (Table 1). All of the sugar residues to which these lectins bind are common to mammalian membrane-associated glycoconjugates.

The biological effects of these 4 lectins have been investigated in a variety of cellular systems both in vivo and in vitro. However, few studies have used lectins to evaluate the structure and function of the skeletal system. Wang et al. (1982) demonstrated that the in vivo administration of PHA-P caused a significant increase in the number and size of long bone osteoclasts. Horton et al. (1982) demonstrated a decrease in resorption of long bones cultured in the presence of high concentrations of con A (50 and 100 µg/ml). Subsequent transmission electron microscopy of these con A-treated osteoclasts revealed ultrastructural changes associated with inhibition of osteoclastic activity (Ryder et al., 1982).

The studies involving the skeletal system indicate that some lectins can induce changes in the structure and function of bone cells, particularly osteoclasts. The osteoclast is a large multinucleate cell that is widely accepted as the primary effector cell responsible for normal bone resorption (Marks, 1983). In a previous study, we conducted a series of in vitro <sup>45</sup>Ca bone release assays to evaluate the effect of various concentrations of con A on the bone resorbing capacity of osteoclasts (Popoff and Schneider, 1983). The bone mineral release assays demonstrated that con A has a biphasic effect; at a low concentration (0.1 µg/ml) con A increased



bone resorption, whereas at higher concentrations (50 and 100  $\mu\text{g}/\text{ml}$ ) con A inhibited bone resorption. In this study we were interested in comparing the effects of the lectins con A, WGA, SBA and PNA, each with different saccharide binding specificities, on bone resorption. We used a bone-organ culture of  $^{45}\text{Ca}$  pre-labeled calvariae to quantitate bone resorption and tested each lectin at a concentration of 50  $\mu\text{g}/\text{ml}$ .

## MATERIALS AND METHODS

The procedure employed in these bone mineral release assays is similar to that described in a previous study (Popoff and Schneider, 1983). The calvariae of 7-day old Norway Hooded rats were pre-labeled by injecting the animals with 40  $\mu\text{Ci}$  of  $^{45}\text{Ca}$  per rat (specific activity 10-40 mCi/mg calcium, Amersham, Arlington Heights, Il.). Three days later, the animals were anesthetized with ether and sacrificed. The calvariae were removed under sterile conditions and placed into Hanks' Balanced Salt Solution (HBSS). The dura mater and periosteum were dissected off the endocranial surface and the calvariae were rinsed in HBSS. Each calvaria was split longitudinally down the sagittal suture, and corresponding halves were placed into adjacent wells of a 24-well Costar tissue culture cluster. Each well contained 2 ml of BGJb medium supplemented with 5% Fetal Bovine Serum, 100 units/ml Penicillin-Streptomycin and 0.292 mg/ml Glutamine. The calvarial halves were incubated at 37°C for 24 hours in 5% CO<sub>2</sub> and air to remove the labile  $^{45}\text{Ca}$ . After 24 hours, the medium was discarded and the wells were rinsed with HBSS to remove any residual  $^{45}\text{Ca}$ .

At this point, the calvarial samples were divided into 5 groups with each group containing 6 matching calvarial halves. The 5 groups correspond to the 4 lectins, con A, WGA, SBA, and PNA, to be tested and one group for the control release assay. In the experimental cultures, one half of each particular calvaria was incubated in 2 ml

of supplemented BGJb medium containing one of the four lectins at a concentration of 50 µg/ml, while the other half was incubated in 2 ml of supplemented BGJb medium without the addition of any lectin and used as an internal control. In the control cultures both halves of each pair were incubated in supplemented BGJb medium in the absence of lectin. At 24, 48 and 72 hours, 0.2 ml samples of culture medium were pipetted off each well and added to 10 ml Biocount liquid scintillation counting cocktail (Research Products International, Mount Prospect, Il.). Duplicate samples were taken from each well and the remaining medium was discarded at 24 and 48 hours. Each well was rinsed with HBSS and replenished with 2 ml of fresh medium. The cultures were terminated at 72 hours and the remaining bone samples were placed into scintillation vials and digested in 0.5 ml formic acid at 100°C for 30 minutes. Following the bone digest, 10 ml counting cocktail was added to each vial. All samples were counted in a Beckman LS 7000 Liquid Scintillation System. The bone digest was performed so that the percent of  $^{45}\text{Ca}$  released from each calvarial sample with respect to the total  $^{45}\text{Ca}$  initially present in that sample could be calculated.

## RESULTS

The  $^{45}\text{Ca}$  bone release assays were conducted to evaluate the effects of the lectins, con A, WGA, SBA and PNA, on bone resorption. Bone resorption was quantitated by the amount of  $^{45}\text{Ca}$  released into the tissue culture medium during three 24 hour time periods after the release of labile  $^{45}\text{Ca}$ . Values for the level of radioactivity (cpm) were obtained by counting duplicate samples from each well at each time period. The counts per minute obtained from duplicate samples never differed by more than 3%. The percent  $^{45}\text{Ca}$  released from each calvarial sample (% release) at each sampling time point was calculated from the cpm values after the bone digest was performed. The value (% release) for each calvarial half incubated in the presence of a specific lectin was divided by the value (% release) of the corresponding half incubated in control medium (no lectin added) to give a treated/control (T/C) ratio for each calvaria. The mean T/C ratio for each of the four experimental groups was calculated and compared to the mean control/control (C/C) ratio obtained from the control assay to determine whether the observed changes in bone resorption were significantly different. Significance was evaluated by a two-tailed Student t-test for the difference between two means.

The mean C/C ratios obtained from paired calvarial halves incubated in the absence of lectin varied slightly from the expected value of 1.00 (24 hr. T/C =  $1.02 \pm 0.02$ ; 48 hr. T/C =  $1.04 \pm 0.02$ ; 72

hr.  $T/C = 1.02 \pm 0.03$ ). The calvarial samples incubated in the presence of con A showed a significant decrease in bone resorption at 24 ( $T/C = 0.78 \pm 0.06$ ), 48 ( $T/C = 0.66 \pm 0.05$ ), and 72 ( $T/C = 0.55 \pm 0.06$ ) hours (Fig. 1). In a previous study (Popoff and Schneider, 1983), we have shown that the reduction in mineral mobilization mediated by con A at a concentration of 50  $\mu\text{g}/\text{ml}$  is reversible, indicating that this concentration is inhibitory rather than cytotoxic to the bone resorbing osteoclasts. In the samples incubated in 50  $\mu\text{g}/\text{ml}$  WGA, there was a slight decrease in bone resorption at 24 hours ( $T/C = 0.95 \pm 0.03$ ), which became significant at 48 ( $T/C = 0.84 \pm 0.04$ ) and 72 ( $T/C = 0.71 \pm 0.05$ ) hours (Fig. 1). The decrease observed in the WGA-treated samples was not as pronounced as that observed in the con A-treated samples at each of the sampling time points. On the contrary, the  $^{45}\text{Ca}$  released from the calvarial halves incubated in the presence of either SBA or PNA did not differ significantly from that of control halves at any sampling time point (Fig. 1). The  $T/C$  ratios obtained from the SBA-treated assay were  $1.00 \pm 0.04$  at 24 hours,  $0.98 \pm 0.05$  at 48 hours and  $0.98 \pm 0.05$  at 72 hours. The  $T/C$  ratios obtained from the PNA-treated samples were  $1.01 \pm 0.08$  at 24 hours,  $0.97 \pm 0.01$  at 48 hours and  $0.94 \pm 0.06$  at 72 hours.

## DISCUSSION

The lectins con A, WGA, SBA and PNA are highly specific in their cell surface saccharide binding requirements and the membrane-associated saccharides to which they bind are distinctly different from one another (Table 1). Con A has a broad biological applicability primarily because it recognizes 2 commonly occurring cell surface saccharides,  $\alpha$ -D-mannose and  $\alpha$ -D-glucose. Although it was originally postulated that the specificity of con A is directed toward a single sugar residue, studies by So et al. (1968) demonstrated that the con A binding site is more complex in both its primary structure and three-dimensional configuration than originally proposed. WGA binds to oligosaccharides containing terminal N-acetylglucosamine (GluNAc) residues, with preferential binding to dimers and trimers of this saccharide. SBA preferentially binds to oligosaccharides containing  $\alpha$ - or  $\beta$ -linked N-acetylgalactosamine (GalNAc) residues, and to a lesser extent to D-galactose residues. PNA binding is directed toward D-galactose residues, although this lectin binds to the disaccharide, galactosyl  $\beta$ -1,3GalNAc (T-antigen), with greatest affinity. Con A, WGA, SBA and PNA are multimeric lectins; con A and PNA are each composed of 4 identical subunits, whereas WGA and SBA are each composed of 2 identical subunits (Table 1). SBA and PNA are glycoproteins and the sugars which have been isolated from these lectins are common to animal cell membranes. In

contrast, con A and WGA are devoid of covalently bound carbohydrate side chains (Table 1).

In this study, a series of  $^{45}\text{Ca}$  bone release assays were conducted using a lectin concentration of 50  $\mu\text{g/ml}$  for each of the 4 lectins to be tested. The results demonstrate that both con A and WGA inhibit the release of  $^{45}\text{Ca}$  in cultures of pre-labeled calvarial explants, although the WGA-mediated effect is not as pronounced as that of con A. On the contrary, neither SBA nor PNA have any effect on bone resorption in comparison to the control assay run in the absence of any lectin. These findings indicate that the inhibition of bone resorption mediated by con A, and to a lesser extent by WGA, at a concentration of 50  $\mu\text{g/ml}$  is not a generalized phenomenon that occurs subsequent to the binding of any lectin at this concentration. This conclusion would suggest that the specificity of lectin binding to cell surface receptors is an important factor in the regulation of cellular function that is mediated by a particular lectin. Studies should be conducted to evaluate possible mechanisms that could be responsible for the differential effects of these lectins on bone resorption.

Because of the difficulty in obtaining pure populations of osteoclasts, most of the studies on bone resorption are performed on intact animals or bone-organ cultures. These systems do not permit detailed analysis of the cellular mechanisms of bone resorption (Teitelbaum et al., 1979). Studies by Mundy et al. (1977), Kahn et

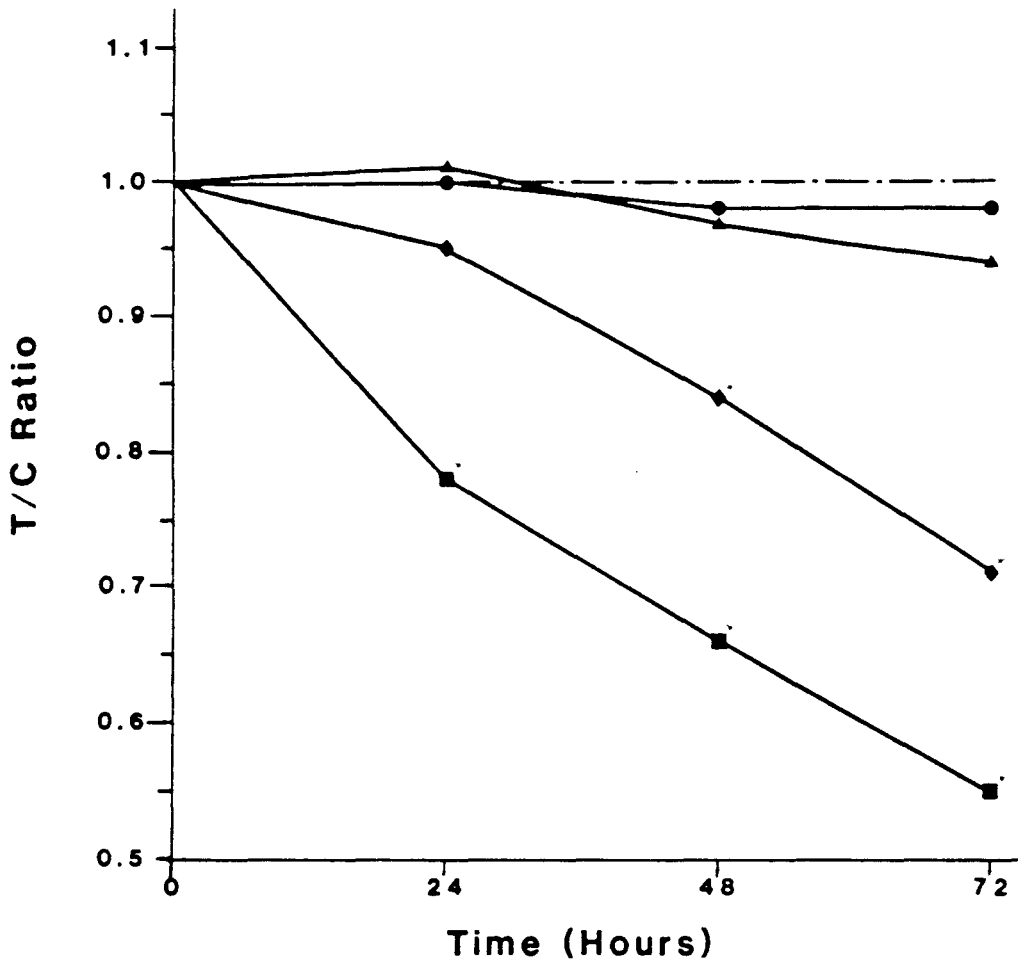
al. (1978) and Teitelbaum et al. (1979) have demonstrated that monocytes and macrophages are capable of resorbing devitalized bone in vitro. The studies by Kahn et al. (1978) and Teitelbaum et al. (1979) also demonstrated that optimal macrophage-mediated mineral mobilization requires cell-matrix contact. Since macrophages are capable of attaching to and subsequently degrading a bone matrix, the macrophage-devitalized bone culture system serves as a useful model to study the mechanisms of bone resorption in tissue culture (Bar-Shavit et al., 1983a). Recent studies by Bar-Shavit et al. (1983b,c) have shown that the attachment of macrophages to bone is a regulated process that is mediated, in part, by certain saccharides located on the cell and/or bone surfaces. These findings indicate that the attachment of resorbing macrophages to the bone matrix is an important component of the process of bone resorption and that the oligosaccharide-mediated binding mechanism that links the resorbing macrophages to bone is essential for normal resorptive activity. The macrophage-devitalized bone culture system could be used to evaluate the effects of lectins, that have differential effects on  $^{45}\text{Ca}$  release in cultures of pre-labeled calvariae, on the morphological and functional aspects of the macrophage-bone interaction.



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Fig. 1.  $^{45}\text{Ca}$  bone release assay. SBA and PNA had no significant effect on bone resorption at a concentration of 50  $\mu\text{g/ml}$ , whereas con A and WGA inhibited bone resorption at this concentration.



● - Soybean Agglutinin

◆ - Wheat Germ Agglutinin

▲ - Peanut Agglutinin

■ - Concanavalin A

\* - Significantly different from controls (p < .002)

Table 1

Lectin Specificity

Lectin	Source	Saccharide Specificity and Binding Affinity	Cation Requirement	Structure		
				Chemical Composition	# of Subunits	MW (daltons)
Concanavalin A (Con A)	Canavalia ensiformis (Jack bean) seeds	$\alpha$ -D-mannose $\alpha$ -D-glucose	+	protein	tetramer	104,000
Peanut Agglutinin (PNA)	Arachis hypogaea (peanuts)	D-galactose galactosyl $\beta$ -1,3 GalNAc	±	glycoprotein	tetramer	110,000
Soybean Agglutinin (SBA)	Glycine max (soybean) seeds	D-GalNAc D-galactose	+	glycoprotein	tetramer	120,000
Wheat Germ Agglutinin (WGA)	Triticum vulgare (wheat germ)	(D-GluNAc) <sub>3/2/1</sub>	-	protein	dimer	36,000

CHAPTER V

THE EFFECTS OF LECTINS ON THE INTERACTION  
BETWEEN MACROPHAGES AND BONE IN VITRO:  
A MORPHOLOGICAL AND FUNCTIONAL STUDY

## SUMMARY

Recent studies have demonstrated that the attachment of elicited rat macrophages to bone is mediated by specific saccharides located on the cell and/or bone surfaces. We have used a macrophage-bone culture system to study the effects of 2 lectins, concanavalin A (con A) and soybean agglutinin (SBA), on the morphology of macrophage attachment to a devitalized bone surface and subsequent functional activity. Macrophages were obtained from 3-4 week old rats by peritoneal lavage and the adherent pool was used to prepare cell suspensions. Con A-treated, SBA-treated or control cell suspensions were aliquoted onto the endocranial surface of devitalized rat calvariae. The cells were allowed to attach for 1h at 37°C, after which, the bone samples were removed from culture and prepared for scanning electron microscopy (SEM). The morphology of con A-treated macrophages attached to bone was markedly different from that of control or SBA-treated cells. Con A altered the attachment and subsequent spreading of macrophages on bone as visualized by SEM. Furthermore, the number of con A-treated cells that attached to bone and the average surface area of cell membrane apposed to the matrix was significantly different from that of control or SBA-treated cells. A  $^{45}\text{Ca}$  bone release assay was performed to evaluate the functional significance of the morphological findings. Lectin-treated or control cell suspensions were allowed to attach to the endocranial surface of  $^{45}\text{Ca}$  pre-labeled calvariae for

1h. Following attachment, the samples were cultured for 72h. The con A-treated cultures demonstrated a significant decrease in the release of  $^{45}\text{Ca}$  after 48 and 72h in comparison to control cultures, while the  $^{45}\text{Ca}$  released from SBA-treated cultures did not differ significantly from controls. These results suggest that certain sugar residues common to membrane-associated glycoconjugates and the organic component of the bone matrix regulate the attachment of macrophages to bone and their subsequent bone resorbing activity.

## INTRODUCTION

The hypothesis that the osteoclast precursor is extraskeletal in origin has been recently accepted (Marks 1983). Although the definitive origin of the osteoclast remains uncertain, there is abundant evidence to support the hypothesis that osteoclasts are derived from mononuclear cells of hemopoietic origin, possibly of the monocytic lineage (Walker 1975; Marks and Walker 1981; Schneider and Byrnes 1983). In addition to the investigative effort to establish a common lineage between the osteoclast and the macrophage mononuclear cell, it has been well-documented that cells of the mononuclear phagocyte system are capable of resorbing devitalized bone in vitro (Mundy et al. 1977; Kahn et al. 1978; Teitelbaum et al. 1979). The studies by Kahn et al. (1978) and Teitelbaum et al. (1979) also demonstrated that optimal macrophage-mediated mineral mobilization requires cell-matrix contact. These findings indicate that the attachment of resorbing mononuclear cells to the bone matrix is an important component of this process.

More recently, Bar-Shavit et al. (1983a,b) have shown that the attachment of macrophages to bone is a regulated process that is mediated, in part, by saccharides located on the cell and/or bone surfaces. These investigators have also shown that glucocorticoids enhance the attachment of macrophages to bone in a dose-dependent manner which parallels the dose-dependent stimulation of



macrophage-mediated bone resorption by glucocorticoids (Bar-Shavit et al. 1984). The increase in macrophage attachment involves glucocorticoid-induced alterations of cell surface oligosaccharides. Bar-Shavit et al. (1982, 1983c) also demonstrated that macrophages attach to osteomalacic bone with significantly less avidity than normal matrix. They attributed this reduction in binding to the absence or inaccessibility of the appropriate bone matrix oligosaccharides. From these studies it was concluded that the oligosaccharide-mediated binding mechanism which links the resorbing mononuclear cells to bone is essential for normal resorptive activity.

Since monocytes and macrophages are capable of attaching to and subsequently degrading a mineralized bone matrix, the macrophage-bone culture system serves as a useful model to study the interaction between macrophage mononuclear cells and bone. Therefore, we used a macrophage-bone culture system to study the effects of two lectins, concanavalin A (con A) and soybean agglutinin (SBA), on the morphology of cell attachment and subsequent functional activity. Lectins are proteins that are highly specific in their saccharide binding requirements. Lectins have been used extensively in biological assays, particularly as probes to study the structure and activity of membrane components. The two lectins employed in this study have saccharide binding specificities that are distinctly different from one another. Con A binds to oligosaccharides with  $\alpha$ -linked D-glucose and D-mannose residues, whereas SBA preferentially binds to

oligosaccharides with  $\alpha$ - or  $\beta$ -linked N-acetylgalactosamine (GalNAc) residues, and to a lesser extent to D-galactose residues. For this study, scanning electron microscopy (SEM) was used to assess the qualitative and quantitative differences in the attachment of macrophages to bone in the presence and absence of lectin. Furthermore, a  $^{45}\text{Ca}$  bone release assay was performed to evaluate the functional significance of the morphological findings.

## MATERIALS AND METHODS

Mononuclear cells for all experiments were obtained from 3 to 4 week old Norway Hooded rats by intraperitoneal injection of cold saline (4°C), as described by Schneider and Byrnes (1983). The abdomen was massaged for a few minutes and the fluid was aspirated from the peritoneal cavity. Adherent cells were separated from nonadherent cells by incubating the peritoneal washes in RPMI 1640 medium supplemented with 10% Fetal Bovine Serum in 35 mm plastic tissue culture dishes ( $5 \times 10^6$  cells/dish). After 2h, the nonadherent pool was decanted and the cultures were rinsed in phosphate buffered saline (PBS). The remaining attached cells were incubated in calcium-, magnesium-free Hanks' Balanced Salt Solution (HBSS) for 15 min at 4°C. The cells were removed from the plastic surface by gentle scraping using a rubber policeman. The resulting cell suspensions were counted on a hemocytometer. Approximately 95% of the adherent cells were macrophages, as assessed by their ability to phagocytose the particulate dye, neutral red.

Morphology and quantitation of macrophage attachment to bone in the presence of con A and SBA

The cell surface morphology of macrophages attached to bone in the presence of con A and SBA was visualized by scanning electron microscopy (SEM). Three cell suspensions containing equivalent

numbers of macrophages were prepared for this experiment: 1) a con A treated cell suspension in BGJb medium containing 50 µg/ml con A (Pharmacia, Piscataway, N.J.); 2) an SBA treated cell suspension in BGJb medium containing 50 µg/ml SBA (Pharmacia, Piscataway, N.J.); 3) and a control suspension of cells in BGJb medium without the addition of lectin. The BGJb medium used in all tissue culture procedures was supplemented with 10% Fetal Bovine Serum, 100 units/ml Penicillin-Streptomycin and 0.3mg/ml Glutamine. The lectin concentration of 50 µg/ml was selected based upon previous results which demonstrated that con A significantly inhibits the release of <sup>45</sup>Ca from a bone organ culture system at this concentration, whereas the release of <sup>45</sup>Ca from SBA-treated cultures is not significantly different from controls (Popoff et al. 1984).

Devitalized bone chips were prepared from the calvariae of 10 day old Norway Hooded rats by freeze-thawing and sonication. Three devitalized bone chips were placed onto the bottom of each of 6 tissue culture wells, the wells being divided into 3 groups corresponding to the three cell suspensions. The cell suspensions were then layered over the bone chips and the cells were allowed to attach for 1h. Bar-Shavit et al. (1983) have shown that elicited rat peritoneal macrophages bind to bone in a temperature-dependent and saturable manner with maximal attachment occurring at 60 min at 37°C. Following attachment the bone chips were removed from culture and rinsed in PBS.

After the bone attachment assay, all bone samples were fixed in

2.5% glutaraldehyde in 0.1 M cacodylate buffer for 1h. Following fixation, the samples were rinsed in cacodylate, dehydrated through a graded series of ethanol, critical point dried via CO<sub>2</sub> using a Denton DCP-1 (Denton Vacuum, Cherry Hill, N.J.), attached to aluminum stubs and coated (20 nm thickness) with Gold/Palladium using a Hummer VI sputter-coating apparatus (Technics, Alexandria, Va.). The specimens were scanned using an ISI SX40 (Santa Clara, Ca.) at an accelerating voltage of 15kV and either a 0° or 45° tilt. These samples were used to evaluate cell surface morphology and to quantitate the attachment of macrophages to bone.

To quantitate the number of macrophages attached to the bone surface, 5 different fields of each bone sample were selected using identical x:y coordinates for each sample. These fields were photographed at 300X, a magnification that allows for the accurate identification of individual cells. The cells were then counted on a Zeiss Videoplan Image Analyzer (Carl Zeiss, New York, N.Y.). To validate this method of quantitation by SEM, a <sup>51</sup>Cr bone binding assay similar to that described by Bar-Shavit et al. (1983d) was employed. Macrophages were radiolabeled by incubation for 1h at 37°C in a solution containing Na<sup>51</sup>CrO<sub>4</sub> in RPMI 1640 medium at a concentration of 50 µCi/10 X 10<sup>6</sup> cells. After washing the radiolabeled cells in PBS, they were resuspended in the following media; control cells in supplemented BGJb medium and treated cells in supplemented BGJb medium containing 50 µg/ml of con A. The control and treated cell

suspensions, each containing an equivalent number of radiolabeled macrophages, were aliquoted onto the endocranial surface of 6 devitalized calvarial chips and the cells were allowed to attach for 1h at 37°C. All calvarial chips used in this experiment were identical in size. After rinsing, the bone chips, with their attached radiolabeled cells, were counted in a Beckman Gamma 4000 system. A pre-determined number of radiolabeled cells were also counted, allowing for an approximate determination of the number of attached macrophages from the counts per minute (cpm) obtained from the gamma counter for each bone chip.

To quantitate the average cell surface area apposed to the bone surface in the presence or absence of either con A or SBA, fields of attached macrophages were photographed at 1000X. From these micrographs, the perimeter of individual cells on the bone surface could accurately be traced using a Zeiss Videoplan Image Analyzer (Carl Zeiss, New York, N.Y.). 100 cells in each group were traced on the image analyzer, which was calibrated to convert this measurement parameter into the corresponding surface area in  $\mu\text{m}^2$ . The individual surface area measurements in each group were averaged and significance was evaluated by a two-tailed student t-test.

#### Macrophage mediated release of bone mineral

The calvariae of twelve 7-day old Norway Hooded rats were pre-labeled by injecting the animals with 40  $\mu\text{Ci}$  of  $^{45}\text{Ca}$  per rat.

Three days later, the calvariae were removed and the dura mater was dissected off the endocranial surface. After being devitalized, each calvaria was split longitudinally down the sagittal suture and corresponding halves were placed into adjacent tissue culture wells. The culture plates were placed under an ultraviolet light source for 12h to assure that all calvariae were sterile. Following sterilization, 2 ml of PBS was added to each well for 24h to remove the labile  $^{45}\text{Ca}$ .

After the washout period, lectin-treated and control macrophage cell suspensions were prepared in supplemented BGJb medium as previously described. The calvarial samples were divided into 2 groups (con A and SBA), each containing 6 matching calvarial pairs. Two ml of either the con A-treated or SBA-treated cell suspension was aliquoted into the wells containing one half of each calvarial pair, while 2 ml of the control cell suspension was aliquoted into the wells containing the corresponding half of each calvarial pair. An equivalent number of cells (treated or control) was aliquoted into each well. At 24, 48 and 72h, duplicate 0.2 ml samples of culture medium were pipetted off each well and added to 10 ml Ready-Solv liquid scintillation counting cocktail (Beckman, Fullerton, Ca.). The remaining medium was discarded at 24 and 48 hours and replenished with 2 ml of fresh medium. Cell viability in control, con A-treated and SBA-treated cultures after 72h was determined by the trypan blue dye exclusion method.

After terminating the cultures at 72h, the calvarial samples were rinsed in PBS, placed into scintillation vials, and digested in 0.5 ml formic acid at 100°C for 30 min. Following calvarial digest, 10 ml counting cocktail was added to the vials. All samples were counted in a Beckman LS 7000 Liquid Scintillation System. The calvarial digest was performed so that the percent of  $^{45}\text{Ca}$  released from each calvaria with respect to the total  $^{45}\text{Ca}$  initially present in that calvaria could be calculated.



## RESULTS

Morphology of macrophage attachment to bone

Macrophages that are allowed to attach to a bone surface at 37°C in the absence of lectin, will interact with the bone surface and spread beyond the original site of attachment. After 1h in culture, the macrophages are in various stages of the spreading process; some cells exhibit a proportionately large membrane surface area that is apposed to the bone surface, while others are in a more immature phase of the spreading process (fig. 1). Many filopodia as well as some larger lamellipodia are present at the cell periphery (fig. 2). The dorsal, unapposed surface exhibits numerous microspikes (fig. 2).

Macrophages that are allowed to attach to bone in the presence of con A exhibit a cell surface morphology that is markedly different from that of controls (fig. 3). Although the con A-treated macrophages do attach to the bone surface, they are hindered in their ability to spread on the substrate. A much smaller proportion of the total membrane surface area of con A-treated macrophages is apposed to the bone matrix in comparison with controls. Membrane specializations at the cell periphery are present in the form of a few blunt lamellipodia, while filopodia are absent; the dorsal surface is ruffled (fig. 4). In addition, some of the macrophages are present in aggregates, most likely the result of the cross-linking of cell membranes by con A. Macrophages attaching to bone in the presence of

SBA exhibit an extensive membrane-substrate interaction similar to controls. Many of the SBA-treated macrophages have a large membrane surface area that is apposed to the bone matrix, while others are less advanced in the spreading process (fig. 5). The surface morphology of SBA-treated macrophages includes large peripheral pseudopodia and a ruffled dorsal surface (fig. 6). Macrophage agglutination by SBA is not nearly as pronounced as with con A; most of the cells are present either singly or in small clusters.

#### Quantitation of macrophage attachment to bone

The number of macrophages attaching to bone in the presence or absence of either con A or SBA was quantitated via SEM as described in Methods. Significance was evaluated by a two-tailed Student t-test for the difference between two means. The number of macrophages attaching in the con A system is significantly greater than the number of cells attaching in the absence of lectin (fig. 7). On the contrary, the number of attached SBA-treated cells is not significantly different from that of controls (fig. 7). A  $^{51}\text{Cr}$ -labeled macrophage-bone binding assay was performed to confirm the significant increase in the number of con A-treated macrophages attaching to bone. The mean level of radioactivity (cpm) obtained from control samples was  $18,025 \pm 3097$ , while the mean cpm for con A-treated samples was  $34,285 \pm 6118$  ( $p < .005$ ). The level of radioactivity for a fixed number of  $^{51}\text{Cr}$ -labeled macrophages was also

obtained ( $1.6 \times 10^6$  cells = 267,118 cpm). From this data, the relative cpm values were converted to the number of cells per  $32 \text{ mm}^2$ , the surface area of each bone sample (control  $108,151 \pm 18,614$ ; con A  $205,709 \pm 36,603$ ).

The surface area of cell-matrix contact in the presence or absence of either con A or SBA was quantitated per cell by tracing the perimeter of attached cells on a Zeiss Videoplan Image Analyzer. The average cell surface area of con A-treated macrophages apposed to the bone surface is significantly less than the average cell surface area of control macrophages (con A  $24.8 \pm 6.5 \mu\text{m}^2$ ; control  $55.6 \pm 17.4 \mu\text{m}^2$ ;  $p < .001$ ). On the contrary, the average membrane surface area apposed to bone in the SBA-treated system is not significantly different from that of controls (SBA  $58.4 \pm 20.1 \mu\text{m}^2$ ).

#### Macrophage mediated release of bone mineral

The morphological findings confirm the hypothesis that specific saccharides common to cell surface and/or matrix associated glycoconjugates are involved in the interaction between macrophages and bone. Therefore, lectins specific for these saccharides can both qualitatively and quantitatively modify the macrophage-bone interaction. A  $^{45}\text{Ca}$  bone release assay was performed to determine whether the observations made by SEM could be correlated to any functional differences in the ability of lectin-treated macrophages to

release  $^{45}\text{Ca}$  in vitro.

The ability of macrophages to degrade a mineralized bone matrix was determined by the amount of  $^{45}\text{Ca}$  released into the culture medium during three 24h periods after the release of labile  $^{45}\text{Ca}$ . Values for the level of radioactivity (cpm) were obtained by counting duplicate samples from each well at each time period. The mean value (cpm) for each calvarial half incubated in either con A or SBA was divided by the mean value of the corresponding half incubated in control medium to give a treated/control (T/C) ratio for each calvaria. The mean T/C ratio for the two experimental groups was calculated. These values were compared to the mean control/control (C/C) ratio to determine whether the observed changes in bone resorption were significantly different. The C/C ratios were obtained from an assay in which both calvarial halves of each pair were incubated in the absence of either lectin. Significance was evaluated by a two-tailed Student t-test for the difference between two means.

In the macrophage-bone samples incubated in 50  $\mu\text{g}/\text{ml}$  con A, the same concentration that was utilized for the morphological studies, there was a marked decrease in the release of  $^{45}\text{Ca}$  from bone at 48h (T/C =  $0.83 \pm .07$ ;  $p < .005$ ) and 72h (T/C =  $0.72 \pm .09$ ;  $p < .002$ ) (fig. 8). On the other hand, the  $^{45}\text{Ca}$  released from the macrophage-bone samples incubated in 50  $\mu\text{g}/\text{ml}$  SBA did not differ significantly from that of controls at 24, 48 and 72h (fig. 8). In addition, the actual C/C values never differed by more than 5% from

the ideal value of 1.0 at any of the three sampling time points. The viability of macrophages incubated in con A, SBA and control medium was also determined after 72h using the trypan blue dye exclusion method. Cell viability ranged between 75% to 80% in all wells tested. This excludes the possibility that the reduction in the release of <sup>45</sup>Ca from con A-treated samples may have been the result of a selective increase in cell death at 48 and 72h.

## DISCUSSION

The hypothesis that mononuclear phagocytes are the precursors of osteoclasts has created an intense investigation to determine if these two distinct cell types share a common lineage and function. Most studies have focused on ultrastructural and functional similarities between cells belonging to the family of mononuclear phagocytes and osteoclasts on a bone matrix. It has been well-documented that cells of the mononuclear phagocyte system resorb devitalized bone in vitro (Mundy et al. 1977; Kahn et al. 1978; Teitelbaum et al. 1979). Glowacki et al. (1981) recently developed a model system in which they implanted devitalized bone fragments into calvarial defects in rats, thereby initiating a rapid cell-mediated resorptive sequence. A histological and ultrastructural study of these implants by Holtrop et al. (1982) demonstrated that the cells surrounding the bone fragments belong to the mononuclear phagocyte system. However, none of these cells, even the multinucleate giant cells, had ruffled borders, a morphological hallmark of osteoclasts, and therefore, could not be classified as osteoclasts. Other studies have also demonstrated that macrophages attached to bone acquire a number of the morphological characteristics of osteoclasts in culture, with the exception of ruffled borders (Kahn et al. 1978; Rifkin et al. 1979). All of these studies indicate that cells of the mononuclear phagocyte system are capable of resorbing bone both in vivo and in vitro.

More recently, Bar-Shavit et al. (1983a,b) have used a macrophage-bone binding assay to show that the attachment of macrophages to bone can be inhibited by certain sugars, while others have little or no effect. In a separate study, they have demonstrated that the glucocorticoids, dexamethasone and cortisol, enhance the attachment of macrophages to bone in a dose-dependent manner by causing alterations of cell surface oligosaccharides (Bar-Shavit et al. 1984). On the other hand, these investigators have shown that macrophage-bone attachment is greatly reduced in both vitamin D deficiency and hypophosphatemia (Bar-Shavit et al. 1982; Bar-Shavit et al. 1983c). They attribute this reduction in binding to the absence or inaccessibility of the bone matrix and/or cell surface oligosaccharides that are essential to the attachment process. These studies suggest that the reduction in bone resorption accompanying certain disease states or the stimulation of resorption induced by glucocorticoid treatment may, at least in part, depend on the ability of osteoclasts and their precursors to properly attach to the bone matrix, a step that appears to be essential for normal resorptive activity and osteoclast differentiation.

Lectins, proteins that selectively bind carbohydrates but do not have enzymatic activity, were initially used to study the surface structure and function of lymphocytes. More recently, the biological effects of different lectins on a variety of cellular systems have been investigated both in vivo and in vitro. Based on hapten

inhibition studies, which demonstrate that lectin-ligand binding can be inhibited by simple sugars, most lectins are highly specific in their cell surface saccharide binding requirements. In this study we used 2 lectins, each having distinctly different saccharide binding specificities, to study the importance of specific oligosaccharides on the attachment of macrophages to bone, and the subsequent release of  $^{45}\text{Ca}$  from the matrix. Con A binds to  $\alpha$ -linked D-glucose and D-mannose residues, whereas SBA preferentially binds to  $\alpha$ - or  $\beta$ -linked GalNAc residues, and, with lesser avidity, to D-galactose residues. Therefore, cell surface and matrix associated oligosaccharides containing these particular sugars will bind the appropriate lectin, and, as a consequence, the lectin bound oligosaccharides will be unable to directly participate in the macrophage-bone interaction.

The morphological findings indicate that con A does hinder the ability of macrophages to interact with the bone matrix. The membrane surface area that is apposed to the bone surface in the con A-treated system is far less than that of control macrophages; the con A-treated cells exhibit a minimal degree of attachment to the substrate. On the contrary, SBA does not appear to change the attachment and subsequent spreading of macrophages on bone. However, the dorsal, unapposed surface of both con A- and SBA-treated macrophages is ruffled; the microspikes present on control cells are absent. In addition, the morphology of the cell periphery is also different in lectin-treated cells. These differences in cell surface morphology may be due to the



processing of membrane-bound lectin complexes. Many of the biological effects of lectins are subsequent to binding, which in turn, alters the structure and activity of membrane components. Con A has been used extensively to study the redistribution and endocytosis of lectin-binding site complexes in a variety of cell types. An ultrastructural study by Welsch and Schumacher (1983) demonstrated that rat pulmonary macrophages bound and incorporated large amounts of con A in vivo. Additional studies should be conducted, to assess the manner in which membrane-bound con A and SBA are processed by these resident peritoneal macrophages in vitro, including the possible redistribution and/or endocytosis of these complexes.

The quantitative data demonstrates that approximately twice the number of con A-treated macrophages attached to the bone surface in comparison with the number of untreated cells. Although these results seem to conflict with the <sup>45</sup>Ca release data, this quantitative difference is most likely the result of the extensive aggregation of macrophages by con A. The cross-linking of cell membranes by con A may significantly increase the number of cells that appear to attach to the bone matrix, since those cells that would not attach under normal conditions appear to be attached to the bone surface by cross-linking to the cells that are capable of attachment. The quantitation of membrane surface area apposed to the bone surface, demonstrates that the average cell surface area of con A-treated macrophages that are in direct contact with the bone matrix is

significantly less than that of control or SBA-treated cells. The surface area values presented in the results were measured from SEM micrographs, and therefore, are less than the actual surface areas of cell-matrix apposition in the unfixed state. Schneider (1976) demonstrated that during routine SEM tissue processing, cell shrinkage, due primarily to critical point drying, results in a greater than 50% reduction in the total cell surface area.

In addition to the morphological observations, the  $^{45}\text{Ca}$  bone release assay was performed to evaluate the functional effects of con A and SBA on the macrophage-bone system. Previous studies on macrophage-mediated  $^{45}\text{Ca}$  release from pre-labeled bone in vitro, have shown that optimal mobilization of the radioactive label requires cell-matrix contact (Kahn et al. 1978; Teitelbaum et al. 1979). Morphological studies have also demonstrated localized areas of matrix removal immediately underlying bone-resorbing mononuclear cells both in vivo (Holtrop et al. 1982) and in vitro (Kahn et al. 1978). Based on these studies and our own findings, we conclude that the decreased surface area of cell-matrix contact in the con A cultures may, at least in part, account for the reduction in macrophage-mediated mineral mobilization from these cultures.

There also exists an important correlation between the effects of these 2 lectins on bone mineral mobilization by macrophages and their effects on  $^{45}\text{Ca}$  release in our bone organ culture system. In previous studies, we have shown that con A, at a concentration of 50  $\mu\text{g/ml}$ ,

will significantly reduce the release of  $^{45}\text{Ca}$  from a pre-labeled bone-organ culture system, while SBA, at this same concentration, has no functional effect on mineral mobilization (Popoff and Schneider 1983; Popoff et al. 1984). The results obtained from this study demonstrate that con A, but not SBA, alters the morphology and function of macrophages on a bone surface. These findings suggest that cell surface and bone matrix oligosaccharides with  $\alpha$ -D-glucosidic and  $\alpha$ -D-mannosidic residues are more likely to be involved in the macrophage-bone interaction, than oligosaccharides with GalNAc or D-galactose residues. However, the possibility that membrane-bound con A may cause changes in cell activity and processes that play an important role in mineral mobilization must be assessed. Although there is substantial evidence that specific saccharides mediate the attachment of macrophages to bone, it is not yet clear how much this affects the function of these cells on a mineralized bone matrix.

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Fig. 1. Control macrophages attached to a devitalized bone surface. The cells are in various stages of the spreading process (X 1000).

Fig. 2. High magnification of control macrophages demonstrating numerous microvilli on the unapposed surface. Many filopodia as well as some larger lamellipodia are present at the cell periphery (X3000).

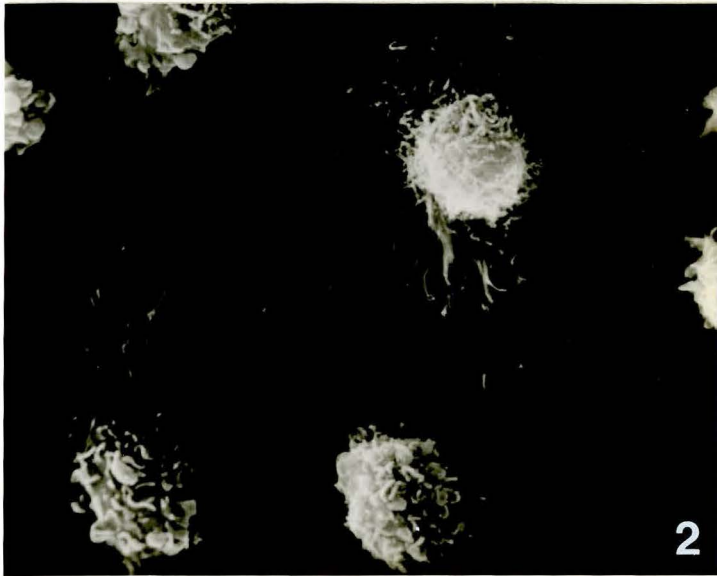
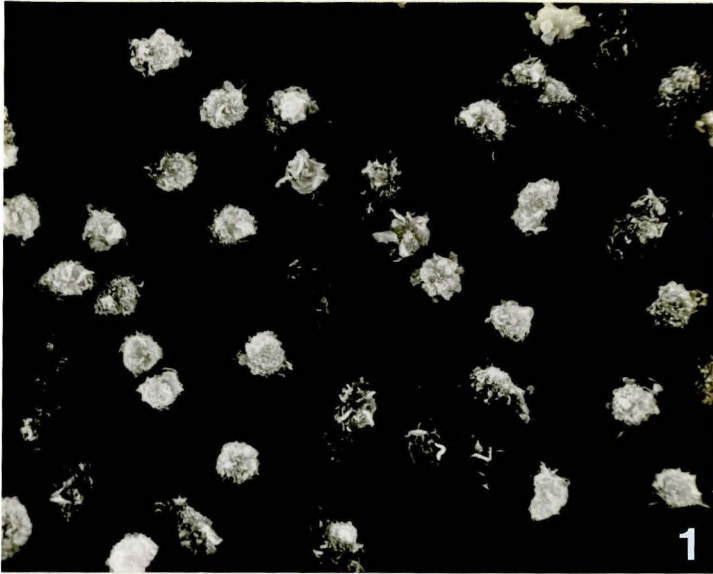


Fig. 3. Con A-treated macrophages attached to bone, illustrating the minimal cell-matrix interaction (X 1000).

Fig. 4. High magnification of con A-treated macrophages, as in fig. 3. The surface morphology demonstrates little evidence of cell spreading. The dorsal (unapposed) surface has a ruffled appearance (X 3000).



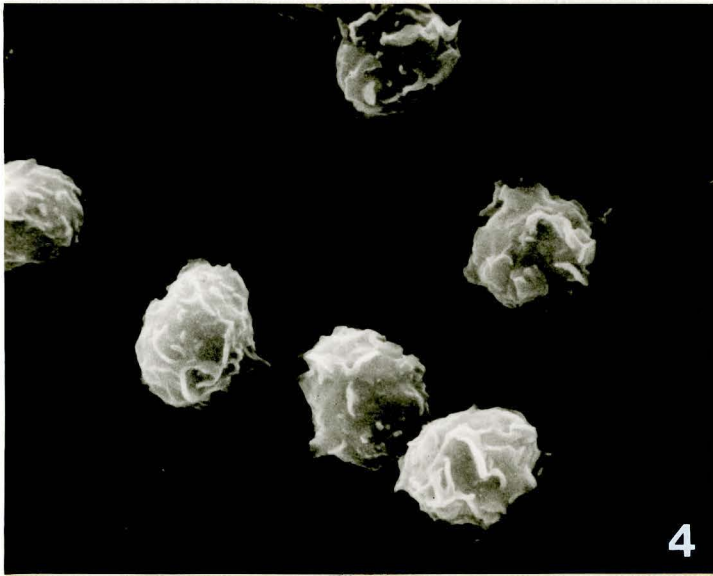
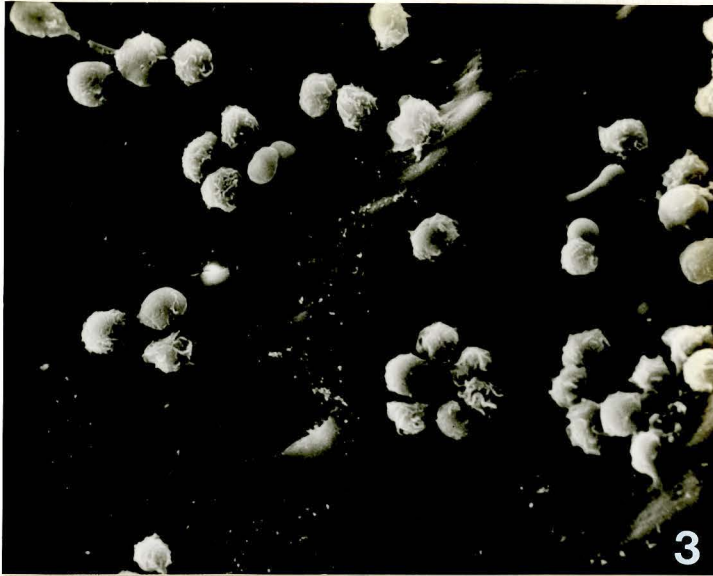


Fig. 5. Field of SBA-treated macrophages attached to and subsequently spreading on a bone surface. The SBA does not appear to inhibit the membrane-substrate interaction (X 1200).

Fig. 6. SBA-treated cells, as in fig. 5, at a higher magnification. Some cells exhibit an extensive membrane-substrate interaction (single arrow), while others are less advanced (double arrows) (X 2400).

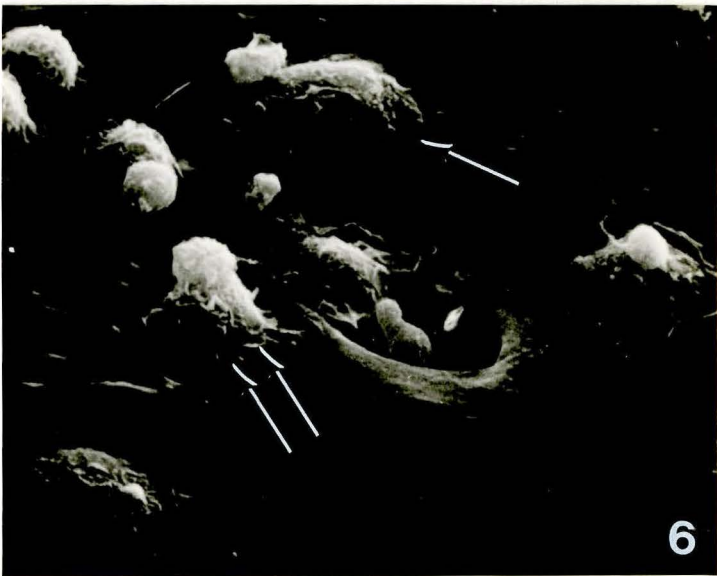


Fig. 7. Quantitation of macrophage attachment to bone. The number of con A-treated macrophages that attached to the bone surface is significantly greater than the number of control or SBA-treated cells ( $p < .001$ ).

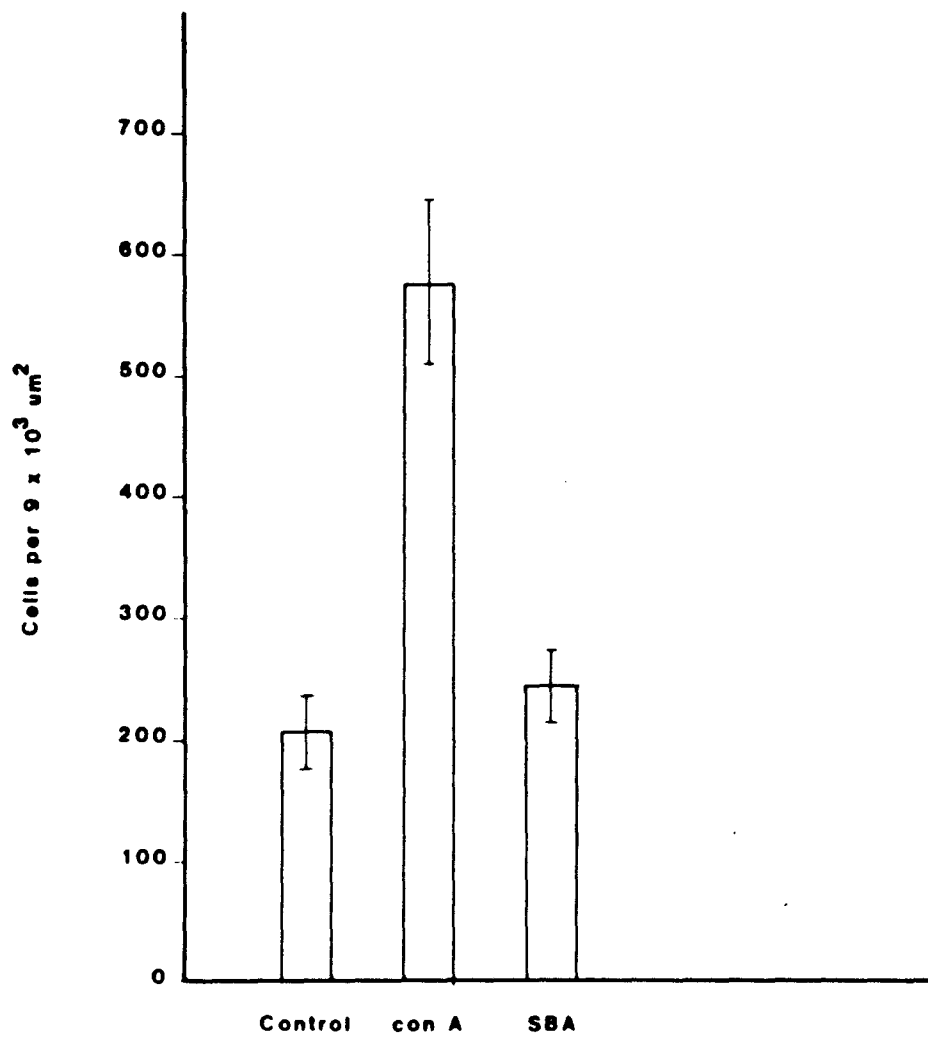
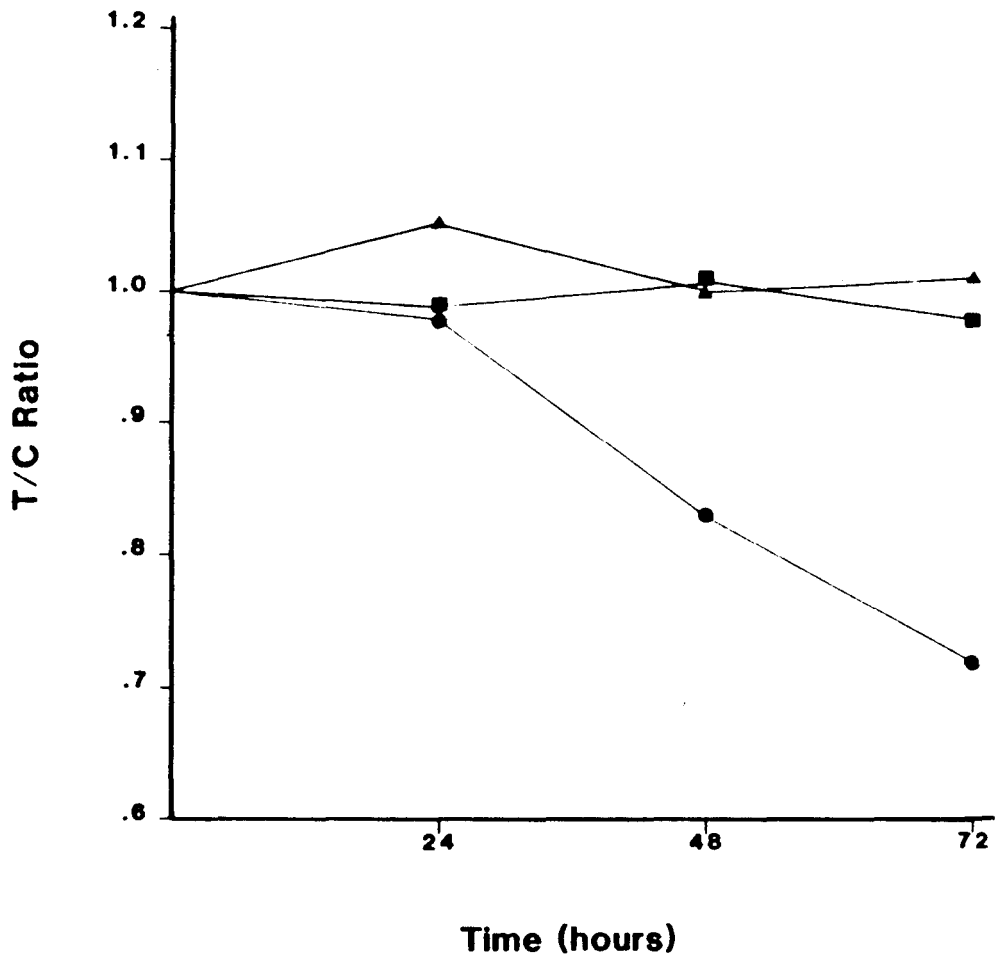


Fig. 8.  $^{45}\text{Ca}$  bone release assay. C/C ratios; 24h ( $0.99 \pm .02$ ), 48h ( $1.01 \pm .03$ ), 72h ( $0.98 \pm .03$ ): SBA T/C ratios; 24h ( $1.05 \pm .06$ ), 48h ( $1.00 \pm .06$ ), 72h ( $1.01 \pm .04$ ): con A T/C ratios; 24h ( $0.98 \pm .04$ ), 48h ( $0.83 \pm .07$ ); 72h ( $0.72 \pm .09$ ).



▲ - Soybean Agglutinin

● - Concanavalin A

■ - Control

CHAPTER VI

PROCESSING OF CONCAVALIN A-RECEPTOR  
COMPLEXES BY OSTEOCLASTS IN VITRO



## ABSTRACT

The osteoclast is a large multinucleate cell that is widely accepted as the primary effector cell responsible for normal bone resorption. In a previous study, we demonstrated that concanavalin A (con A) has a dose-dependent biphasic effect on the bone resorbing capacity of osteoclasts using a  $^{45}\text{Ca}$  bone-organ culture system; bone resorption was stimulated at low concentrations and inhibited at high concentrations. The mitogenic property of con A in lymphocyte cultures is well-documented, and therefore, con A has been used extensively to study the manner in which lymphocytes and other mononuclear cells process the cell-bound lectin. In this study, we have investigated the processing of con A-receptor complexes by osteoclasts in culture, using con A-FITC to evaluate the redistribution of cell-bound con A via epifluorescence microscopy and using con A-ferritin to determine whether the lectin-receptor complexes are internalized. The osteoclasts were obtained from the long bones of newborn rats and allowed to attach to glass coverslips at  $37^{\circ}\text{C}$ . Following attachment, the non-adherent cells were removed by rinsing. The adherent osteoclasts were pre-incubated in  $50\ \mu\text{g/ml}$  con A-FITC or con A-ferritin at  $4^{\circ}\text{C}$  for 10 minutes, washed to remove unbound con A, and incubated at  $37^{\circ}\text{C}$  for 15 or 30 minutes in the absence of con A. Positive controls were fixed immediately after pre-incubation at  $4^{\circ}\text{C}$ ; negative controls were pre-incubated in

con A-FITC and  $\alpha$ -methyl mannoside, the haptenic inhibitor of con A binding. The results demonstrate that redistribution and endocytosis of con A-receptor complexes occurs within 30 min. These findings confirm the hypothesis that cell-bound con A can alter the structure and activity of osteoclast membrane components in a manner similar to that observed in mononuclear cell cultures. The internalization of con A may be important in altering osteoclastic activity by mediating intracellular mechanisms involved in the bone resorbing process.

## INTRODUCTION

Lectins are proteins that have been used in a variety of biological assays to study the surface structure and function of cells and tissues. Based on hapten inhibition studies which demonstrate that the interactions of lectins with cells can be inhibited by simple sugars, lectins are highly specific in their cell surface saccharide binding requirements (Sharon and Lis, 1972). In addition, many lectins exhibit differential binding affinities for various mammalian cells due to cell-specific differences in the expression of membrane glycoconjugates. These properties promote the use of lectins as probes to study the structure and activity of cell surface components in both normal and pathologically-altered tissues. In biological assays, lectins have demonstrated the ability to mimic the functional effects of documented, physiological mediators of cellular activity, such as hormones and lymphokines. Therefore, lectins have been used as in vitro model systems to evaluate the mechanisms of cellular activation and/or inhibition.

A variety of agents, including certain lectins, such as concanavalin A (con A), phytohemagglutinin (PHA) and pokeweed mitogen (PWM), are capable of activating DNA and RNA synthesis in cultured lymphocytes (Janossy and Greaves, 1972). Subsequent to the binding of these mitogenic lectins, the lymphocytes undergo a sequence of morphological and metabolic transitions culminating in the formation

of blast-like cells. In addition, the mitogenic lectins are particularly useful since they are capable of activating cell division in a large, heterogeneous population of resting lymphocytes, in comparison to the mitogenic capacity of specific antigens that can only activate cell division in a small subpopulation of sensitized lymphocytes (Takada et al., 1983). This mitogenic property created a surge of interest in the use of lectins in the early 1970's, to study the morphological and biochemical events involved in the conversion of a resting cell into an active, dividing cell.

Con A is the most widely used lectin in biological assays and its mitogenic property in lymphocyte cultures has been well-documented. Therefore, con A has been used extensively to study the manner in which lymphocytes process cell-bound mitogenic lectins. Studies by Unanue et al. (1972), Yahara and Edelman (1973) and Bhalla et al. (1979) have demonstrated that con A bound to lymphocyte surface receptors can induce patch and cap formation of its own receptor sites under certain conditions of incubation. The endocytosis of con A-receptor complexes following cap formation has also been described (Unanue et al., 1972; Bhalla et al., 1979). The formation of lectin-receptor caps and their subsequent internalization are metabolically dependent events (Yahara and Edelman, 1973; Ryan et al., 1974; dePetris, 1975). It has been postulated that the processing of lectin-receptor complexes subsequent to lectin binding is necessary for mitogenic activation of lymphocytes (Taylor et al., 1971; Greaves

et al., 1972).

Aside from the mechanisms involved in lectin-cell surface interactions, the biological effects of many lectins on a variety of cellular systems have been investigated both in vivo and in vitro. However, few studies have used lectins to evaluate the structure and function of the skeletal system. Wang et al. (1982) demonstrated that the in vivo administration of PHA-P caused a significant increase in the number and size of osteoclasts. Horton et al. (1982) demonstrated a decrease in resorption of long bones cultured in the presence of high concentrations of con A (50 and 100  $\mu\text{g/ml}$ ). Subsequent TEM of these con A-treated osteoclasts revealed ultrastructural changes associated with inhibition of osteoclastic activity (Ryder et al., 1982).

The studies involving the skeletal system indicate that some lectins can induce changes in the structure and function of bone cells, particularly osteoclasts. The osteoclast is a large multinucleate cell that is widely accepted as the primary effector cell responsible for normal bone resorption (Marks, 1983). Although the definitive origin of the osteoclast remains uncertain, there is abundant evidence to support the hypothesis that osteoclasts are derived from mononuclear cells of hemopoietic origin, possibly of the monocytic lineage (Walker, 1975, Marks and Walker, 1981, Schneider and Byrnes, 1983). In a previous study, we demonstrated that the plasma membrane of resident osteoclasts on the endocranial surface of rat

calvariae possess specific binding sites for con A (Popoff and Schneider, 1983). In addition, we conducted a series of in vitro <sup>45</sup>Ca bone release assays to evaluate the effect of various concentrations of con A on the bone resorbing capacity of osteoclasts. The bone mineral release assays demonstrated that con A has a biphasic effect on osteoclastic activity; at a low concentration (0.1 µg/ml) con A increased bone resorption, whereas at higher concentrations (50 and 100 µg/ml) con A inhibited bone resorption. We concluded that con A may directly alter osteoclastic activity subsequent to its binding to specific membrane glycoconjugates. Alternately, con A may mediate osteoclastic activity indirectly, through the intermediary of another cell type. These mechanisms of action have been proposed for other endogenous agents that regulate osteoclastic activity (Rodan and Martin, 1981; Chambers and Magnus, 1982; Chambers and Dunn, 1983).

It has been well-documented that there are a variety of agents that regulate osteoclast function both in vivo and in vitro. Although the morphological changes associated with the stimulation or inhibition of osteoclastic activity have been well-documented (King et al., 1978; Holtrop et al, 1979; Wezeman et al., 1979), the nature of the intracellular mechanisms responsible for these changes is poorly understood. Therefore, the use of an exogeneous biologically active agent, such as con A, in conjunction with a bone organ culture system, is of interest as an in vitro model to study the biological control of osteoclast function. Since many of the biological activities of con A

appear to involve the initial binding of con A to the cell surface, the fate of the cell-bound con A has been the focus of numerous investigations. In this study, we investigated the processing of con A-binding site complexes by osteoclasts in vitro. Con A conjugated to fluorescein isothiocyanate (con A-FITC) was used to evaluate the redistribution of cell-bound con A via epifluorescence microscopy. In addition, an ultrastructural study using con A-ferritin was conducted to determine whether these lectin-receptor complexes are internalized.

## MATERIALS AND METHODS

Preparation of osteoclasts

The procedure used to obtain osteoclasts from neonatal rat long bone is similar to that described by Chambers et al. (1984). Newborn Norway Hooded rats (less than 24 hr) were sacrificed and the femurs and tibiae were removed and dissected free of soft tissue. The long bones were cut across at their epiphyses to separate the bony diaphyses from the cartilaginous ends. The four diaphyses obtained from each rat were then placed into shallow (2 mm) glass wells of a Boerner test slide containing approximately 500  $\mu$ l of Medium 199 supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin. The bones were curetted with a scalpel and the resulting suspension of cells and bone fragments was agitated and then pipetted onto a 22 mm glass coverslip using a siliconized glass pipette. The cells were incubated for 1 hour at 37°C. The non-adherent cells and bone fragments were removed by thoroughly washing the coverslips in 0.05M Tris buffer. The coverslips were examined via phase contrast microscopy and osteoclasts were identified on the basis of their multinucleation, since these are the only multinucleate cells in normal bone. This procedure yields an average of 80 osteoclasts per 4 long bones and a sparse population of attached mononuclear cells. Chambers et al. (1984) have shown that the long bones from newborn animals reduces the presence of contaminating



mononuclear cells in these osteoclast cultures, since the cellular component of the bone marrow is poorly developed at this age. The following epifluorescent (con A-FITC) and ultrastructural (con A-ferritin) experiments were performed on the attached osteoclasts within a few hours.

#### Phase contrast - epifluorescence microscopy

Eight coverslips were used for this experiment. The conditions of incubation employed in this study have been used to study the redistribution of cell-bound con A-FITC in mononuclear cell cultures (Unanue et al., 1972; Yahara and Edelman, 1973; Bhalla et al., 1979). The osteoclasts were pre-incubated in 0.05M Tris buffer containing 50 µg/ml con A-FITC (E-Y Labs, San Mateo, Ca.) for 10 minutes at 4°C. The Tris buffer used in all of our procedures was supplemented with 0.1M NaCl and 0.01M CaCl<sub>2</sub> and adjusted to a pH of 7.2 to 7.4. To insure specific binding of tetravalent con A to cell surface glycoconjugates, both divalent calcium ions and a narrow pH range from 7.0 to 7.4 are required (Sharon and Lis, 1972). The osteoclasts on 2 of the coverslips were negative controls that were pre-incubated in 50 µg/ml con A-FITC and -methyl mannoside, the haptenic inhibitor of con A binding. Following pre-incubation, the cells were sequentially rinsed to remove unbound con A-FITC molecules. After washing, the cells on 2 of the remaining 6 coverslips and the negative controls were fixed in 2.5% glutaraldehyde for 15 minutes. The osteoclasts on

the remaining 4 coverslips were incubated in supplemented Medium 199 at 37°C in the absence of con A for 15 or 30 minutes prior to fixation. Following fixation, all coverslips were inverted and placed onto glycerol-coated glass slides. The osteoclasts were immediately examined with a Leitz Orthoplan epifluorescence microscope.

#### Transmission electron microscopy

The procedure for pre-incubation (4°C) and incubation (37°C) of osteoclasts with con A-ferritin was identical to that used for con A-FITC with the following exception. The negative controls were pre-incubated in 50 µg/ml native ferritin (Sigma, St. Louis, Mo.) at 4°C for 10 minutes in the absence of con A to demonstrate the specificity of the conjugated ferritin label for cell-bound con A. Following pre-incubation in 50 µg/ml con A-ferritin (E-Y Labs, San Mateo, Ca.) at 4°C for 10 minutes, the cells were rinsed and the osteoclasts on 2 coverslips were fixed immediately, while the remaining osteoclasts were incubated in supplemented Medium 199 at 37°C in the absence of con A for 15 or 30 minutes. Following fixation in 2.5% glutaraldehyde for 15 minutes, the cells were rinsed and post-fixed in 1% osmium tetroxide for 15 minutes. The cells were dehydrated through a graded series of ethanol and placed into propylene oxide for 2 changes at 10 minutes each. The cells were infiltrated in a mixture of propylene oxide and Epon 812 and embedded in beam capsules. After the epoxy resin polymerized, the beam

capsules and attached coverslips were immersed in liquid nitrogen. The coverslips were snapped free of the Epon capsule which resulted in a smooth surface on one end of the capsule containing the monolayer of cells. The cells were sectioned parallel to the plane of attachment. A single 1  $\mu\text{m}$  thick section was cut from the smooth block face and stained with toluidine blue for light microscopy. Thin sections displaying silver or gold interference colors (60-80 nm thickness) were obtained using a diamond knife on an LKB Ultratome V. The ultrathin sections were collected on 200 mesh thin-bar grids, stained with uranyl acetate and lead citrate for 2 minutes each and examined using a Hitachi H600 electron microscope.

## RESULTS

Phase contrast - epifluorescence microscopy

Osteoclasts attached to glass coverslips were easily identified via phase contrast microscopy on the basis of their multinucleation. Control osteoclasts that were labeled with 50  $\mu\text{g/ml}$  con A-FITC at 4°C for 10 minutes exhibit a fluorescence that is evenly distributed over the entire cell surface (Fig. 1). Frequently, the fluorescence at the perimeter of the cell is accentuated, thereby outlining the peripheral extent of these attached cells (Fig. 1). A faint fluorescence over the filopodia extending from the periphery can also be observed. The negative control osteoclasts that were labeled at 4°C in the presence of 50  $\mu\text{g/ml}$  con A-FITC and 0.1 M  $\alpha$ -methyl mannoside, the specific inhibitor of con A-ligand binding, do not exhibit any fluorescence (Fig. 2). These negative controls indicate that the binding of con A-FITC to osteoclast membrane glycoconjugates containing  $\alpha$ -linked D-glucose and D-mannose residues is specific.

The osteoclasts that were rinsed and incubated at 37°C for 15 minutes in the absence of con A following cold labeling with con A-FITC, demonstrate a localization of membrane fluorescence that is confined to the central, nuclear area of the cells (Fig. 3). The membrane peripheral to the nuclear region and the peripheral membrane specializations, such as lamellipodia and filopodia, showed minimal fluorescence. The fluorescent "caps" observed in the 15 minute

osteoclasts have an uneven or patchy appearance in comparison to the fluorescence observed on control osteoclasts. Some areas of the "cap" fluoresce brightly while other areas fluoresce less intensely. The size of the fluorescent "caps" varied depending upon the degree to which the cells were spread; the "caps" were smaller in those osteoclasts that were spread more extensively. The osteoclasts that were rinsed and incubated at 37°C for 30 minutes in the absence of con A following pre-incubation with con A-FITC also exhibit a fluorescent pattern that is observed in the central regions of the cells (Fig. 4). However, in comparison to the 15 minute osteoclasts, the fluorescence after 30 minutes is more evenly distributed and occupies a larger area, thus extending further out toward the periphery. In addition, the negative images of the nuclei are clearly visible suggesting that at 30 minutes some fluorescent label is located in the cytoplasm of these cells (Fig. 4).

#### Transmission electron microscopy

By transmission electron microscopy (TEM), the cultured osteoclasts possessed many of the ultrastructural features that have been well-documented for osteoclasts apposed to bone in vivo (Holtrop and King, 1976)(Fig. 5). The nuclei in 1  $\mu$ m sections range in number from 5 to 20 per cell. Most of the nucleoplasm is euchromatic with a peripheral clumping of heterochromatin along the inner nuclear membrane. The cytoplasm is densely populated with pleomorphic

mitochondria. Dense bodies and vacuoles of various sizes are also uniformly scattered throughout the cytoplasm. Numerous investigators have described these dense bodies in the osteoclast and have associated them with the lysosomal system of the osteoclasts (Lucht, 1972; Thyberg et al., 1975). In addition, some of the osteoclasts remain attached to fragments of bone surrounded by an organelle-free region of the cytoplasm, the clear zone (Fig. 5).

The control osteoclasts that were pre-incubated at 4°C with 50 µg/ml con A-ferritin exhibit an even distribution of ferritin particles along the entire outer leaflet of the plasmalemma (Figs. 6 & 7). There is no evidence of internalization of the ferritin label in these control osteoclasts. In contrast, the osteoclasts that were first labeled with con A-ferritin at 4°C and then incubated at 37°C for 15 minutes in the absence of con A, demonstrate active endocytosis of the con A-ferritin receptor complexes (Fig. 8). Some areas of the membrane show tubular invaginations forming a system of canaliculi that are continuous with the cell surface (Figs. 8 & 9). Clathrin-coated pits are also frequently observed along the plasmalemma (Fig. 10). The ferritin label is concentrated in these canaliculi and coated pits (Figs. 9 & 10). The plasmalemma surrounding these membrane specializations is either devoid of label or sparsely labeled in an uneven fashion. In addition, some endocytic vesicles (endosomes) labeled with ferritin are found in the cytoplasm immediately underlying the cell surface (Fig. 8). Frequently,

channels containing the ferritin label are also observed in areas of the cytoplasm that do not exhibit continuity with the cell surface. This indicates that the canalicular system may be more extensively developed than is apparent from the tubular invaginations that show continuity with the cell surface. In the osteoclasts incubated at 37°C for 30 minutes, most of the ferritin label is found in the cytoplasm, primarily in endosomes and occasionally in channels (Fig. 11). The ferritin labeling density within the vesicles and the size of the vesicles vary considerably. Frequently, smaller endosomes appear to be fusing with larger ones (Fig. 11). Some of the channels within the cytoplasm are also continuous with the larger endosomes. Osteoclasts that were pre-incubated in 50 µg/ml native ferritin at 4°C in the absence of con A, do not exhibit a ferritin label along their cell surface. This indicates that the conjugated ferritin label is specific for membrane-bound con A.

## DISCUSSION

In the early 1970's, the mitogenic property of certain lectins, such as con A, PHA and PWM, created an interest in the use of these lectins to study blast transformation in resting lymphocyte cultures. Many of the ensuing investigations that focused on the morphological and biochemical events involved in the activation of cell division used con A as the lymphocyte activating agent because it offered a number of attractive features as a biological probe. Con A is a well-defined tetravalent protein that specifically binds to cell surface glycoconjugates containing  $\alpha$ -linked D-glucose and D-mannose residues. The haptenic inhibitor of con A binding,  $\alpha$ -methyl mannoside, is used in control cultures to demonstrate the specificity of con A binding to cell surface receptors. A variety of con A-conjugates are commercially available in high purity and can be used to visualize membrane-bound con A via ultrastructural and cytochemical techniques.

Con A is the most widely used lectin in biological assays and it has been used extensively to study the manner in which lymphocytes process cell-bound mitogenic agents. Unanue et al. (1972) used con A-FITC to demonstrate that patching and capping of con A binding sites occurred when lymphocytes were labeled with con A at 4°C, then washed to remove unattached con A molecules, followed by incubation at 37°C. Yahara and Edelman (1973) showed that lymphocytes incubated at



37°C in concentrations of con A above 10 µg/ml did not exhibit patch or cap formation, whereas at concentrations below 10 µg/ml some of the lymphocytes capped. These observations were confirmed by Bhalla et al. (1979), who demonstrated that if washing is omitted after pre-incubation at 4°C or if con A is added back to the medium after washing, the percentage of cells forming caps is drastically reduced. Once the caps are formed, the con A-receptor complexes are rapidly endocytosed (Unanue et al., 1972; Bhalla et al., 1979). The formation of lectin-receptor caps and their subsequent internalization are metabolically dependent events (Yahara and Edelman, 1973; Ryan et al., 1974; dePetris, 1975). It has been postulated that the redistribution and endocytosis of cell-bound con A may be necessary events for mitogenic induction in lymphocyte cultures (Taylor et al., 1971; Greaves et al., 1972).

The results of the con A-redistribution studies indicate that depending on the conditions of incubation employed, con A exhibits 2 antagonistic properties when bound to lymphocytes; it can induce or inhibit cap formation of its own binding sites. To induce cap formation of con A-receptor complexes it is necessary to wash the cells following pre-incubation with con A to remove unbound con A molecules. In this study we have demonstrated the redistribution of con A-receptor complexes in cultured osteoclasts in a manner similar to that described for lymphocytes and other mononuclear cells. The osteoclasts that were pre-incubated with con A-FITC and α-methyl

mannoside do not fluoresce when examined via epifluorescent microscopy. This indicates that the fluorescent label is specific for con A binding sites on the osteoclast membrane. The positive controls that were fixed immediately after pre-incubation with con A-FITC at 4°C, exhibit a fluorescent pattern suggesting that the cell-bound con A is evenly distributed over the entire cell surface. When the cells were washed to remove the unbound con A molecules following pre-incubation at 4°C and subsequently warmed to 37°C for 15 minutes in the absence of any con A in the culture medium, the con A-binding site complexes had moved to occupy the membrane overlying the centrally located nuclei, visualized by the central localization of con A-FITC. This movement is similar to the formation of ligand-receptor caps in adherent mononuclear cell cultures. Although the intensity of the fluorescence in these osteoclast "caps" was greater than the intensity in the control osteoclasts, the fluorescent pattern within each "cap" was uneven. The cells that were washed and incubated in con A free-medium at 37°C for 30 minutes, exhibit a cytoplasmic localization of the fluorescent label as evidenced by the negative nuclear images. The cytoplasmic fluorescence observed in the 30 minute osteoclasts suggests that the con A-receptor complexes have been endocytosed.

The plasma membrane of most animal cells contains a variety of specific receptors for external macromolecular ligands, including hormones, vitamins, lymphokines and pathological agents, such as

bacterial toxins and viruses. Most of the surface receptors are glycoproteins. The induction of many of the functional effects by cell-specific ligands requires the internalization of the receptor-bound ligands. Subsequent to ligand binding, the endocytosis of the ligand-receptor complexes is rapid and highly specific (Willingham and Pastan, 1984). The general pathway of endocytosis involves the clustering of ligand-receptor complexes in clathrin-coated pits along the plasma membrane. The coated pits pinch off from the membrane, thus forming intracellular endocytic vesicles (endosomes).

Since lectins bind specifically to cell surface glycoproteins and subsequently enter cells via the endocytic cycle, they have been used to study the mechanisms of endocytosis in cell cultures (Gonatas et al., 1980; Welsch et al., 1982; Welsch and Schumacher, 1983). In this study we used con A conjugated to ferritin to determine whether osteoclasts are capable of internalizing cell-bound con A. We performed a preliminary study to determine what concentration of con A-ferritin would give us a sufficient ferritin label so that we could accurately assess the redistribution and endocytosis of cell-bound con A-ferritin. From this study, we found that a concentration of 50 µg/ml was optimal. The control cells that were fixed immediately after pre-incubation at 4°C with con A-ferritin demonstrate a uniform ferritin label along the cell surface. The osteoclasts that were pre-incubated at 4°C, washed and incubated at

37°C for 15 minutes exhibit a clustering of ferritin in coated pits and in tubular invaginations of the cell surface. In addition, ferritin-labeled vesicles are present in the cytoplasm subjacent to the cell surface. The system of canaliculi in these osteoclasts forms in response to the processing of cell-bound con A, since tubular profiles are absent in the control cells. In addition, these canaliculi have not been described by other investigators who have examined the ultrastructure of cultured osteoclasts (Allen et al., 1981; Testa et al., 1981; Suda et al., 1983). Although the continuity between the cell surface and some of the tubular invaginations is clearly evident, we frequently observed sections of ferritin labeled tubules that did not exhibit continuity with the cell surface. Therefore, the complexity of this tubular labyrinth cannot be determined from our ultrathin sections. Abe et al. (1981) showed that the examination of thick sections (1.0 - 1.5  $\mu\text{m}$ ) of macrophages by high voltage electron microscopy provides an image with three-dimensional features of the cell surface configuration. Employing this procedure to examine thick sections would enable us to establish the complexity of the canalicular system in these osteoclasts. The changes we observed in the structure and activity of the osteoclast membrane in this study were induced by the cell-bound con A and not the ferritin. This conclusion is based on the observation that osteoclasts pre-incubated with native ferritin at 4°C do not bind the ferritin molecule.

In the osteoclasts that were incubated at 37°C for 30 minutes following the 4°C pre-incubation with con A-ferritin, the ferritin label is primarily located intracellularly contained within endosomes. Occasionally, the ferritin label is also observed within tubular profiles. Frequently, the larger ferritin-labeled endosomes are surrounded by smaller ferritin-labeled vesicles, some of which appear to be fusing with the larger endosomes. Although we were able to observe a spatial relationship between ferritin-labeled endosomes and the lysosomes, the lysosomal contents and the ferritin label are similar in density, and therefore, ferritin cannot be visualized within the lysosomes. To determine whether the endocytosed con A-ferritin is ultimately routed into the lysosomes, possibly via the Golgi apparatus as described in some cell types, a study employing a different con A conjugate, such as con A-colloidal gold, should be conducted.

Ultrastructural studies by Takata et al. (1984) and Abe et al. (1979) demonstrated that murine peritoneal macrophages possess an extensive three-dimensional tubular labyrinth underneath the cell surface. These channels are continuous with the cell surface. This tubular labyrinth appears similar to the tubular profiles we observed in the osteoclasts, with the exception that the macrophage tubular network even exists in cells that are not exposed to a specific ligand. In a recent study by Takata et al. (1984), they demonstrated the redistribution and endocytosis of con A-receptor complexes in

murine peritoneal macrophages. They labeled macrophages with 50  $\mu\text{g}/\text{ml}$  con A-colloidal gold at  $0^{\circ}\text{C}$ , washed the cells and warmed them to  $37^{\circ}\text{C}$ . This resulted in the movement of con A-receptor complexes into the tubular network beneath the cell surface. The subsequent localization of colloidal gold in endosomes verified the endocytosis of con A-receptor complexes. They concluded that the tubular labyrinth is involved in the processing of cell-bound con A by these macrophages.

Since the phenomenon of capping was originally described in 1971 by Taylor et al. on B lymphocytes in suspension, its relation to cell motility and the endocytic cycle has been studied on cells attached to a substrate. Surface receptors are cross-linked by a specific ligand resulting in the formation of small two-dimensional aggregates of ligand-receptor complexes called patches. If a cell is motile, the patches will move to and cap in a region of the cell membrane that is remote from the cell's leading edge of attachment and migration. The process of cap formation in migrating mononuclear cells that exhibit a bipolar structural organization is well documented (dePetris and Raff, 1972; Edidin and Weiss, 1972; dePetris and Raff, 1973; Vasilieu et al., 1976). Bretscher (1984) described a model of membrane flow that relates the process of cap formation with the endocytic cycle in migrating cells. On the cell surface, membrane components flow from the cell's leading edge to the rear of the cell, the region that corresponds to the eventual site of cap formation. Patches of ligand-receptor complexes exhibit a preferential mobility resulting in

cap formation. The subsequent endocytosis of the capped ligand-receptor complexes results in a large amount of plasma membrane that is being internalized and must be recycled back to the cell surface. According to Bretscher's model, this internal membrane pool is transported through the cell and reinserted into the plasma membrane at the cell's leading edge. In this study, the osteoclasts are spreading evenly in all directions on the substrate, and therefore do not exhibit a bipolar organization. In these cells the leading edge occurs along the entire cell perimeter. According to the membrane flow hypothesis, the cross-linked con A-receptor patches should move away from the free edge toward the center of the cell. Our observations on the localization of cell-bound con A-FITC coincides with this hypothesis. Endocytosis of the cell-bound lectin results in the internalization of large amounts of plasma membrane which may then be transported through the cell to be reinserted at the leading edge. Although the phenomena of capping and endocytosis have been studied in relation to one another, capping of ligand-receptor complexes is not a necessary prerequisite for endocytosis. Endocytosis can occur in the absence of cap formation and it may also occur during cap formation.

There are a variety of physiological agents that are capable of altering the bone resorbing activity of osteoclasts both in vivo and in vitro. These include parathyroid hormone (PTH) and calcitonin (CT), the two primary hormones regulating osteoclastic activity in

vivo. In addition studies have demonstrated that osteoclast activating factor, a lymphokine produced by activated lymphocytes, several prostaglandins, 1,25 dihydroxyvitamin D<sub>3</sub> and interleukin-1 can stimulate bone resorption in vitro (Klein and Raisz, 1970; Raisz et al., 1972; Horton et al., 1974; Gowen et al., 1983). Some of these agents (i.e. CT) bind directly to specific osteoclast membrane receptors (Warshawsky et al., 1980; Roa et al, 1981; Chambers and Magnus, 1982), while others (i.e. PTH) lack specific receptors on the osteoclast membrane (Silve et al., 1982; Chambers and Dunn, 1983). These results support the hypothesis that some biologically active ligands may act directly on osteoclasts, while others may regulate osteoclastic activity indirectly through the intermediary of another cell type (Rodan and Martin, 1981; Chambers and Dunn, 1983). In a previous study, we demonstrated specific con A binding sites on the osteoclast membrane (Popoff and Schneider, 1983). We also showed that con A has a dose-dependent biphasic effect on the bone resorbing capacity of osteoclasts using a <sup>45</sup>Ca bone organ culture system.

The ultrastructural changes associated with the stimulation or inhibition of osteoclastic activity have been well-documented both in vivo (Holtrop et al., 1979) and in vitro (King et al., 1978; Wezeman et al., 1979). However, the manner in which the osteoclast processes ligand-receptor complexes subsequent to the binding of a functional ligand and the intracellular mechanisms responsible for changes in cell activity are poorly understood. Under the proper conditions of



incubation, we have shown that cell-bound con A causes an alteration of membrane structure and activity in cultured osteoclasts. The processing of con A-receptor complexes includes the redistribution and endocytosis of cell-bound con A in a manner similar to that described in a variety of mononuclear cell cultures. These metabolically dependent events may represent a general mechanism that is used by the osteoclast to process other endogenous mediators of physiological function that directly bind to specific membrane receptors. The endocytosis of ligand-receptor complexes may regulate intracellular events that ultimately results in an increase or a decrease in osteoclastic activity.

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Fig. 1. a) Phase contrast of an osteoclast pre-incubated with con A-FITC at 4°C prior to fixation (X 600).

b) corresponding fluorescent image demonstrating a fluorescent label that is evenly distributed over the entire cell surface.

Fig. 2. a) Phase contrast of an osteoclast pre-incubated with con A-FITC and  $\alpha$ -methyl mannoside at 4°C (X 600).

b) the fluorescent image demonstrates a lack of fluorescent label associated with the osteoclast.

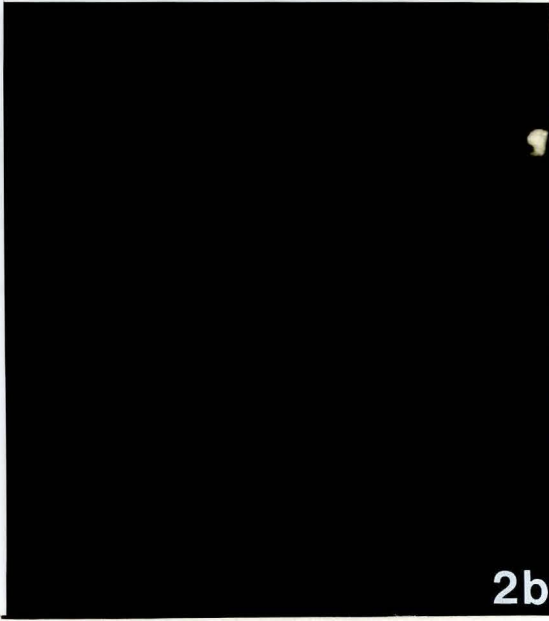
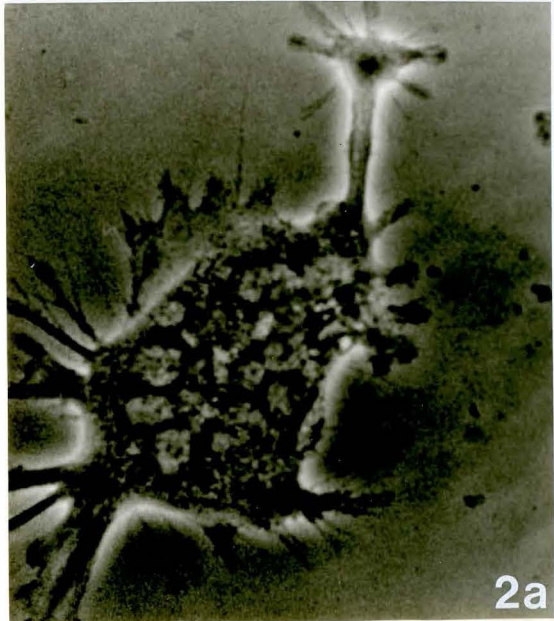
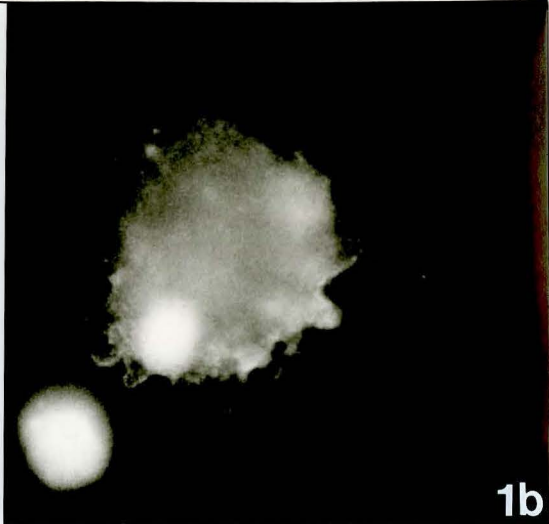
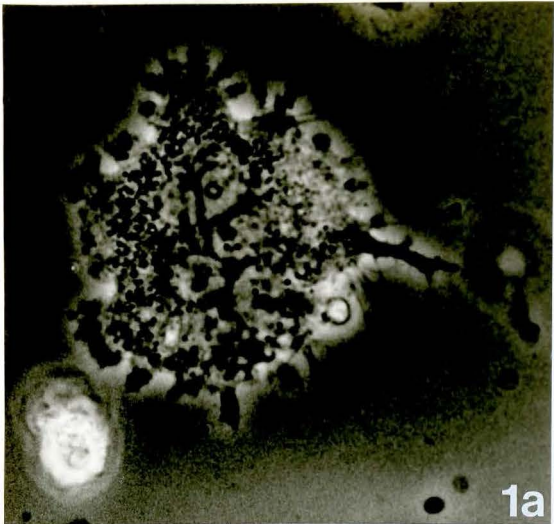
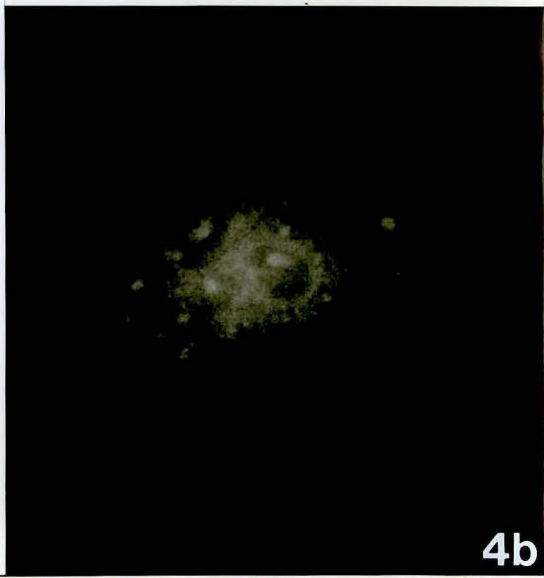
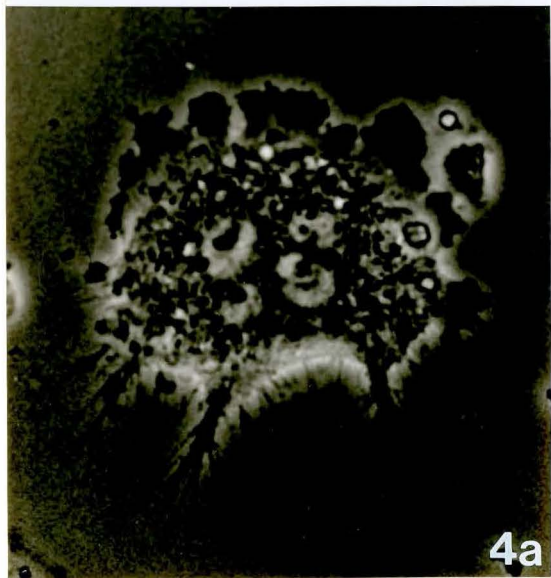
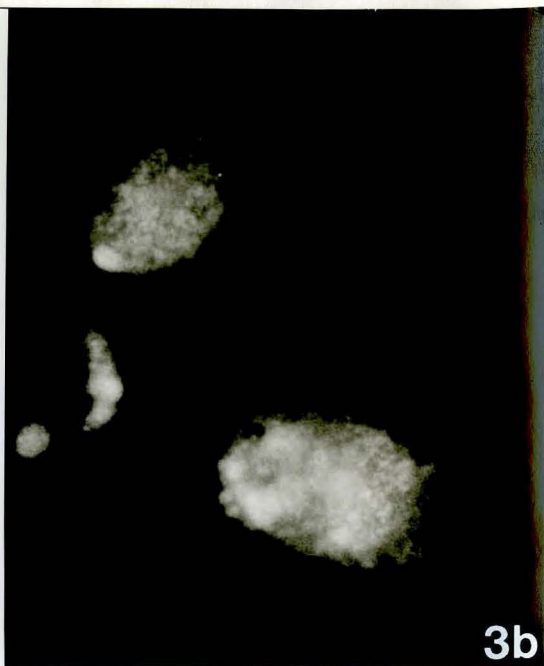
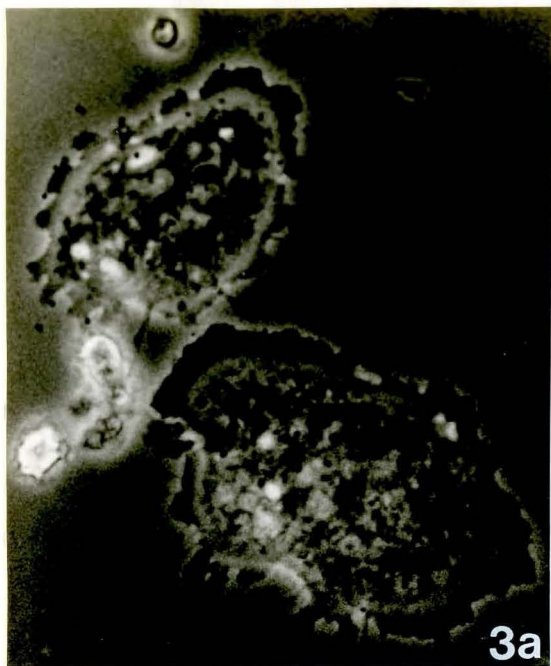


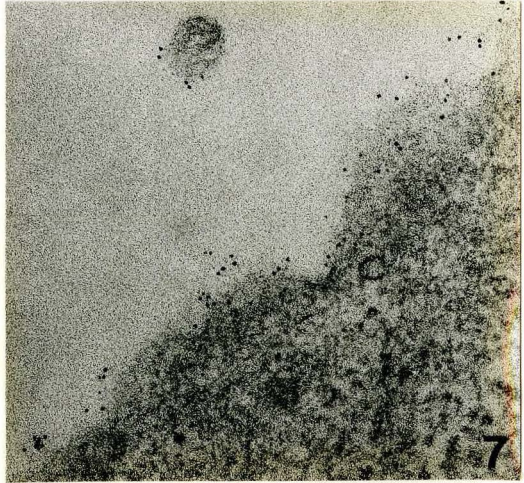
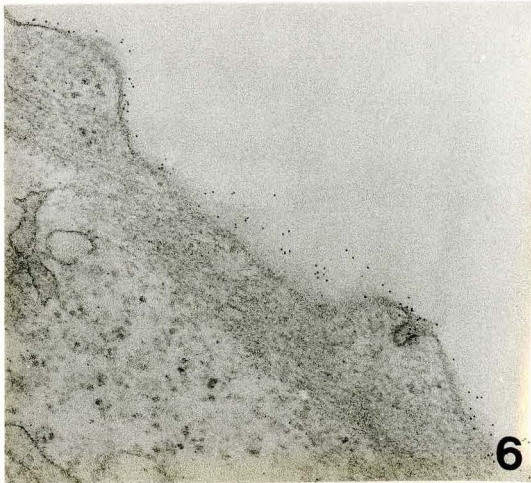
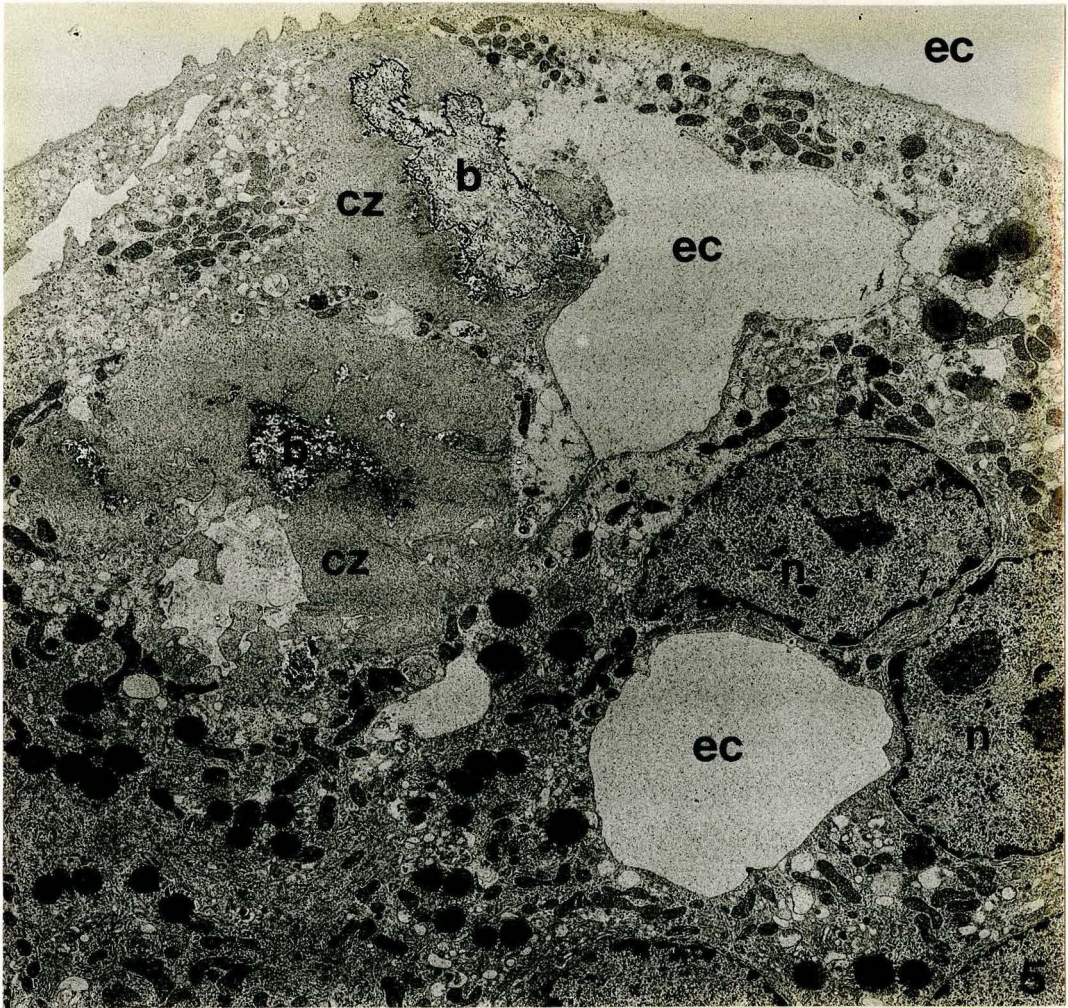


Fig. 3. a) Phase contrast of 2 osteoclasts pre-incubated in con A-FITC at 4°C, washed, and incubated at 37°C for 15 min (X 600). b) the fluorescent images demonstrate central localization of the membrane-bound label.

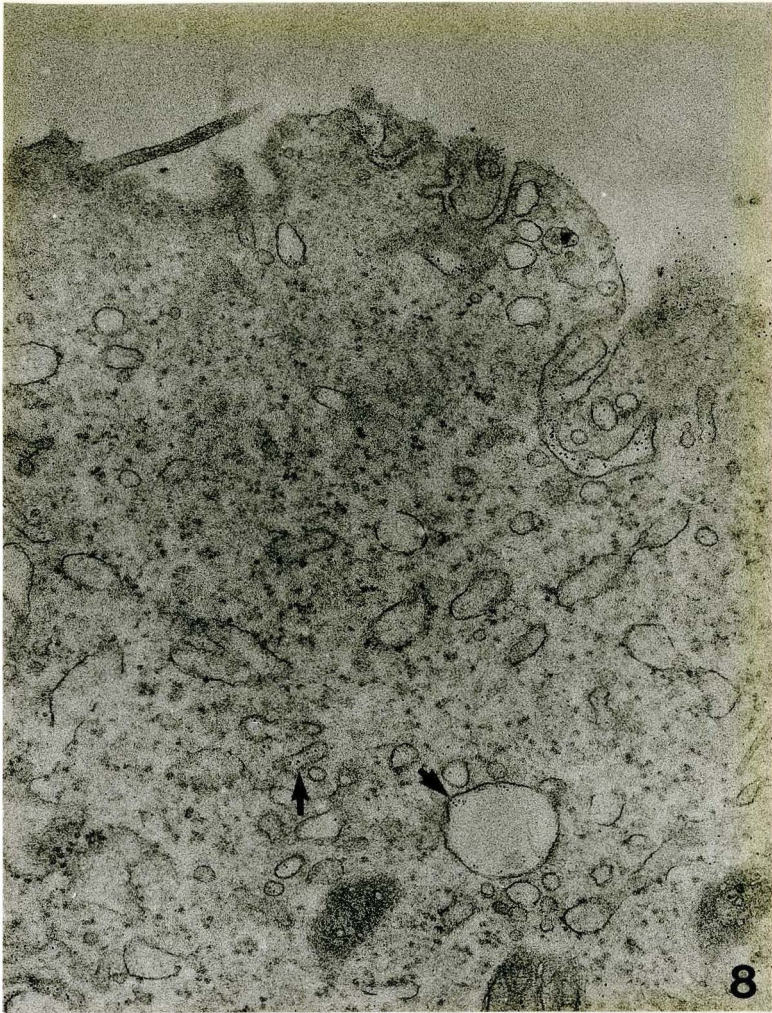
Fig. 4. a) Phase contrast of an osteoclast incubated at 37°C for 30 min. following pre-incubation with con A-FITC at 4°C (X 600). b) the negative images of the nuclei suggest that the fluorescent label is located inside the cell.



- Fig. 5. Portion of a cultured osteoclast that exhibits cytoplasmic features characteristic of osteoclasts. The cytoplasm contains many pleomorphic mitochondria, vacuoles and dense bodies. n - nucleus; ec - extracellular space; b - bone fragment; cz - clear zone (X 5500).
- Fig. 6. Cell surface of an osteoclast pre-incubated with con A-ferritin prior to fixation. The ferritin label is evenly distributed along the outer aspect of the plasma membrane (X 40,000).
- Fig. 7. High magnification of the cell membrane of a control osteoclast. This tangential section demonstrates the even distribution of ferritin particles (X 80,000).



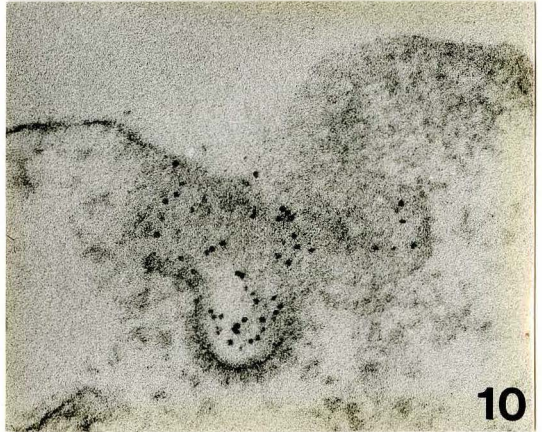
- Fig. 8. Portion of an osteoclast incubated at 37°C for 15 min after pre-incubation with con A-ferritin at 4°C. The ferritin label at the cell surface is concentrated in the tubular invaginations of the plasmalemma. There are also some ferritin-labeled endocytic vesicles (arrows) within the cytoplasm (X 40,000).
- Fig. 9. High magnification of a tubular invagination from the osteoclast in Figure 8, demonstrating the localization of the ferritin label (X 120,000).
- Fig. 10 High magnification of a coated pit at the surface of a 15 min. osteoclast. The ferritin label is confined to the coated pit area, while the surrounding membrane is free of label (X 120,000).



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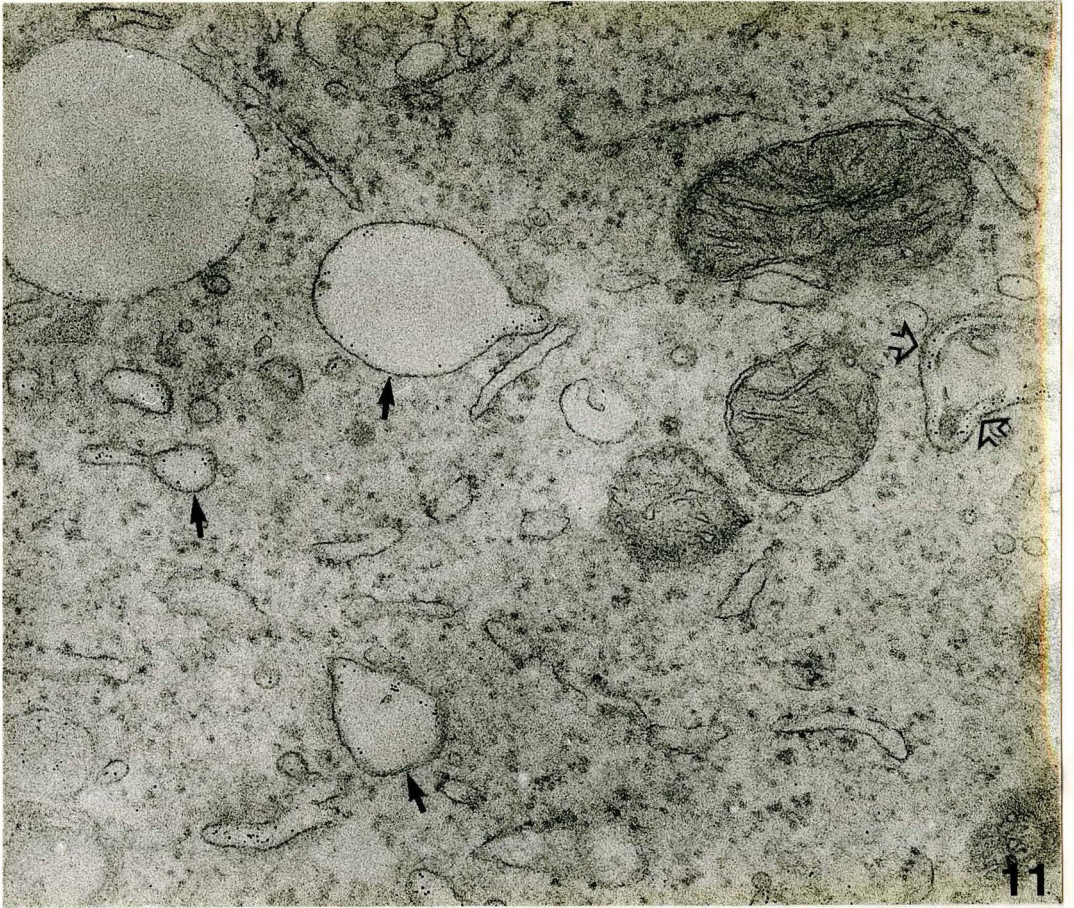


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Fig. 11 Cytoplasm of an osteoclast incubated at 37°C for 30 min. after con A-ferritin pre-incubation at 4°C. Ferritin-labeled endosomes of various sizes (solid arrows) and occasional ferritin-labeled tubules (open arrows) are present in the cytoplasm (X 50,000).



11



## CHAPTER VII

### DISCUSSION

Lectins have been used in a variety of biological assays to study the surface structure and function of cells and tissues. However, few studies have used lectins to evaluate the structure and function of the skeletal system. Wang et al. (1982) demonstrated that the in vivo administration of PHA-P caused a significant increase in the number and size of long bone osteoclasts. Horton et al. (1982) demonstrated a decrease in resorption of long bones cultured in the presence of high concentrations of con A (50 and 100 µg/ml). Subsequent TEM of these con A-treated osteoclasts revealed ultrastructural changes associated with inhibition of osteoclastic activity (Ryder et al., 1982).

The studies involving the skeletal system indicate that some lectins are capable of inducing changes in bone cells, particularly osteoclasts. Therefore, we were initially interested in demonstrating the existence of specific con A binding sites on the surface of osteoclasts and in evaluating the functional effects of various concentrations of con A on bone resorption and osteoclastic activity. For these studies we used the calvariae of 10-day old rats for the following reasons. The distribution of osteoclasts and osteoblasts on

the endocranial surface of the flat bones of the growing rodent skull is well-documented and the number of osteoclasts reaches its peak at approximately 10-days post-partum (Barnicott, 1947; Jones and Boyde, 1976; Jones and Boyde, 1977; Abe et al., 1983). In addition, the culture of these calvarial bone-organ explants is a resorbing culture system that can be used as an in vitro model to quantitatively evaluate the functional effects of con A on resident osteoclasts attached to their natural substrate.

The culture of bone-organ explants such as calvariae is an organ culture system in which the resorption of bone occurs without the addition of any stimulating agents. The fetal bovine serum that is used to supplement the tissue culture medium contains a variety of serum components, some of which are probably capable of influencing osteoclastic bone resorbing activity. The release of  $^{45}\text{Ca}$  from pre-labeled calvariae can be quantitated with respect to the total amount of  $^{45}\text{Ca}$  initially present in each calvaria, yielding the percent of  $^{45}\text{Ca}$  released over a specified period of time. The average percent  $^{45}\text{Ca}$  release from calvariae that have been devitalized and cultured in BGJb medium for a period of 72 hrs. (dead bone release) is  $3.1 \pm 0.4\%$  (fig. 1). In comparison, the average percent  $^{45}\text{Ca}$  release from live calvarial explants cultured under identical conditions for 72 hrs. (live bone release) is  $11.2 \pm 0.6\%$  (fig. 1). The difference of approximately 8.2% is due to the resident cellular component which is eliminated from the calvarial explants by devitalization. The

cellular component is primarily composed of the 3 bone cell types: namely osteoclasts, osteoblasts and osteocytes. Other mononuclear cells, such as macrophages and periosteal fibroblasts, also constitute a small fraction of the cells present in normal calvariae. The osteoclast is widely accepted as the primary effector cell responsible for normal bone resorption (Marks, 1983). Therefore, the osteoclasts are largely responsible for the cell-mediated  $^{45}\text{Ca}$  release in the live bone-organ cultures. However, the other resident cells are also capable of mobilizing  $^{45}\text{Ca}$  and they may also contribute to the overall release of  $^{45}\text{Ca}$  from the live bone-organ cultures. (Rodan and Martin, 1981; Hamilton et al., 1984; Heath et al., 1984; Otsuka et al., 1984).

Using con A and hemocyanin, a marker molecule used to visualize cell-bound con A via SEM, we demonstrated that resident calvarial osteoclasts have specific binding sites for con A on their cell surface. The specificity of these con A receptors was determined by using  $\alpha$ -methyl mannoside, the haptenic inhibitor of con A binding, in control cultures. A series of  $^{45}\text{Ca}$  bone release assays were conducted using pre-labeled calvariae to determine whether or not con A, at various concentrations, has any effect on  $^{45}\text{Ca}$  release. The ability of osteoclasts to resorb bone was determined by the amount of  $^{45}\text{Ca}$  released into the tissue culture medium during three 24 hr. periods after the release of labile  $^{45}\text{Ca}$ . The results demonstrate that con A has a dose-dependent biphasic effect on the bone resorbing capacity of osteoclasts; at a low concentration (0.1  $\mu\text{g}/\text{ml}$ ) con A increased bone

resorption, whereas at higher concentrations (50 and 100  $\mu\text{g/ml}$ ) con A inhibited bone resorption. We also showed that the con A-mediated reduction in the release of  $^{45}\text{Ca}$  at 50  $\mu\text{g/ml}$  is reversible, indicating that con A is inhibitory rather than cytotoxic to osteoclasts at the higher concentrations.

After observing the effects of con A, we were interested in the effects of three other lectins, WGA, SBA and PNA, on  $^{45}\text{Ca}$  release in the calvarial culture system. The four lectins, con A, WGA, SBA and PNA, have cell surface saccharide binding specificities that are distinctly different from one another. All of the sugar residues to which these four lectins bind are common to mammalian membrane-associated glycoconjugates. Another series of  $^{45}\text{Ca}$  bone release assays were performed using a lectin concentration of 50  $\mu\text{g/ml}$  for each of the three lectins to be tested. At this concentration, WGA inhibited the release of  $^{45}\text{Ca}$ , although its effect was not as pronounced as that of con A at the same concentration. On the contrary, neither SBA nor PNA had any effect on bone resorption in comparison with the control assay run in the absence of any lectin. These findings indicate that the reduction in bone resorption mediated by con A, and to a lesser extent WGA, at a concentration of 50  $\mu\text{g/ml}$  is not a generalized phenomenon that occurs subsequent to the binding of any lectin at this concentration. This conclusion would suggest that the specificity of lectin binding to cell surface receptors is an important factor in the induction of an alteration of cellular

function that is mediated by a particular lectin.

There are a variety of agents that regulate osteoclastic activity in vivo and in vitro. These include the two primary hormones that regulate osteoclastic function in vivo, PTH and CT. In vitro a variety of agents influence the ability of osteoclasts to resorb bone, including the vitamin  $1,25(\text{OH})_2\text{D}_3$ , several prostaglandins, and the lymphokines OAF and IL-1. Numerous investigators have probed the osteoclast membrane to determine whether or not specific receptors for PTH and CT are present (Warshawsky et al., 1980; Rao et al., 1981; Chambers and Dunn, 1982; Chambers and Magnus, 1982; Silve et al., 1982; Chambers and Moore, 1983). The results of these studies support the hypothesis that some agents may act directly on osteoclasts (i.e. CT), while others may regulate osteoclastic activity indirectly (i.e. PTH) through the intermediary of another cell type (Rodan and Martin, 1981; Chambers and Dunn, 1983). Although the ultrastructural changes associated with the stimulation or inhibition of osteoclastic activity have been well-documented both in vivo (Holtrop and King, 1976; Holtrop et al., 1979) and in vitro (King et al., 1978; Wezeman et al., 1979), the mechanisms responsible for changes in osteoclastic activity are poorly understood. Functional ligands that directly bind to specific membrane receptors and subsequently alter cellular activity can do so via two general mechanisms; the cell-bound ligand can regulate cell function through an alteration of the cell surface structure or the cell-matrix interaction (external mechanism), or the

ligand-receptor complexes can be internalized (internal mechanism).

The external mechanism whereby a ligand can regulate a cellular activity includes the following possibilities. The binding of a particular ligand to specific membrane receptors can either enhance or inhibit the binding of another functional ligand to the cell surface. In the con A-treated bone-organ culture system this is possible since the tissue culture serum supplement contains a variety of components, some of which are probably capable of regulating osteoclastic function. The ability of a particular ligand to influence the binding of another functional ligand can only be evaluated in a culture system in which all of the components of the culture medium are defined. On the other hand, in tissue and organ culture systems where a morphological and functional interaction between the cells being studied and the matrix to which the cells are attached is essential for normal cell function, a cell-bound ligand may modify the cell-matrix interaction thereby altering cell function.

It is well-documented that there is a morphological and functional interaction between the osteoclast and the bone matrix that is essential for normal resorptive activity to occur. The clear zone and ruffled border membrane specializations apposed to the bone matrix are the ultrastructural hallmarks of active osteoclastic bone resorption. We were interested in evaluating the effects of 2 lectins, con A and SBA on the interaction between the osteoclast and the bone matrix. However, this type of experiment is technically

impossible for a number of reasons. The procedure would involve the isolation of a pure population of osteoclasts in large numbers. No one to date has successfully isolated a pure population of osteoclasts. If it were possible to isolate osteoclasts in large numbers the cells would then be treated with either con A or SBA and allowed to attach to a devitalized bone surface. However, osteoclasts are extremely sensitive to any type of mechanical trauma and the manipulation of these cells required in this type of experiment would probably result in cell death. Even if it were possible to establish a viable pool of osteoclasts attached to a devitalized bone surface, the maintenance of these cells in long term culture and the ability to quantitatively assess their capacity to resorb bone is, at best, questionable.

Because of the difficulty in obtaining pure populations of osteoclasts, most studies on bone resorption are performed on intact animals or bone-organ cultures. These systems do not permit detailed analysis of cellular mechanisms of bone resorption or of the means whereby resorbing cells attach to the bone surface (Teitelbaum et al., 1979). Since monocytes and macrophages are capable of attaching to and subsequently resorbing devitalized bone in vitro (Mundy et al., 1977; Kahn et al., 1978; Teitelbaum et al., 1979), the macrophage-devitalized bone culture system serves as a useful model to study the morphological and functional interaction between the resorbing macrophages and the bone matrix. The studies by Kahn et al.

(1978) and Teitelbaum et al. (1979) also demonstrated that optimal macrophage-mediated mineral mobilization requires cell-matrix contact. Recent studies by Bar-Shavit et al. (1983b,c,d) have demonstrated that the attachment of macrophages to bone is a regulated process that is mediated, in part, by certain saccharides located on the cell and/or bone surfaces. These findings indicate that the attachment of resorbing macrophages to the bone matrix is an important component of the process of bone resorption and that the oligosaccharide-mediated binding mechanism that links the resorbing macrophages to bone is essential for normal resorptive activity.

The importance of the cell-matrix interaction in the macrophage-bone culture system justifies its use to evaluate the effects of con A and SBA on the interaction between a bone matrix and cells that are capable of attaching to and subsequently degrading it. We chose these 2 lectins based on the results from the  $^{45}\text{Ca}$  bone release assays in which con A at a concentration of 50  $\mu\text{g}/\text{ml}$  inhibited bone resorption, whereas SBA had no effect on bone resorption at the same concentration. The results demonstrate that macrophages allowed to attach to bone in the presence of con A exhibit a cell surface morphology that is markedly different from that of control macrophages as visualized by SEM. Although the con A-treated macrophages do attach to the bone surface, they are hindered in their ability to spread on the substrate. The membrane surface area that is apposed to the bone surface in the con A-treated system is only one-half that of



control cells. The quantitative results demonstrate that approximately twice the number of con A-treated macrophages attached to the bone surface in comparison with the number of untreated cells. Although these results seem to conflict with the  $^{45}\text{Ca}$  functional data, this quantitative difference is most likely the result of the extensive aggregation of macrophages by con A. The cross-linking of cell membranes by con A may significantly increase the number of cells that appear to attach to the bone matrix, since those cells that would not attach under normal conditions appear to be attached to the bone surface by cross-linking to the cells that are capable of attachment. On the contrary, SBA does not appear to change the attachment and subsequent spreading of macrophages on bone either morphologically or quantitatively.

A series of  $^{45}\text{Ca}$  bone release assays were conducted to determine whether the morphological observations could be correlated to any functional differences in the ability of con A-treated or SBA-treated macrophages to release  $^{45}\text{Ca}$  in vitro. The con A-treated macrophage-bone samples demonstrated a significant decrease in mineral mobilization at 48 and 72 hours, whereas the  $^{45}\text{Ca}$  released from the macrophage-bone samples in the presence of SBA did not differ significantly from the control assay at any of the sampling time points.

The results obtained from this macrophage-devitalized bone culture system demonstrate that con A, but not SBA, alters the

morphology and function of macrophages on a bone surface. The morphological findings support the hypothesis that specific saccharides common to cell surface and/or matrix associated glycoconjugates are involved in the macrophage-bone interaction. Oligosaccharides containing  $\alpha$ -linked D-glucose and D-mannose residues are more likely to be involved in the macrophage-bone interaction, than oligosaccharides containing GalNAc or D-galactose residues. The functional data suggest that the decreased surface area of cell-matrix contact in the con A-treated cultures may, at least in part, account for the reduction in macrophage-mediated mineral mobilization.

There also exists a correlation between the effects of con A and SBA on bone mineral mobilization in the macrophage-bone cultures and their effects on  $^{45}\text{Ca}$  release in the bone-organ cultures. At the lectin concentration of 50  $\mu\text{g/ml}$ , con A causes a reduction in the release of  $^{45}\text{Ca}$  in both systems, whereas SBA has no effect on mineral mobilization in either system. In both culture systems, cell-matrix contact and the subsequent functional interaction are important components of the process of bone degradation. Therefore, it is possible that the con A-mediated inhibition of bone resorption in the bone-organ culture system at higher concentrations may, in part, be the result of the ability of cell-bound con A to interfere with the interaction between the osteoclast and the bone matrix, an essential prerequisite for normal resorptive activity.

When comparing these 2 culture systems it is important to use the

term "resorption" correctly. The regulated process of normal bone breakdown by osteoclasts is called bone resorption. The ruffled border and clear zone are ultrastructural hallmarks indicative of active resorption of underlying bone by osteoclasts. Although there are similarities in the degradation of bone by macrophages, the development of clear zones and ruffled borders have never been demonstrated in the macrophage-bone culture system. Therefore, when referring to macrophage-mediated bone breakdown terms such as mineral mobilization and bone degradation should be used and the word "resorption" should be limited to the process mediated by osteoclasts.

The induction of many of the functional effects by cell-specific ligands requires the internalization of the cell-bound ligands. The internal mechanism that was alluded to earlier in this discussion involves the processing of ligand-receptor complexes, including the redistribution and endocytosis of these complexes. Once the ligand has been internalized it can follow a variety of intracellular pathways thereby influencing one or more intracellular events that will ultimately result in altered cell function. A general summary of the events related to receptor mediated endocytosis is schematically presented in fig. 2. Subsequent to ligand binding to specific membrane receptors, the ligand-receptor complexes become clustered in coated pits along the membrane. These coated pits have a clathrin-protein coat attached to the inner leaflet of the plasmalemma. The coated pits pinch off from the cell surface forming

endocytic vesicles (endosomes) that rapidly lose their protein coat which is recycled back to the cell surface. The endosomes migrate deeper into the cytoplasm and frequently fuse with one another. At some point along the intracellular pathway, the ligand-receptor complex is dissociated and the receptors are usually recycled back to the surface. The intracellular routing of a particular ligand and the ultimate localization of that ligand within a specific subcellular compartment depends on the type of ligand that has been internalized. The lysosomal system, nucleus and Golgi apparatus are frequent sites of ligand localization.

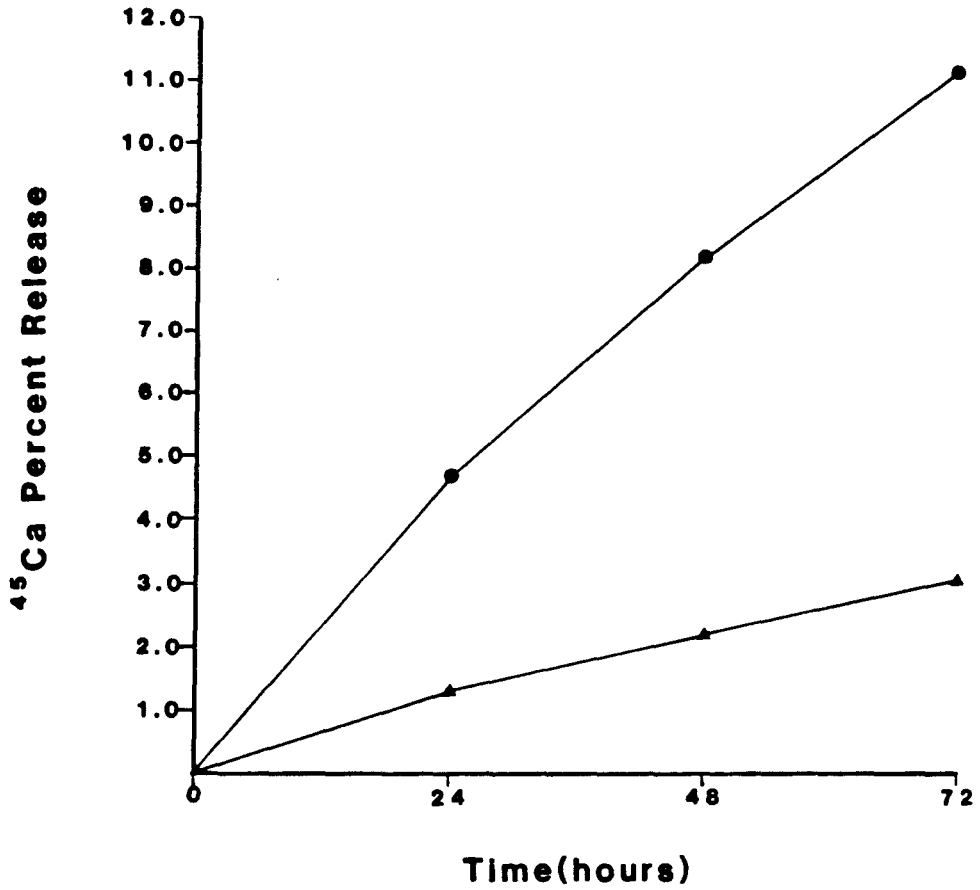
Since many of the biological activities of con A appear to involve the initial binding and subsequent processing of cell-bound con A, the fate of the con A-receptor complexes has been investigated in a variety of mononuclear cell cultures. The mitogenic property of con A in lymphocyte cultures is well-documented, and therefore, the manner in which lymphocytes process cell-bound con A has been studied extensively. Studies by Unanue et al. (1972), Yahara and Edelman (1973) and Bhalla et al. (1979) demonstrated that con A bound to lymphocyte surface receptors can induce patch and cap formation of its own receptors under certain conditions of incubation. The endocytosis of con A-receptor complexes following cap formation has also been described (Unanue et al., 1972; Bhalla et al., 1979). The capping and endocytosis of ligand-receptor complexes are metabolically dependent events (Yahara and Edelman, 1973; Ryan et al., 1974; dePetris, 1975).

It has been postulated that the processing of ligand-receptor complexes in these lymphocyte cultures is necessary for mitogenic activation of lymphocytes (Taylor et al., 1971; Greaves et al., 1972).

Since the phenomenon of capping was originally described in 1971 by Taylor et al. on B lymphocytes in suspension, its relation to cell motility and the endocytic cycle has been studied in a variety of substrate-attached mononuclear cells (dePetris and Rff, 1972; Edidin and Weiss, 1972; Vasilieu et al., 1976). We investigated the processing of con A-receptor complexes by osteoclasts in culture, using con A-FITC to evaluate the redistribution of cell-bound con A via epifluorescence microscopy and using con A-ferritin to determine whether the lectin-receptor complexes are internalized via electron microscopy. The results demonstrate that redistribution and endocytosis of con A-receptor complexes occurs within 30 minutes. In comparison to control osteoclasts that exhibit a fluorescent pattern which is evenly distributed over the entire cell surface, after 15 minutes the fluorescence is localized to the membrane overlying the central, nuclear region of the substrate-attached osteoclasts. The membrane peripheral to the nuclear region and membrane specializations such as filopodia and lamellipodia were cleared of the fluorescent label. The redistribution of membrane-bound con A-FITC on substrate-attached osteoclasts is similar to the movement of con A-FITC on substrate-attached epithelial cells and fibroblasts described by Vasilieu et al. (1976). Electron microscopy of con A-ferritin

treated osteoclasts revealed active endocytosis of the con A-receptor complexes. The ferritin label is concentrated in coated pits and in tubular invaginations of the cell surface (fig. 2). The tubular invaginations found along some areas of the membrane form a system of canaliculi that are continuous with the cell surface. These tubular invaginations are membrane specializations that form in response to the binding and processing of con A, since they are absent in control osteoclasts. The internalization of con A may be an important event in altering osteoclastic activity by mediating intracellular mechanisms involved in the bone resorbing process. Our findings confirm the hypothesis that cell-bound con A can alter the structure and activity of osteoclast membrane components in a manner similar to that observed in some mononuclear cell cultures. The redistribution and endocytosis of con A-receptor complexes may represent a general mechanism that is used by the osteoclast to process other endogenous mediators of physiological function that directly bind to specific membrane receptors.

Fig. 1. The percent of  $^{45}\text{Ca}$  released at 24 hour intervals in cultures of live calvarial explants versus devitalized bone. Live bone percent release: 24 hr =  $4.7 \pm 0.3$ ; 48 hr =  $8.2 \pm 0.5$ ; 72 hr =  $11.2 \pm 0.6$ . Dead bone percent release: 24 hr =  $1.3 \pm 0.3$ ; 48 hr =  $2.2 \pm 0.2$ ; 72 hr =  $3.1 \pm 0.4$ .

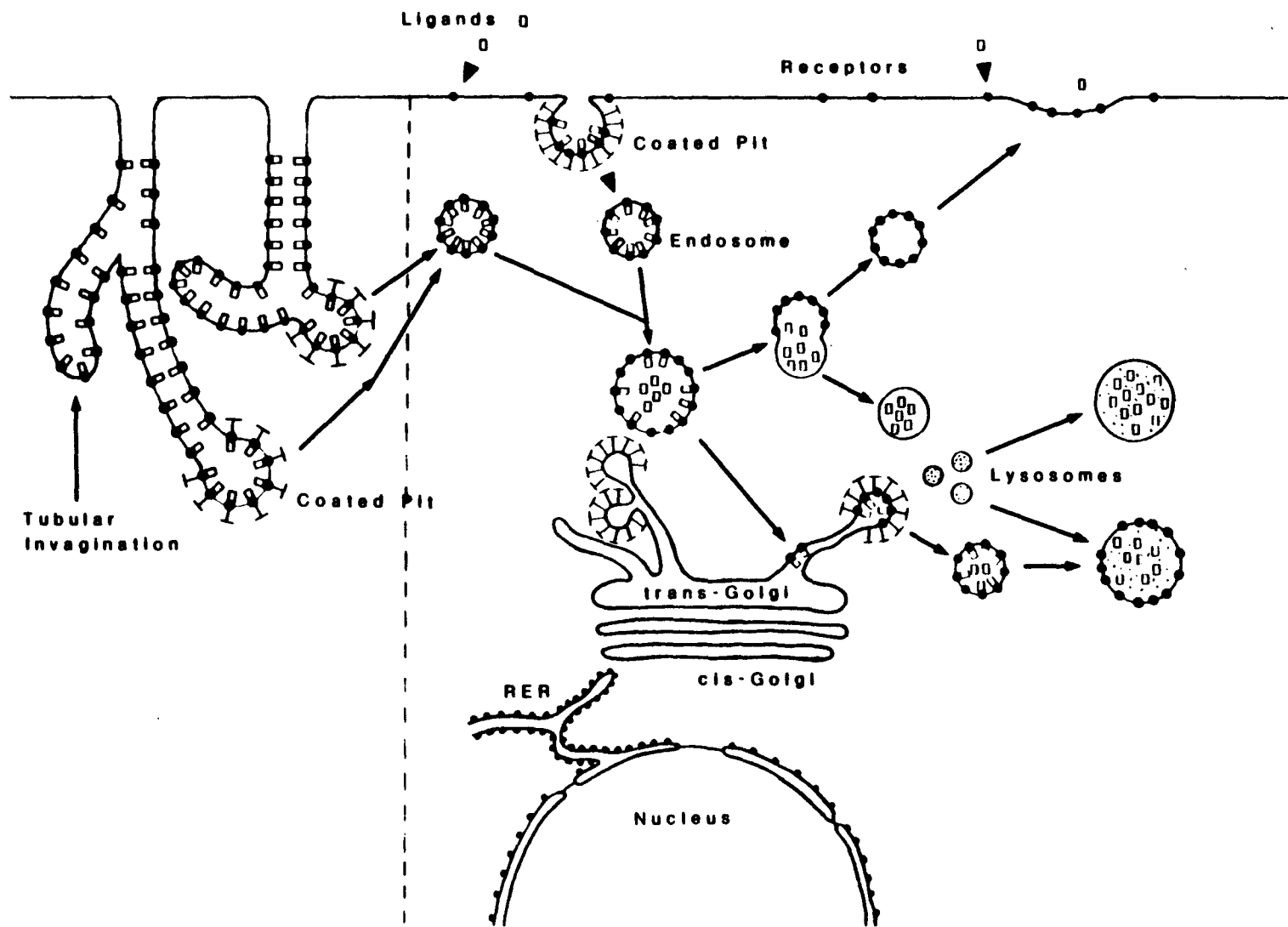


● - Live Bone Release

▲ - Devitalized Bone Release



Fig. 2. Diagram showing some of the general events that occur as a part of the endocytic mechanism following ligand binding to specific surface receptors (right side of broken line). To the left are the membrane specializations (tubular invaginations) that we demonstrated on the surface of osteoclasts processing cell-bound con A in vitro.



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**APPENDIX A**

## Appendix to Chapter III

Discussion with Reviewers

T.J. Chambers: The authors should give some idea of the counts per minute upon which their bone resorption assay figures are based.

S.C. Marks: The calcium-45 release data might be even more significant if younger (newborn vs. 7 day old) rats and higher doses (50-100 vs. 40  $\mu$ Ci) of calcium-45 were used. This would have made the assay even more sensitive.

Authors: The data presented in Table 1 demonstrates some typical values of radioactivity in counts per minute (cpm) generated from a 200  $\mu$ l sample of tissue culture supernatant at 48 hours. The value generated by a treated calvarial half is shown above the value generated by the corresponding control half. Values for two calvarial samples at each of the four concentrations of con A are shown. The relatively high counts validate the sensitivity of our  $^{45}\text{Ca}$  bone release assay.

T.J. Chambers: What is the contribution of con A toxicity to osteoclastic inhibition as demonstrated in this paper?

M.M. Miller: How do you propose to distinguish between inhibition of cytotoxicity at high concentrations of con A?

Authors: To determine whether the decrease in bone resorption at 50  $\mu\text{g/ml}$  con A is due to con A induced inhibition of osteoclastic activity or whether this high concentration is merely cytotoxic to osteoclasts, we conducted another  $^{45}\text{Ca}$  bone release assay. The experimental protocol was identical to that which has already been described in the text, with the exception that the media containing con A was removed from the treated wells after 12 hours. These wells were then rinsed and replenished with fresh media in the absence of any con A. After 12 hours of incubation in 50  $\mu\text{g/ml}$  con A, the decreased release of  $^{45}\text{Ca}$  from treated calvarial halves was significantly different from that of control calvarial halves (fig. 1). 24 hours after con A was removed, the mean T/C ratio returned to 1.0 and this reversal continued at 48 hours (fig. 11). We also took a sample 120 hours after con A removal and this preliminary data suggests that we have a rebound effect on bone resorption. This result showing that the decrease in bone resorption at 50  $\mu\text{g/ml}$  is



reversible, indicates that con A is inhibitory rather than cytotoxic to osteoclasts at this concentration.

S.C. Marks: The significance of the differences between  $^{45}\text{Ca}$  release at the lowest concentration of con A and the concentration (2.0  $\mu\text{g}/\text{ml}$ ) with peak mitogenic activity is puzzling. That the former is higher than the latter suggests that lymphokine production is probably not involved in elevated  $^{45}\text{Ca}$  release.

Authors: We agree with this conclusion and furthermore evaluated the calvarial samples to determine the extent of cells present in the culture system that could produce lymphokines. The calvarial samples were digested with collagenase and the cellular pools evaluated for T lymphocytes, B lymphocytes, monocytes and macrophages, utilizing fluorescence microscopy and fluorescein-conjugated antibodies directed against specific cell surface markers. The calvarial system did not contain enough cells to produce significant amounts of lymphokines.

T.J. Chambers: The bidirectional effect of con A suggests that there may be both a direct and an indirect effect on osteoclastic activity. Have you tested these possibilities using isolated osteoclasts? Has anyone reported an effect of con A on osteoblast activity?

Authors: We have been unable to isolate a homogeneous population of osteoclasts which could be tested in vitro. The osteoclast isolate always contains mononuclear cells which may include lymphocytes and osteoblasts, both of which are known to be able to influence osteoclasts. We plan to evaluate the effects of con A on osteoblast activity by monitoring such parameters as  $^3\text{H}$ -proline incorporation, alkaline phosphatase levels and cAMP levels.

R.S. Molday: Have you studied the effect of con A concentration on the density and distribution of binding sites using the hemocyanin method?

Authors: We have only looked at con A binding at the concentration of 100  $\mu\text{g}/\text{ml}$ . The hemocyanin particles appear densely packed at this concentration and may be causing steric hindrance. The density and distribution of binding sites at different concentrations of con A should be studied using a smaller marker and a TEM as well as an SEM evaluation.

S.C. Marks: What is the functional significance of con A binding?

That a lectin would bind to the surface of an animal cell might not be surprising. That this represents some specific receptor site remains to be determined. We know of no evidence that these cells encounter con A in vivo. Therefore, other than the fact that certain carbohydrate moieties appear to be uniformly distributed on the external surface of osteoclasts and osteoblasts, what does con A binding mean? Can one make a case for its physiological significance in bone?

Authors: We are using lectin binding and its stimulatory and/or inhibitory effects as a model system for osteoclasts, much the same as other investigators have used lectins to activate lymphocytes and initiate their proliferation. Although the activation may be quite non-specific, the way in which the lectin is handled by the cell, i.e., the possible redistribution and endocytosis of binding sites, may give some insight into the mechanisms by which physiologically active substances exert their effects on bone cells.

M.M. Miller: What are those fibrous structures associated with the osteoblasts and would you comment on the apparent association of con A-hemocyanin with them?

Authors: The fibrous structures are collagen fibrils. The con A-hemocyanin is binding to the mannosyl and glucosyl residues of the glycosaminoglycans associated with the collagen.

S.C. Marks: Is it possible that reduced calcium-45 release observed with high doses of con A is caused by activation of the calcitonin receptors through a local distortion of the osteoclast membrane by the high density binding of con A? If so, one might not be surprised if a measurement of bone formation at this dose were also affected.

Authors: We plan to investigate the fate of the con A binding sites at various time intervals after treatment with the lectin. If the binding sites demonstrate redistribution, then this movement may cause the local distortion that you suggest. We are going to evaluate osteoblastic activity at all of the concentrations originally tested via the incorporation of  $^3\text{H}$ -proline.

M.M. Miller: How do you imagine that con A stimulates  $^{45}\text{Ca}$  release?

Authors: In order to approach this question we have to study the fate of the con A-binding site complexes at various time intervals after treatment. Is there redistribution of plasma membrane components; is

the con A internalized by the bone cells; and is this internalization necessary for stimulation?

R.S. Molday: In control labeling experiments,  $\alpha$ -methyl mannoside was successfully used to reverse the labeling of cells with con A. Why doesn't the hemocyanin also compete with the cell binding sites and cause the con A to dissociate?

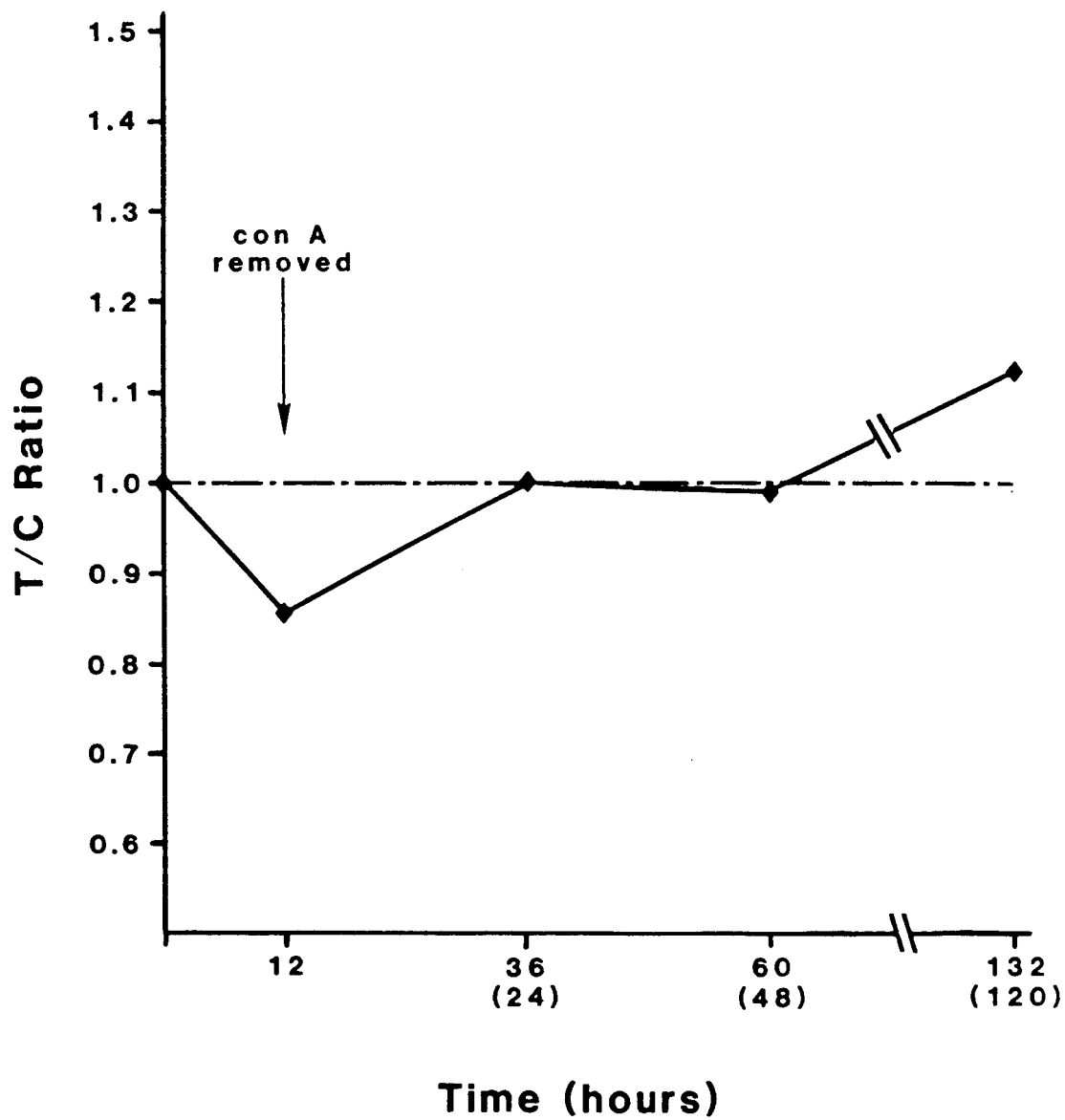
Authors:  $\alpha$ -methyl mannoside competes for the same sites by which con A binds to cell surface macromolecules. Since  $\alpha$ -methyl mannoside has a much greater affinity than cell surface glucosyl and mannosyl residues for the same sites on the con A molecule, con A dissociates from the cell surface when  $\alpha$ -methyl mannoside is present in the culture media. Hemocyanin, on the other hand, binds to sugar residues on the con A molecule which are structurally different than those involved in cell surface binding and, therefore, does not compete with cell surface binding sites.

Table 1                       $^{45}\text{Ca}$  Bone Release Assay  
CPM at 48 Hours

con A (0.1 $\mu\text{g/ml}$ )	2550*	1922
control	1867	1533
con A (2.0 $\mu\text{g/ml}$ )	2007	1471
control	1823	1569
con A (50 $\mu\text{g/ml}$ )	1219	1056
control	1948	1655
con A (100 $\mu\text{g/ml}$ )	1165	1153
control	2066	2149

\*counts per minute generated from a 200  $\mu\text{l}$   
sample of tissue culture supernatant

Fig. 1. Reversal of con A-mediated inhibition of bone resorption. The mean T/C ratio is plotted on the y-axis and the time of sampling on the x-axis. The numbers in parentheses represents the time of sampling following con A removal (indicated by arrow), whereas the number above represents the total time in culture following the 24 hour washout of labile  $^{45}\text{Ca}$ .



**APPENDIX B**

This appendix supplements the data presented in Chapter IV. <sup>45</sup>Ca bone release assays were conducted to determine the effect of wheat germ agglutinin (WGA), soybean agglutinin (SBA) and peanut agglutinin (PNA) on bone resorption. The data presented in Chapter IV only includes the treated/control (T/C) ratios for each lectin at a concentration of 50 µg/ml. However, the <sup>45</sup>Ca bone release assays were conducted at 2 or more concentrations for each of the lectins tested. The following table lists the mean T/C ratios at 24, 48 and 72 hours for all concentrations tested. The T/C ratios were compared to the control/control (C/C) ratio obtained from the control release assay to determine whether the observed changes in bone resorption were significantly different. Significance was evaluated by a two-tailed Student t-test for the difference between two means.



Table 1      Effects of various lectins on bone resorption

<u>Lectin</u>	<u>Concentration (<math>\mu\text{g/ml}</math>)</u>	<u>T/C ratios</u>		
		<u>24 hr.</u>	<u>48 hr.</u>	<u>72 hr.</u>
WGA	0.1	1.08 $\pm$ .06	1.06 $\pm$ .04	1.09 $\pm$ .05
	1.0	1.06 $\pm$ .06	1.03 $\pm$ .05	1.05 $\pm$ .04
	10	1.01 $\pm$ .09	0.95 $\pm$ .06	0.95 $\pm$ .09
	50	0.95 $\pm$ .09	*0.84 $\pm$ .05	*0.71 $\pm$ .04
SBA	50	0.98 $\pm$ .06	0.96 $\pm$ .07	0.97 $\pm$ .06
	100	1.00 $\pm$ .04	0.98 $\pm$ .02	0.97 $\pm$ .03
PNA	1.0	1.00 $\pm$ .03	1.01 $\pm$ .02	1.01 $\pm$ .06
	10	1.03 $\pm$ .03	0.96 $\pm$ .06	1.01 $\pm$ .05
	50	1.01 $\pm$ .08	0.97 $\pm$ .01	0.94 $\pm$ .06

\* Significantly different from control assay ( $p < .001$ )

APPROVAL SHEET

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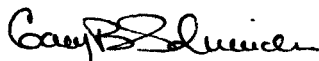
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The final copies have been examined by the director of the dissertation and the signature which appears below verifies the fact that any necessary changes have been incorporated and that the dissertation is now given final approval by the Committee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

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

Sept. 11, 1985

Date



Director's Signature