

STUDIES ON GUT MACROPHAGES, BLOOD MONOCYTES
AND ALVEOLAR MACROPHAGES IN THE DOG

by

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CHAPTER I

INTRODUCTION

Blood monocytes, tissue macrophages (mø) of the intestinal lamina propria and alveolar macrophages are members of the mononuclear phagocyte system.¹ This system also includes the Kupffer cells of the liver, the lining møs of the spleen and bone marrow sinusoids, the free møs in the peritoneal cavity and the microglial cells of the central nervous system.¹

Traditionally, these cells were known for their ability to phagocytose foreign particles and dead or dying tissues. In recent years, this view has been expanded to accommodate the functional diversity of the mø system. It has been shown that møs can act in a regulatory role to enhance or suppress immune responsiveness. They have the ability to secrete a diverse array of biologically active substances and in certain situations are known to be tumoricidal. Mø have a complex involvement in the inflammatory response and interact with both the complement and clotting systems.

While each of these functions plays an important role in host defense against micro-organisms and injurious agents, these processes may secondarily damage normal structures by amplification and perpetuation of the inflammatory response. It is for this reason that the mononuclear phagocyte system is implicated in the pathogenesis of chronic inflammatory diseases of man and animals.

Most of the extensive literature dealing with mø physiology and function is referable to cells derived from laboratory animals and human sources. Little work has been done in the characterization of mø populations from other species.

The objectives of this project were to:

1. enumerate and characterize møs from the normal canine intestine.

2. define functionally and morphologically two reference populations of canine mononuclear phagocytes: the blood monocyte and the resident alveolar mØ.

It is a possibility that the canine may provide a model for the study of chronic inflammatory disease. The characterization of mØ populations from the normal animal is an essential prerequisite for the study of these clinical disease syndromes.

CHAPTER II

LITERATURE REVIEW

Mononuclear phagocytes are spread widely throughout the body, although it is generally believed that the origin of the mφ is the bone marrow.¹ The most convincing data for this concept derives from chimeric experiments in animals in which a unique genetic marker in bone marrow is later observed in various tissue mφs.² In adult animals, mφs arise as pro-monoblasts and mature through monoblast and promonocyte stages.³ They emerge as monocytes the representative of the mononuclear phagocyte system in the peripheral circulation. After a period ranging from 1.5 to 4 days,¹ they migrate to body tissues where they continue their morphological and functional differentiation to become characteristic for that site. Tissue populations of mφs are maintained by recruitment of blood monocytes in most cases.^{1,3} However, human alveolar mφs are capable of replication in situ although in small numbers and under limited circumstances.⁴

The eventual fate of mφs is uncertain. Animal studies suggest they are eliminated through epithelial surfaces such as the bronchoalveolar tree^{5,6} and the gastrointestinal mucosa.⁷ Tissue mφs are relatively long-lived cells unless they ingest foreign material. Their life-span in this case depends on the nature of the ingested material.⁵

Alveolar mφs are found in the interstitial connective tissues of the alveolar wall, in the lining epithelium of the alveolus and free within the alveolar lumen.⁶ Intestinal mφs reside within the wall of the intestine. These include mφs situated interepithelially,^{8,9,10} in the villus cores, in the loose connective tissue surrounding the crypts of Leiberkuhn and those in the gut-associated lymphoid tissue, especially Peyer's patches.⁷

Blood monocytes and alveolar mØ have morphological features common to the mononuclear phagocyte system. Information regarding intestinal mØs is relatively sparse in this respect. Monocytes, alveolar mØs and intestinal mØs are large spherical cells, with diameters ranging from 20-30 microns for monocytes,¹¹ 40-50 microns for alveolar mØs¹² and 14-15 microns for intestinal mØs.¹³ The nucleus of the monocyte and alveolar mØ is oval to kidney-shaped and is usually eccentrically placed in the latter cell.¹¹ The chromatin structure of the nucleus is described as an open lacy network.¹¹ Nuclear morphology has not been described for the intestinal mØ. On a sub-cellular level, monocytes contain mitochondria, several types of vesicles, rough endoplasmic reticulum and ribosomes.¹⁴ Alveolar mØs have more mitochondria than mØs in other locations.⁶ They contain moderate amounts of smooth endoplasmic reticulum but relatively little rough endoplasmic reticulum.⁶ Many lysosomes are present often containing ferritin, carbon particles and other digested material.¹¹ Like the alveolar mØ, intestinal mØs are granular cells with a large array of internal organelles including mitochondria, endoplasmic reticulum and many lysosomes.^{13,15} The surface membrane has been extensively studied with both the scanning and transmission electron microscope, and is composed of "veil-like" ruffles and ridges which are in constant motion in living cells.⁵ Alveolar mØs are capable of sending out far-reaching cytoplasmic extensions.¹¹ MØs situated beneath the intestinal basement membrane or totally within the epithelium of the intestinal mucosa have been reported to extend pseudopodia to the mucosal surface.^{8,16}

The structure of mØs may alter dramatically when exposed to non-specific irritants or certain specific stimulants such as lymphokines. The cells enlarge, their lysosomes increase in number,^{5,11} and they exhibit a greater degree of surface irregularity.¹⁴

Macrophages obtain their energy from both anaerobic glycolysis and aerobic respiration. Alveolar mφs normally function in an environment where the partial pressure of oxygen approximates 100mmHg. Because they have adapted to an aerobic environment, these cells utilize significantly greater quantities of oxygen and demonstrate a lower rate of glycolysis than mφs from other locations.^{11,14}

Mφs from different tissue sites and from a variety of species have been studied with respect to enzymatic activity. Although the list of enzymes is incomplete, lysozyme, acid phosphatase, lipase, cathepsin, acid ribonuclease, neuraminidase, B-glucuronidase, hyaluronidase, aryl sulfatase, phospholipase, collagenase, elastase and non-specific esterases are present.⁵ Activation of mφs produces variably increased enzyme activities, although some enzymes are decreased in activity.^{6,14} Intrinsically, alveolar mφs are very active metabolically and they contain more hydrolytic enzymes than their peritoneal counterparts.⁶

The presence of non-specific esterases in mφ cytoplasm has been exploited as a marker identifying a cell as a member of the mononuclear phagocyte family. The significance of mφ and monocyte esterases is not understood. (X naphthyl acetate esterase may be lysosomal in origin as its presence in membrane-bound organelles and vesicles has been reported.¹⁸ Methodology for the demonstration of non-specific esterases in monocytes using (X naphthyl acetate and butyrate as substrates has been described in detail for several species including the dog.^{17,19,20} The enzymatic reaction is strongly dependent on pH and incubation time,^{17,20} and the optimum requirements for staining are species specific.¹⁷ In the dog, an optimum pH of 6.0 and a 90 minute incubation period is recommended.¹⁷ When correctly stained using Yam's method for (X naphthyl acetate esterase^{19,20} monocytes will show heavy

reddish-brown cytoplasmic staining.¹⁷⁻²¹ Platelets, megakaryocytes, basophils and plasma cells show some reaction.²¹ T lymphocytes show a characteristic single red-brown cytoplasmic nodule adjacent to the cell membrane.¹⁸ This has been advocated as a useful marker of the T cell in peripheral blood and lympho node aspirates.¹⁸ In humans, α naphthyl acetate esterase staining for peripheral blood monocytes is considered more accurate than morphology alone and it has been estimated the technique correctly identifies 98% of monocytes in the peripheral blood.^{22,23}

Alveolar and intestinal m ϕ s are reported to be strongly α naphthyl acetate esterase positive.^{9,12,24} Methodology for the demonstration of activity using frozen tissue sections has been reported.^{9,25} This allows a study of mononuclear phagocytes in situ without interference to spatial relationships with other cells. Prior to the use of non-specific esterase staining in tissue, periodic acid-Schiff²⁶ and Gomori's acid phosphatase²⁷ staining were used to demonstrate m ϕ s in histological preparations. These methods lack sensitivity and specificity for the mononuclear phagocytes.⁷

M ϕ s are constantly interacting with their environment through receptors on their external surface. Recognition mechanisms are well developed and the phagocyte can distinguish between self and non-self or altered self. Initial recognition at binding sites is a non-energy requiring step and can occur at several receptors or at a variety of ill-defined recognition sites.

Receptors for the Fc portion of IgG and the C3b and C3d fragments of complement have been identified on monocytes and m ϕ s from a variety of sources and in a number of species including man,²⁰ dog,^{29,30} pig,³¹ and cattle.³² In normal m ϕ s, Fc receptors mediate both attachment and endocytosis of IgG-coated particles. Complement receptors mediate only the attachment of complement-coated particles to the cell membrane. However, in activated m ϕ s,

complement receptors mediate endocytosis as well.³³ Moreover, receptor density and affinity change with the state of activation.³¹ Fc receptors express variability in their affinity for different sub-classes of IgG.^{32,34} Although in many cases the structure of these receptors is unknown, the proteins for binding IgG2a and IgG2b on mouse peritoneal mφs have been isolated and characterized.³⁴ While the Fc receptor is present on all mononuclear phagocytes, it is also present on mammalian granulocytes and B lymphocytes.³⁵ Fc and complement receptors are usually detected by means of rosette formation with opsonized sheep red blood cells.^{28-31,35} Control samples to estimate the degree of non-specific attachment of red cells to the mφ cell membrane should be included in this assay.³⁵

Receptors of IgM and IgA have not been described in monocytes or mφs.^{25,28,31} However, mφs may react indirectly with IgM through C3b receptors.²⁸ Receptors for IgE have recently been described,³⁴ as have those for histamine, glucagon, insulin and lactoferrin.³ Mφs reportedly have binding sites for macrophage inhibition factor,³⁶ lipoproteins,³⁷ and chemotactic factors.³⁸

Poorly understood non-specific binding sites are responsible for the attachment of other particles such as Leishmania, latex beads and tumor cells.³⁹ It is likely that there are a number of different mechanisms and adhesive structures involved in this process.³⁹

One of the major characteristics of mφs is their ability to engulf particulate matter, thereby constituting an indispensable host defense mechanism against invasion by microorganisms and other foreign materials.

Optimal phagocytosis is mediated by opsonins and is an energy requiring step when receptors are involved in binding.¹⁴ Subsequent to the attachment of the opsonized particle, the cell's plasma membrane invaginates and encloses the particle forming a phagocytic vacuole within the cell cytoplasm. The

membrane of the phagocytic vacuole then fuses with the membranes of the neighboring lysosomes. These in turn discharge their enzymatic contents into the phagocytic vacuole via the process of degranulation.^{5,11} On a sub-cellular level, an intact system of microfilaments and microtubules is essential for endocytic activity.⁴⁰ Optimum calcium and magnesium ion concentrations appear to be necessary for efficient phagocytosis.⁴⁰

The effectiveness of the process depends on the characteristics of the antigen ingested and on the degree of activation of the mφ.¹⁴ Gram negative bacteria may survive longer because their outer wall is more resistant to lysosomal digestion.⁵ Brucella abortus and Listeria monocytogenes are so resistant to the lethal effects of the lysosomal enzymes in normal mφs that they may multiply within the cell.⁵ Toxoplasma gondii and Mycobacterium tuberculosis evade the action of the mφ by blocking the fusion of the phagosome with the lysosome, thus preventing the release of hydrolytic enzymes.^{5,11} Activated mφs were found to destroy not only a number of bacteria but also viruses, fungi and protozoa.^{5,11}

The mechanisms by which mφs kill bacteria are largely unknown. Observations suggest that these cells utilize an oxygen-dependent system as an important bacteriocidal mechanism.⁴¹ In neutrophils, the process of killing involves peroxidation of H₂O₂ and an interaction with myeloperoxidase and iodide derived from tyrosine. This combination destroys microbial cell membranes and is followed by dissolution involving the integrated action of hydrolytic enzymes.^{11,41} Myeloperoxidase is only present in mφ precursors up to and including the monocyte. It appears, therefore, that in the monocyte, as in the neutrophil, myeloperoxidase provides an ancillary killing mechanism.⁴¹ In the mature tissue mφ, however, microbicidal mechanisms are independent of myeloperoxidase.⁴¹

The ability to phagocytose foreign material and microorganisms is impaired under certain environmental conditions including the exposure of cells to post-burn serum factors,^{42,43} ethanol⁴⁴ and high molecular weight hyaluronate.⁴⁵

A number of useful methods are available to measure the endocytic activity of mononuclear phagocytes. These methods have been extensively reviewed.⁴⁰ Once harvested, phagocytic cells are usually studied in suspension or as an adherent monolayer. Prior to a phagocytic assay, it is necessary to quantify and identify the cells present and to determine the percent of viable phagocytes.⁴⁰ Likewise, it is essential to be able to arrest phagocytosis and separate cells from unphagocytosed particles. These two criteria are satisfied simultaneously in a monolayer system by thorough rinsing of the culture dish.⁴⁰

Particles used in phagocytic assays include microorganisms -- live or dead such as Candida albicans^{42,43,46-48} and Staphylococcus aureus,⁴⁹ non-biological particles such as latex polystyrene beads,^{44,45,46,48} and paraffin oil droplets,⁵¹ and immune complexes such as IgG-coated sheep red blood cells.^{31,40} The particles are exposed to the monolayer for a standard period of time after which the monolayer is rinsed and the amount of ingestion measured.⁴⁰ Phagocytic assays often exploit the ease with which monolayers are prepared for light microscopy. However, a disadvantage of this method is the tendency for particles to aggregate and adsorb to the outside of the cell without ingestion.⁴⁰

Following fixation and staining, a number of indices of uptake can be measured. The phagocytic rate or index expresses the velocity or rate of uptake. Phagocytic rates should approach a maximal value as the particle concentration increases.⁴⁰ It is necessary to identify that the rate is constant with time and that the assay commences after a lag period, if one

exists.⁴⁰ Uptake should be zero at time zero.⁴⁰

The percent phagocytosis reflects the extent of phagocytosis and is used especially when investigating the fraction of a population of cells which are phagocytic. This method is commonly used to assess the degree of monocyte contamination in blood lymphocyte suspensions.⁴⁰

The best assays for ingestion are considered to involve direct measurement in phagocytes of particles that permit detection by chemical means. Such systems have been developed with polystyrene particles, labelled bacteria, starch granules and immune complexes.⁵²

While the more familiar role for the m ϕ is in an effector cell capacity, it is becoming increasingly obvious that this cell plays a crucial part in the afferent limb of the immune response. Macrophages interact with other cell types by direct contact and by means of soluble mediators such as monokines, interferons, and prostaglandins.¹⁴ These interactions are important in the maturation of T and B lymphocytes, mitogen-induced T lymphocyte blastogenesis, syngeneic tumor immunity, mixed leucocyte culture reactivity, antigen recognition and subsequent proliferation of T lymphocytes.¹⁴

M ϕ s are involved in the uptake, catabolism and presentation of antigens to responding T and B lymphocytes. This results in the activation of the immune system and the development of the characteristic antibody or cell-mediated immune response.^{53,54} This process involved the presentation of antigen on the surface of m ϕ s bearing determinants (Ia) specified by the I-region of the major histocompatibility complex, such that both antigen and Ia are recognized by the lymphoid cells.^{53,54} The manner in which a m ϕ presents an antigen may be important in determining the balance between helper and suppressor T cells, which in turn is critically important in determining the extent of the immune response.^{53,56}

Another area of macrophage function is that of resistance to tumor growth. Mø can be stimulated by bacterial endotoxin,⁵⁷⁻⁵⁹ perfluorochemical emulsions,⁶⁰ and T cell derived lymphokines,^{59,61} to express specific or non-specific activity against tumors. The anti-tumor activity is cytostatic and often cytolytic. The mechanisms of mø tumor cytotoxicity are unclear, but suggestions include complement cleavage products, hydrogen peroxide, interferons, prostaglandins and cell-to-cell contact with transfer of lysozyme.⁵⁷

Mø are known to secrete a variety of biologically active substances. These include complement components,⁶² lysosomal hydrolases especially lysozyme,⁵ collagenase,⁶³⁻⁶⁵ elastase,^{65,66} plasminogen-activator,⁶⁸ arachidonic acid oxygenation products especially the prostaglandin PG series,^{66,67} and slow reacting substance of anaphylaxis (SRS-A).⁶⁸ Many of these substances are potent mediators of inflammation. Secretion of soluble mediators with effects on cell substrate metabolism include macrophage insulin-like activity,⁶⁹ and endogenous pyrogen.⁷⁰ Interaction with other cells is mediated in part by the production of monokines such as lymphocyte activating factor,⁷¹⁻⁷³ neutrophil chemotactic factor,^{74,75} platelet activating factor,⁷⁶ a fibroblastic proliferation factor and colony stimulating factor.¹¹ In addition, møs possess inhibitors of enzymes such as α -1-antitrypsin, a potent inhibitor of elastase.⁷⁷ Secretion of soluble mediators is augmented by phagocytosis of immune complexes, inert particulates, zymosan, and by the effects of lymphokines and bacterial endotoxin.

It has become increasingly clear that the mø population is not homogeneous and can be separated into functional subclasses. This functional heterogeneity exists not only between møs from different sites but extends to møs within the same tissue site.⁷⁸ Møs from diverse environments such as the alveolar lining epithelium and peritoneal cavity show great diversity in cell size, phagocytic capacity and surface markers.^{78,79} Mø populations

can be affected by various treatments in vitro to display new functions.⁷⁹

This is most apparent when comparing the morphological, metabolic and functional characteristics of a normal resident population of cells and one that has been stimulated, for example, by C. parvum.⁷⁹

In recent years, four approaches have emerged for the functional definition of $m\phi$ sub-sets. Separation of the $m\phi$ by size on density gradients has yielded sub-sets with differences in phagocytic activity,⁸⁰ anti-tumor activity,⁸⁰ and antigen-presenting ability.⁸¹ The use of cell surface markers to separate functionally different populations has been advocated and reviewed.³ Markers include ectoenzymes, antigens which appear to be specific for activated $m\phi$ s,^{81,82} and Ia antigens. Ia-positive and Ia-negative populations of $m\phi$ s have been described.⁷⁹ The development of $m\phi$ -like cell lines presents an opportunity for functional studies of homogeneous cell populations.^{79,83} However, such cell lines are transformed *in vitro* and any extrapolation of their behavior to that of normal $m\phi$ s must be made with caution. The cultivation of $m\phi$ s from bone marrow or mononuclear phagocyte colony formation^{79,81} permits detailed study of $m\phi$ s at various stages of development from promonocyte to mature $m\phi$.

The presence of sub-classes of $m\phi$ s which are functionally unique is the basis for a controversy surrounding $m\phi$ ontology.⁷⁹ On one hand, $m\phi$ sub-sets are postulated to arise from distinct precursor cells.^{79,81} On the other, the activities of the sub-sets are explained as a function of the stage of differentiation of cells possessing a common precursor.⁷⁹ Current evidence indicates that the latter is at least partially responsible for the observed heterogeneity. It has been suggested by one author⁸¹ that the maturation of monocytes follows a linear sequence from the Ia-positive, antigen-presenting cell to the cytotoxic activated $m\phi$ in the presence of antigenic stimulation by C. parvum. Antigen-presenting activity was shown to develop during the exponential phase of growth of bone marrow colonies under the influence of colony stimulating factor.⁸¹

In order to study the varied facets of mφ function and physiology, reproducible methods of isolating these cells in reasonable numbers must be developed. Mφs are commonly studied in vitro as adherent monolayers or in suspension culture. It has not been ascertained if either of these systems approximates the normal environment of these cells in vivo.⁴⁰

Isolation of relatively pure, functionally active mononuclear phagocyte populations for culture from the various sites within the body presents some unique problems. Peripheral blood monocytes are isolated from heparinized blood using a two-step method. Ficoll-Hypaque^R density centrifugation⁸⁴ allows separation of the mononuclear cells (monocytes and lymphocytes), from the other cellular components of blood. The method has the advantage of being fast, simple, and reproducible with minimal contamination with granulocytes.⁸⁵ Disadvantages may be the variable contamination with platelets and possible toxicity of the reagents attendant on standing or on exposure to light.⁸⁵ Furthermore, Ficoll-Hypaque^R may alter some functions of the cells.⁸⁵ Further purification of monocytes following Ficoll-Hypaque^R is usually achieved by exploiting the ability of mφs and monocytes to adhere to glass and plastic surfaces. Adherence to these surfaces is usually complete within 1 hour at 37°C.⁸⁵ This step eliminates most of the contaminating lymphocytes although some may adhere. Platelets, granulocytes, mesothelial cells and fibroblasts may adhere non-specifically to glass and plastic and should be removed from the suspension prior to plating.⁸⁵ After monolayer formation, adherent cells may be removed to produce a suspension by trypsinization in the presence of E.D.T.A., scraping with a rubber policeman and the application of anesthetics such as lidocaine.⁸⁶ These methods invariably result in decreased yields of mononuclear phagocytes.⁸⁶

The use of the Ficoll-Hypaque^R technique to separate canine monocytes has been reported previously.^{29,30} The dog has a relatively low circulating monocyte count⁸⁷ and there may be some difficulty in obtaining cells in appropriate numbers. Successful attempts have been made to increase the yield of canine monocytes by inducing a monocytosis with intravenously administered lipid extracts of Listeria monocytogenes.³⁰

More recently, techniques using countercurrent centrifugation⁸⁸ and continuous density gradients of Percoll^{89,90} have been described for human monocytes. These techniques result in highly purified monocyte suspensions but are relatively expensive for routine use.

Alveolar mφs may be isolated from the lung by one of two general methods: via bronchoalveolar lavage or from lung biopsy. Bronchoscopic examination and lavage using the fiberoptic bronchoscope is now widely used in man for the collection of cells.¹² In animals, reference to bronchoscopic collection of cytologic specimens are limited, although techniques have been described for the dog^{91,92} cat,⁹² pig,⁹³ and cattle.⁹⁴ In one study in dogs,⁹¹ 95% of all nucleated cells in normal lavages were alveolar macrophages. The average yield from a normal human adult lavage is 93% $\pm$ 5% alveolar mφs.¹² Large numbers of viable mφs can be obtained by mechanical treatment of lung biopsy specimens.^{95,96} Mononuclear cells are isolated from suspensions obtained by either method using Ficoll-Hypaque^R density centrifugation which eliminates contamination by lung parenchymal cells. Purified suspensions and monolayers of mφs are prepared as discussed for blood monocytes.

Macrophages of the small intestine, unlike alveolar mφs and blood monocytes, are not easy to obtain in quantity and in pure cell suspension. This has undoubtedly contributed to the relative lack of published information concerning them. Intestinal macrophages reside within a tissue whose tough

outer wall, mucus secretions and numerous cell types make mØ isolation difficult. Interest in the interepithelial and lamina propria lymphoid cell populations has resulted in the development of techniques for the isolation of these cells.^{9,13,24,97-99} Suspensions of mononuclear cells including mØs have been isolated as a by-product of these techniques but as interest has been focused on the lymphoid components, characterization of mØs has been limited. Mixed cell suspensions containing intestinal mononuclear cells have been prepared from intestinal biopsy specimens using two methods: purely mechanical and enzymatic. Purely mechanical methods range from simple rubbing of the intestinal mucosa to liberate the mononuclear cells,⁹⁹ to homogenizing intestinal mucosal specimens.²⁴ The enzymatic method,^{9,13,97,98} is based on the principle of chemically disrupting the intestinal mucosal layers in systematic fashion. It involves the sequential applications of dithiothreitol as a mucolytic, E.D.T.A. as an agent to disrupt the epithelial layer, and collagenase to digest the connective tissue framework of the lamina propria. The extended exposure of mØs to these various chemical treatments is a disadvantage of this technique,²⁴ and may alter mØ function. However, yields of intestinal mØs from the enzymatic method have been reproducible and based on the ability to phagocytose particles and esterase staining, it has been estimated by several authors that 10% of intestinal mononuclear cells are mØs.^{9,98} Yields from the mechanical methods have varied and estimations of mØ proportion in mononuclear suspensions range from 1%⁹⁹ to 9.5%.²⁴

Contamination of mØ suspensions with other types of cells combined with the relatively small percentage of mØs draws attention to the necessity of developing techniques to concentrate and separate these cells before detailed characterization can begin. With respect to lymphoid cells, this problem has been approached using Ficoll-Hypaque^R,⁹⁸ or bovine serum albumin (B.S.A.)

gradients⁹⁹ to separate the required cell population from the mixed suspension of intestinal mucosal cells. Recent developments in the use of continuous density gradient centrifugation with Percoll^R to separate component cell populations may offer the ultimate solution to this problem.

Various authors have reported a difference in function between inter-epithelial and lamina propria lymphocytes.⁹ It is, therefore, of interest to investigate mφs from interepithelial and lamina proprial locations as separate populations, and to develop methods which will differentiate and concentrate them. Suspensions of interepithelial mφs and lymphocytes may be obtained from techniques which disrupt the epithelial layer only, such as mechanical rubbing and treatment with E.D.T.A.⁹ The fact that no plasma cell contamination occurs in suspensions prepared by these methods indicates minimal contribution by lamina propria mφs to this mφ population and lends support to the claim that these techniques concentrate interepithelial mononuclear cells.⁹

Blood monocytes and tissue mφs can be maintained in vitro under simple culture conditions on the surface of plastic or glass containers. The ability of mononuclear phagocytes to adhere to these surfaces is characteristic. Following attachment, the cells spread along the surface and send out cytoplasmic projections which help anchor the cells. Intracellularly, attachment sites contain organized arrays of microfilaments directly subjacent to the membrane's point of contact with the surface. These locations also display networks of microtubules which appear to originate in the centrosphere region and insert peripherally into the microfilaments.¹⁰⁰ In the absence of serum, mφs and fibroblasts show passive adhesion to plastic or glass that is neither temperature nor divalent cation dependent.¹⁰¹ Therefore, in mφ isolation using adherence techniques, culture medium containing serum has been

regularly used to limit the amount of passive adhesion.¹⁰¹ A modification of this technique advocates the use of serum-coated plastic surfaces to recover mφs from suspension.¹⁰¹ This method is based on the principle that mφs display active adherence to the serum-coated substratum in a divalent cation dependent manner.¹⁰¹ It is a high yielding and reproducible technique. The serum factors promoting active adhesion are unknown.¹⁰¹

Monocytes and mφs do not normally divide in culture.⁸⁵ This produces a complication if mesothelial cells or fibroblasts are present in the suspension to be cultured. These cells actively divide and on prolonged culture may overgrow the mononuclear phagocyte monolayers.⁸⁵ These cells commonly contaminate mφ cultures obtained from solid organs and inflammatory exudates.⁸⁵

There is considerable variation in cell survival in long-term cultures which depends in part on the source and species of the cells employed.⁸⁵ Canine monocytes are functionally active after 10 days of culture in Dulbecco's modified Eagle's medium (MEM) and 10% fetal calf serum.³⁰ Long-term cultures of canine monocytes have been maintained in MEM and 20% canine serum in order to study immunological aspects of Ehrlichia canis infection in dogs.^{102,103} One study showed MEM to be superior to RPMI 1640 in canine cultures.³⁰ MEM has the advantage of being better buffered and thus, controls the pH of the monolayers more accurately. Canine and fetal calf serum proved comparable in supporting canine monocyte cultures while equine serum appeared superior.³⁰

While monocytes tend to be stable in culture, alveolar mφs from both rodents and man demonstrate progressive detachment and subsequent loss to the medium.⁸⁵

It is important to recognize that properties of monocytes and mφs may change in culture. Some cells become progressively more phagocytic and may display new functions. Canine monocytes showed a morphological progression through macrophages to multinucleate giant cells in culture.¹⁰² This must be

taken into account when investigating functional aspects of mφs. Commonly, mφ monolayers are used for functional assays immediately following attachment.

As methods for the culture and study of mφ populations become more sophisticated, the role of the mononuclear phagocyte in various disease processes can be investigated. A variety of human and animal diseases are associated with increased activity and/or numbers of monocytes and tissue macrophages. An increase in the total number of macrophages may result from proliferation of the stem cell pool in the bone marrow. One of the stimuli for such a proliferation is colony stimulating factor produced by the mφ itself.¹¹ Increase in the numbers of mφs within a tissue site, and granuloma formation, may be a sequel to recruitment of blood monocytes in response to chemotactic factors produced by activated T lymphocytes.¹² Mononuclear phagocytes have the ability to synthesize powerful mediators of tissue injury including lysosomal enzymes and plasminogen activator which catalyse fibrinolysis, complement activation and generation of kinins. Furthermore, depending on the nature of the pathologic process, mφ-derived neutrophil chemotactic factor may be produced and is followed by accumulation of these cells, their subsequent degranulation and release of proteolytic enzymes.¹² Thus, the initial mφ response is amplified and may result in the disordering of and damage to parenchymal cells, derangement of structural proteins such as collagen and elastin¹² and the stimulation of fibroblast proliferation.¹²

Parts of this potentially damaging sequence of events may be initiated by phagocytosis of intracellular parasites such as Brucella abortus, Histoplasma capsulatum and Mycobacterium spp.,⁴¹ of chemicals such as beryllium and asbestos,¹² and of Micropolyspora faeni, one of the antigens responsible for hypersensitivity pneumonitis in humans.¹⁰⁴

In many cases of chronic inflammatory or granulomatous disease, the initiating agent is unknown. However, mononuclear phagocytes are incriminated in their pathogenesis. Chronic inflammatory bowel diseases of unknown etiology include Crohn's disease and ulcerative colitis in man,¹⁰⁵⁻¹⁰⁹ granulomatous colitis in the horse,¹¹⁰ and histiocytic colitis in the Boxer dog.¹¹¹ Monocytes isolated from patients with chronic inflammatory bowel disease have reportedly shown several characteristics associated with activation including increased levels of plasminogen activator and lysosomal enzymes,¹⁰⁵⁻¹⁰⁷ enhanced prostaglandin E production,¹⁰⁸ and increased intracellular killing ability for S. aureus.¹⁰⁹

Chronic inflammatory lung diseases of unknown etiology have been investigated in man, and include sarcoidosis, idiopathic pulmonary fibrosis and histiocytosis X.¹² Alveolar mφs isolated by lavage from patients with idiopathic pulmonary fibrosis have been shown to spontaneously secrete low molecular weight chemotactic factor for neutrophils.¹²

Mononuclear phagocytes may also proliferate autonomously as is seen in monocytic leukemia, histiocytic lymphoma, Letterer-Siwe disease, histiocytic medullary reticulosis and Hand-Schuller-Christian disease.⁴¹ The malignant histiocytic diseases can be viewed as different clinically identifiable stopping points along the spectrum of mononuclear phagocyte differentiation.¹¹ The neoplastic diseases of histiocytic cells embrace a wide range, from disorders in which there is extensive proliferation of poorly differentiated cells such as acute monocytic leukemia to disorders in which there is limited and circumscribed proliferation of well-differentiated cells.⁴¹ Clearly, there will be overlap within these diseases, depending on how tightly the neoplastic clone is regulated in its differentiation.¹¹ There is dispute about the malignant nature of some of the syndromes mentioned above. The

clinically chronic disorders characterized by proliferation of well-differentiated histiocytes may represent a reactive response to an unidentified antigen and may be viewed as having more in common with diseases such as pulmonary sarcoidosis than with true malignancy.⁴¹

Another group of disorders in which one observes an increase in the numbers of mononuclear phagocytes is that in which a phagocytizable material accumulates intracellularly more rapidly than it can be disposed of by metabolic processes. The lipid storage diseases, such as Gaucher's disease, Niemann-Pick syndrome, Tay-Sach's disease and gangliosidosis, are characterized by a deficiency in one of the catabolic enzymes involved in the breakdown of sphingolipids.¹¹ The mφs, by virtue of their prominent role in the catabolism of lipid-rich membrane, are particularly prone to accumulate undegraded lipid products.¹¹

In addition to diseases in which there is proliferation of mononuclear phagocytes, there are disorders which are associated with dysfunction of these cells on a genetic or acquired basis. Genetic abnormalities include chronic granulomatous disease in which the defect is probably due to deficient post-phagocytic peroxide and superoxide generation.¹¹ High doses of corticosteroids and ionizing radiation may interfere with mφ defense in animals and probably in man.⁴¹ With high doses of ionizing radiation, the proliferation of mφ precursors in the bone marrow is impaired, but their metabolic activity appears to be intact.¹¹ In patients receiving corticosteroids, susceptibility to opportunistic intracellular infections is increased. The mechanism of the enhanced susceptibility is only partially known but it has been shown that under the influence of corticosteroids, alveolar macrophages have increased lysosomal stability¹² resulting in failure of lysosomes to fuse with phagosomes. More importantly, defective mobilization of phagocytes into areas of inflammation is induced by corticosteroid administration.⁴¹

CHAPTER III

MATERIALS AND METHODSA. Animals

Ten crossbred adult dogs (6 males, 4 females) weighing between 25-30 lbs. were obtained from the Animal Resource Facility at Kansas State University. All dogs were in good health and had been routinely vaccinated for Canine Distemper, Parainfluenza, Hepatitis and Parvovirus. Complete blood counts for all dogs were within normal limits. Fecal examination revealed Dipylidium caninum infestation in 5 dogs, which was confirmed at necropsy.

B. Isolation of blood monocytes

A 15ml blood sample was collected from the jugular vein of each dog into heparinized Vacutainer^R tubes.^a Mononuclear cells were separated from the remainder of the peripheral blood cell population by Ficoll-Hypaque^{Rb} density centrifugation.⁸⁴ Contaminating erythrocytes were removed using osmolar shock with distilled water. Composition of the resulting cell suspension was assessed on the basis of cellular morphology using Diffquik stain^c and α naphthyl acetate esterase activity.^{17,19,20} Viability was estimated using Trypan Blue exclusion. Cells were suspended for culturing in RPMI 1640^d with 50 μ g gentamycin/ml (RPMI/G) with 10% fetal calf serum (F.C.S.). The concentration was adjusted to $3-5 \times 10^6$ cells/ml. Aliquots of 1 ml were placed in plastic culture dishes^e and incubated in 5% CO₂ for 2 hours at 37°C to permit adherence of monocytes. Test monolayers of monocytes and other mononuclear phagocyte populations were prepared as discussed in a later section.

^aVacutainer tubes: Dickinson & Co., Rutherford, NJ 07070

^bFicoll-Hypaque: Div. Pharmacia Inc., Piscataway, NJ 08854

^cHarleco Diffquik: American Scientific Products, N. Kansas City, MO 64116

^dRPMI 1640: Grand Island Biological Co., Grand Island, NY 14076

^eFalcon (35X10mm): Dickinson & Co., Oxnard, CA 93030

C. Isolation of canine alveolar macrophages

Dogs were euthanatized with intravenously administered T61 euthanasia solution^f. The lungs were immediately removed and bronchoalveolar lavage performed using 400ml of cold sterile phosphate buffered saline with 50 µg gentamycin/ml (PBS/G). The collected lavage fluid was filtered through 4 layers of sterile gauze and centrifuged for 20 minutes at 400g in a Damon IEC Model 5000 centrifuge. The cell pellets were pooled and washed 3 times in PBS/G. The morphologic composition of the cell suspension was assessed as mentioned above for blood monocytes. After resuspension in RPMI/G with 10% FCS, the concentration of cells was adjusted to $1-2 \times 10^6$ cells/ml and viability was assessed by Trypan Blue exclusion. As mentioned for blood monocytes, 1 ml of cell suspension was placed in tissue culture dishes and incubated for 2 hours in 5% CO₂ at 37°C to allow for adherence.

D. Isolation of lamina propria macrophages

A 30-40 cm section of ileum was removed from each dog after euthanasia. The lumen was immediately flushed with 1-2 litres of PBS/G to remove mucus and debris. This process was augmented by rubbing the ileal surfaces together during flushing. The intestine was then opened and the mucosal surface scraped with the side of a glass microscope slide to remove residual mucus and to initiate disruption of the epithelial layer. Avoiding Peyer's patches, mucosal strips were separated from the underlying muscularis by firm scraping with the slide held at 45° to the direction of movement. The mucosal strips were dissected into small pieces and incubated with stirring at 20°-22°C for 30 minutes in 100ml of calcium and magnesium free HBSS^g. A suspension of lamina propria cells was obtained using Bull and Bookman's method¹³ of

^fT61 Euthanasia Solution: Taylor Pharmacal Co., Decatur, IL 62525

^gHanks Balanced Salt Solution: K.C. Biological, Lenexa, KS 66215

sequential treatments with D.T.T.^h, E.D.T.A.ⁱ and overnight digestion with purified collagenase^j. After filtering through 4 layers of sterile gauze and washing three times with PBS/G, the viability of the cell suspension was assessed using Trypan Blue exclusion. The proportion of mononuclear phagocytes in the cell suspension was estimated by staining smears for morphology with Diffquik^R and for α naphthyl acetate esterase activity. The ability of the suspended cells to phagocytose neutral red crystals was investigated by placing a drop of suspension on a slide coated with dried 0.1% solution of neutral red. The slide was examined after 3 minutes and the number of phagocytic cells per 200 cells counted was noted.

E. Formation of monolayers and their characterization

Two hour cultures of peripheral blood monocytes and alveolar macrophages were thoroughly rinsed three times with RPMI/G to remove non-adherent cells. All tissue culture dishes were examined with an inverted microscope for monolayer formation. Two monolayers from both the monocyte and alveolar macrophage groups were air-dried and stained for morphology and α naphthyl acetate esterase activity. The remainder of the monolayers were used for EA rosetting and phagocytosis assays.

F. Detection of Fc-IgG receptor bearing cells (EA rosettes)

(i) Sheep red blood cells (SRBC)

Sheep red blood cells were collected in Alsever's solution^k. Prior to use in the EA rosetting technique, the red cells were washed 5 times in PBS/G and then suspended to a final 1% v/v concentration with PBS/G.³¹

^hDithiotreitol: Sigma Chemical Co., St. Louis, MO 63178

ⁱE.D.T.A.: Fischer Scientific Co., Fairlawn, NJ 07410

^jCollagenase 160 units/mg: Grand Island Biol. Co., Grand Island, NY 14076

^kAlsever's Solution: Grand Island Biol. Co., Grand Island, NY 14076

(ii) Sensitized SRBC (EAg)

Rabbit anti-SRBC IgG was prepared by Dr. L.S. Rodkey, Division of Biology, Kansas State University. The rabbit IgG was diluted to a sub-agglutinating titer, 1:1000, with RPMI/G. Equal volumes of 1% washed SRBC and diluted rabbit anti-SRBC IgG were combined and incubated for 30 minutes at 37°C followed by 30 minutes at 4°C. Excess antibody was removed by washing 2 times in PBS/G after incubation.³¹

(iii) Formation of EA rosettes

1 ml of the incubated SRBC suspension was added to duplicate washed monolayers of peripheral blood monocytes and alveolar macrophages. Duplicate controls consisted of monolayers to which had been added 1 ml of 1% v/v unsensitized washed SRBC. All monolayers were incubated for 30 minutes at 37°C in 5% CO₂. Unattached SRBC were carefully rinsed away with PBS/G. The monolayers were air-dried and stained with Diffquik^R. A total of 200 cells per preparation were counted and any cell binding 3 or more SRBC was considered a rosetting cell.

G. Phagocytosis of latex and *Candida albicans* particles

Latex spheres¹ (0.8 μ diam) were washed 2 times in PBS/G and suspended in RPMI/G with 10% F.C.S. in a 1:1000 dilution. The culture medium was removed from the peripheral blood monocyte and alveolar macrophage monolayers and replaced with 1 ml of 1:1000 latex solution. The plates were incubated for 45 minutes at 37°C in 5% CO₂ after which the latex solution was removed and thoroughly rinsed 3 times with PBS/G to remove non-adherent particles. The monolayers were air-dried and stained with Diffquik^R.

¹ Lates Spheres (0.8 μ diam): Sigma Chemical Co., St. Louis, MO 63178

A suspension of heat-killed, Methylene blue stained Candida albicans was prepared by Dr. W. Bailie, Department of Laboratory Medicine, Kansas State University. The particles were washed 3 times in PBS/G and resuspended in RPMI/G with 10% F.C.S. to a concentration of 1×10^9 cells/ml. The culture medium was removed from peripheral blood monocytes and alveolar macrophage monolayers and replaced by 1ml of the Candida suspension. Monolayers were incubated for 45 minutes in 5% CO₂ at 37°C after which they were rinsed 3 times in PBS to remove non-adherent Candida. The monolayers were air-dried and stained with Diffquik^R.

All phagocytosis assays were done in duplicate. Controls consisted of peripheral blood and alveolar macrophage monolayers incubated with 1ml of RPMI/G and 10% F.C.S. only. Two parameters of phagocytosis were measured. A total of 200 cells were counted for each measurement per monolayer.

$$(i) \text{ \% phagocytic cells} = \frac{\# \text{ adherent cells ingesting particles} \times 100\%}{\# \text{ adherent cells}}$$

$$(ii) \text{ Phagocytic index} = \# \text{ particles/phagocytic cell/45 mins}$$

H. Tissue section preparation

Specimens of lung and ileum from each dog were fixed in 10% buffered formalin for histopathological study. The ileum samples were stapled to wooden spatulas to prevent artifact due to fixation and trimming. 5 μ sections were cut in routine fashion for H & E staining. 6-8 μ sections were cryostat^m cut²⁵ for α naphthyl acetate esterase staining.¹⁷ After staining, the coverslip sections were mounted in glycerine jelly-based aqueous mounting medium.²⁵ The paired tissue sections were used to describe the spatial distribution of esterase-positive cells within the lung and mucosa of the ileum.

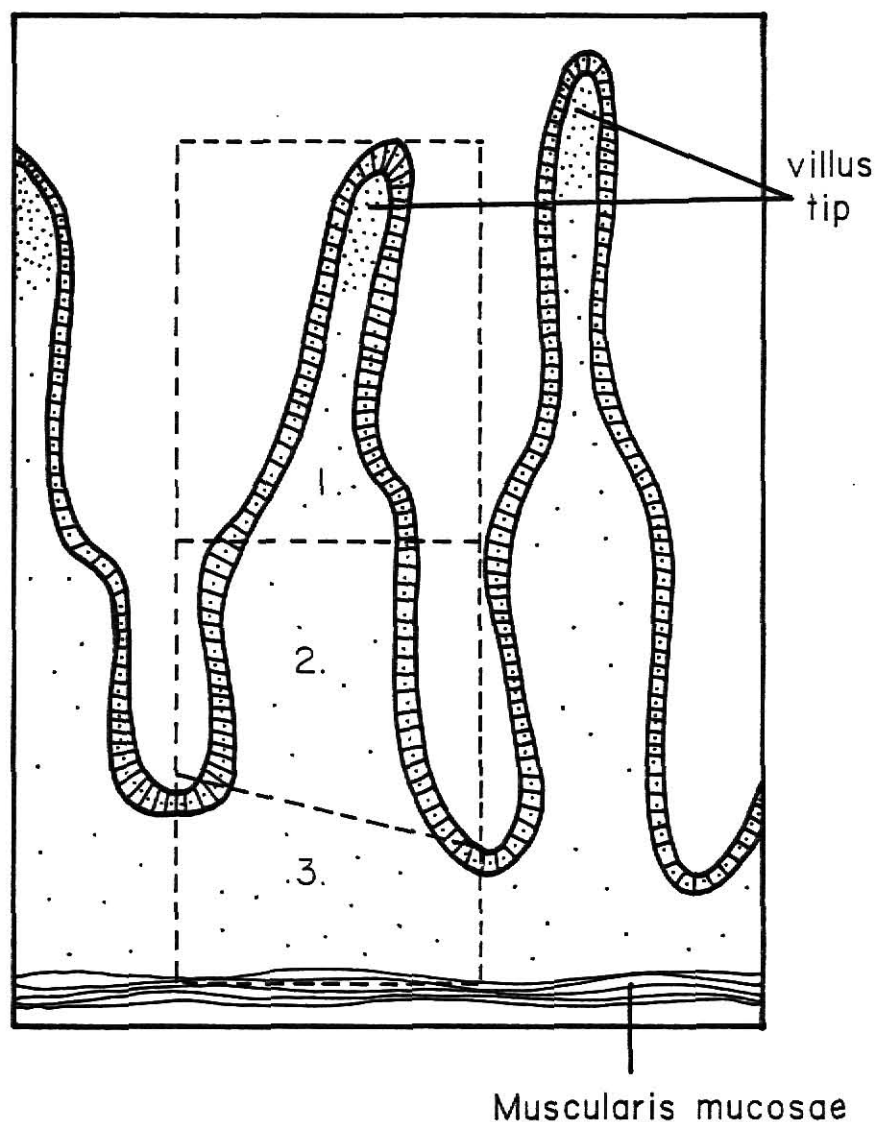
^mAmes cryostat: Elkhart, IN 46514

I. Enumeration of esterase-positive cells within the lamina propria and epithelium of the ileum

All cell counts were made in areas where appropriate longitudinal sections of villi and crypts were present. For enumeration of esterase-positive cells within the epithelial layer, the number of stained cells per 200 epithelial cells was counted. For enumeration of esterase-positive cells, a lamina propria unit was anatomically defined (Figure I). The area enclosed within the unit was sub-divided into three zones as illustrated. The unit extended from the tip of the villus to the muscularis mucosae. The tissue area within the unit was estimated on each specimen using a Nikon microscope with 40X and 100X field objectives. An ocular scale in the eyepiece was calibrated for each objective using a stage micrometer divided into units of 0.01mm. Esterase-positive cells were counted within tissue zones as follows:

1. Villus core lamina propria
2. Crypt-region of the sub-villus lamina propria
3. Deep region of the sub-villus lamina propria.

FIGURE I
NORMAL CANINE ILEAL MUCOSA



Diagrammatic representation of a lamina propria unit in the ileum of a normal dog. The average area of tissue enclosed within the unit was 0.15mm^2 . The diagram indicates the density of esterase-positive cells within the unit. The highest density was found to occur at the tip of the villus. For the purpose of enumerating the esterase-positive cells, 3 tissue zones were defined as illustrated above.

KEY:

1. Villus core lamina propria
2. Crypt-region of the sub-villus lamina propria
3. Deep region of the sub-villus lamina propria

CHAPTER IV

RESULTSA. Initial cell populations

Peripheral blood samples from the 10 dogs in this study contained a mean of $3.8 \pm 1.7\%$ monocytes, $22.8 \pm 2.8\%$ lymphocytes and $72.9 \pm 11.8\%$ neutrophils, eosinophils, bands and basophils. The mean leucocyte count in peripheral blood was $12,460 \pm 2.84$. The mean esterase-positive percentage was $8.6 \pm 2.9\%$. Individual blood count data are shown in Appendix I.

Canine bronchoalveolar lavage samples contained a mean of $76.3 \pm 6.3\%$ macrophages, $11.3 \pm 6.4\%$ lymphocytes, and $13.4 \pm 5.3\%$ other cells including eosinophils, epithelial cells, mast cells and neutrophils. The mean percentage of esterase-positive cells was $86.2 \pm 5.1\%$.

Mixed cell suspension of canine lamina propria cells contained a mean of $3.5 \pm 1.2\%$ esterase-positive cells. Approximately 2% of the lamina propria cells ingested neutral red crystals. More detailed differential counts were difficult to perform due to the large amount of debris and the variety of cell types present.

B. Cell separation

The percentage of mononuclear phagocytes in peripheral blood and bronchoalveolar lavage samples was estimated at 3 stages during the concentration and purification procedure. The cells were identified by 2 methods at each step: non-specific esterase staining and morphology using Diffquik^R stain. The stages were identified as follows:

- I. Initial mixed cell suspension prior to Ficoll-Hypaque^R density centrifugation;
- II. Partially purified mononuclear cell suspension obtained after Ficoll-Hypaque^R density centrifugation;

III. Adherent cell population after 2 hour incubation of the mononuclear population on a plastic surface.

The results obtained were analyzed using a randomized block design and analysis of variance and are illustrated in Tables I and II. At all stages there was a significantly higher percentage of mononuclear phagocytes isolated from bronchoalveolar lavage than from peripheral blood. The trend was present using both methods of identification.

The efficiency of the concentration techniques used in the preparation of the monolayers for function testing was examined statistically using a split plot design and analysis of variance. There was a significant interaction ($P < 0.0001$) increase in the net percentage of blood monocytes harvested. However, neither the Ficoll-Hypaque^R density centrifugation nor adherence to plastic resulted in significant improvement in the yield of alveolar mφs.

A comparison of the methods of identification using non-specific esterase activity and morphology was carried out by analyzing the difference in the mononuclear phagocyte percentage obtained by each method at each concentration step for both populations of cells. (Table III). There was a significant interaction ($P < 0.0001$) between the sources of the mononuclear phagocyte population and the technique. There was a progressive decrease in the advantage of non-specific esterase activity in identifying alveolar macrophages through the 3 isolation steps although it remained superior to morphology alone. Non-specific esterase activity was significantly more efficient ($P < 0.005$) than morphology in estimating the percentage of alveolar mφs in the initial lavage suspension but this efficiency decreased in the adherent cell population. The largest difference in the efficiency of the methods occurred in estimating the percentage of monocytes in the monolayers, where non-specific esterase staining identified a mean of 24.2% more monocytes than morphology alone.

TABLE I

Mean percentage of canine mononuclear phagocytes present at 3 stages of isolation using non-specific esterase activity for identification (n=10)

<u>Stage</u>	<u>A</u> <u>Monocyte</u>	<u>B</u> <u>Alveolar mφ</u>	<u>Significance of difference</u> <u>between A and B</u>
I Initial	8.6 $\pm$ 2.9*	86.2 $\pm$ 5.1	P < 0.0001
II Post-Ficoll	19.4 $\pm$ 7.1	87.3 $\pm$ 7.7	P < 0.0001
III Adherence	66.6 $\pm$ 13.6	86.4 $\pm$ 10.5	P < 0.0003**

* Mean $\pm$ 1 S.D.

**Significant between dog variation (P < 0.05).

TABLE II

Mean percentage of canine mononuclear phagocytes present at 3 stages of isolation using morphology for identification (n=10)

<u>Stage</u>	<u>A</u> <u>Monocyte</u>	<u>B</u> <u>Alveolar mφ</u>	<u>Significance of difference</u> <u>between A and B</u>
I Initial	3.8 ± 2.6*	76.3 ± 6.3	P < 0.0001
II Post-Ficoll	17.8 ± 7.0	80.8 ± 8.5	P < 0.0001
III Adherence	42.4 ± 13.5	82.8 ± 8.9	P < 0.0001

* Mean ± 1 S.D.

TABLE III

Mean difference in percentage of canine mononuclear phagocytes estimated by non-specific esterase staining and morphology at 3 stages of isolation (n=10)

<u>Stage</u>	<u>Monocyte</u>	<u>Alveolar mØ</u>
I Initial	4.8	9.9
II Post-Ficoll	1.6	6.5
III Adherence	24.2	3.6

Concentration of the macrophages of the lamina proprial suspensions was unsuccessful. Consequently, no adherent cell population was obtained and investigation of the function of these cells was not possible.

C. Presence of the Fc receptor for IgG on canine mononuclear phagocytes

$78.2 \pm 10.5\%$ monocytes and $93.4 \pm 6.1\%$ of alveolar m ϕ s showed evidence of Fc receptors for IgG as detected by the EA rosetting technique (Table IV). Neither blood monocytes nor alveolar macrophages formed spontaneous rosettes with unsensitized sheep red blood cells used as the negative control in this study. The data were analyzed using a randomized block design and analysis of variance. The percentage of EA rosette-forming cells was significantly higher ($P < 0.001$) in the alveolar macrophage population than in the blood monocyte population.

D. Phagocytosis

The data obtained from the phagocytic studies with canine mononuclear phagocytes was analyzed using a randomized block design and analysis of variance.

The percentage of alveolar macrophages ingesting C. albicans ($85.5 \pm 8.4\%$) was significantly greater ($P < 0.0001$) than the percentage monocytes ingesting C. albicans ($35.8 \pm 7.5\%$). This trend also occurred when latex beads were used as the test particle. $89.7 \pm 8.5\%$ of alveolar macrophages ingested latex, as did $36.4 \pm 6.1\%$ of blood monocytes. The difference between the population means was significant, ($P < 0.0001$). This data is illustrated in Tables V and VI.

Canine blood monocytes had a phagocytic index of 1.79 ± 0.78 for C. albicans and 4.83 ± 1.91 for latex beads. Alveolar macrophages had a phagocytic index of 6.80 ± 1.64 for C. albicans and 20.91 ± 1.84 for latex beads.

TABLE IV

Mean percentage of canine mononuclear phagocytes displaying Fc receptor (n=10)

<u>Cell type</u>	<u>Mean % of non-specific esterase positive, adherent cells forming EA rosette*</u>
Monocyte	78.1 $\pm$ 10.5** (Range: 58.7 - 94.8)
Alveolar mØ	93.4 $\pm$ 6.2 (Range: 79.6 - 98.9)

* 200 cells counted; cells binding 3 or more IgG-coated sheep red blood cells were considered rosetting cells.

** Mean $\pm$ 1 S.D.

TABLE V

Mean percentage of canine mononuclear phagocytes phagocytic for *C. albicans*
(n=10)

<u>Cell type</u>	<u>Mean % of non-specific esterase positive, adherent cells phagocytic for <i>C. albicans</i>*</u>
Monocyte	35.8 + 7.5** (Range: 25.9 - 46.0)
Alveolar mφ	85.5 + 8.4 (Range: 73.1 - 98.7)

* 200 cells counted per preparation

** Mean $\pm$ 1 S.D.

TABLE VI

Mean percentage of canine mononuclear phagocytes phagocytic for latex beads
(n=10)

<u>Cell type</u>	<u>Mean % of non-specific esterase positive adherent cells</u> <u>phagocytic for latex beads*</u>
Monocyte	36.4 $\pm$ 6.1** (Range: 28.9 - 47.6)
Alveolar mØ	89.7 $\pm$ 8.5 (Range: 70.3 - 98.7)

* 200 cells counted per preparation

** Mean $\pm$ 1 S.D.

The difference in phagocytic ability between the populations was significant irrespective of particle type, ($P < 0.0001$). (See Table VII).

The effect of particle type was examined further by analyzing the difference between phagocytic index for latex and C. albicans for each population of cells. There was an interaction between the type of particle and the source of mononuclear phagocytes. The phagocytic index using latex was consistently higher than that using C. albicans, for both alveolar macrophages and blood monocytes. However, the difference was significantly higher ($P < 0.0001$) in alveolar macrophages as compared to monocytes. Not only do alveolar macrophages have a higher phagocytic index than monocytes, but their avidity for latex particles relative to C. albicans is greater than that of the blood monocyte. The data are illustrated in Table VIII.

E. Interepithelial and lamina propria macrophages in canine ileum

Histological examination of H & E stained sections of the ileum of all dogs in this study revealed no pathological lesions. On tissue sections stained for non-specific esterase activity, counts of esterase-positive cells in the interepithelial position ranged from 6-14 per 200 epithelial cells with a mean of 9.6 ± 2.88 . These cells were predominantly seen at the bases of the crypts and in the surface epithelium at the tips of the villi. They were commonly closely associated with the basement membrane below the level of the epithelial cell nucleus.

In the lamina propria of the ileum, the counts of esterase-positive cells ranged from 17-51 per 0.15mm^2 with a mean of 33.3 ± 9.60 (Table IX). Within the lamina propria counts ranged from 10-32 with a mean of 20.5 ± 6.2 in the villus core; 3-12 with a mean of 7.0 ± 2.8 in the crypt region lamina propria and from 1-10 with a mean of 5.8 ± 2.9 in the deep region of the lamina propria. (Table X).

TABLE VII

Mean phagocytic index for canine mononuclear phagocytes using *C. albicans* and latex particles (n=10)

<u>Cell type</u>	<u>Phagocytic Index*</u>	
	<u><i>C. albicans</i></u>	<u>latex beads</u>
Monocyte	1.79 ± 0.78** (Range: 1.0 - 3.2)	4.83 ± 1.91 (Range: 2.6 - 9.0)
Alveolar mφ	6.8 ± 1.64 (Range: 4.3 - 9.9)	20.91 ± 1.84 (Range: 16.4 - 23.3)

* Phagocytic index = # test particles ingested per phagocytic cell per 45 minutes

** Mean ± 1 S.D.

TABLE VIII

Mean difference in the phagocytic indices* of canine mononuclear phagocytes for *C. albicans* and latex (n=10)

<u>Cell type</u>	<u>Phagocytic index - Phagocytic index</u> <u>(latex) (C. albicans)</u>
Monocyte	3.0
Alveolar mØ	14.1

* Phagocytic index = # test particles ingested per phagocytic cell for 45 minutes

TABLE IX

Distribution of non-specific esterase positive cells within the epithelium of the canine ileum (n=10)

<u>Location</u>	<u>Mean # of esterase positive cells</u>
Interepithelial	$9.6 \pm 2.88^{*\dagger}$ (Range: 6 - 14)
* Interepithelial cells counted per 200 epithelial cells † Mean $\pm$ 1 S.D.	

TABLE X

Distribution of non-specific esterase positive cells within the lamina propria of the canine ileum (n=10)

<u>Location</u>	<u>Mean # of esterase positive cells</u>
Villus tips	$20.5 \pm 6.15^*$ (Range: 10 - 32)
Crypt region lamina propria	7 ± 2.79 (Range: 3 - 12)
Deep lamina propria	5.8 ± 2.97 (Range: 1 - 10)
Total / 0.15mm^2 (lamina propria unit)	$33.3 \pm 9.60^{**}$ (Range: 17 - 51)

* Mean $\pm$ 1 S.D.

** Significant between dog variation ($P < 0.01$)

The data was analyzed using a randomized block design, analysis of variance and LSD mean separation. The mean count of esterase-positive cells in the villus core was significantly greater than the means for other locations, ($P < 0.0001$). There was no significant difference between the means for the crypt and deep regions of the lamina propria. Within the villus core, there was a clustering of esterase-positive cells at the tip of the villus. In other areas of the lamina propria, the esterase-positive cells were relatively evenly distributed throughout the tissue.

CHAPTER V

DISCUSSION

The mean leucocyte count and differential values for peripheral blood samples of the dogs in this study were within the normal range reported by Schalm.⁸⁷ The differential count on the bronchoalveolar lavage samples, however, differed from those described for the dog,⁹¹ the pig,⁹³ and man.¹² The mean macrophage percentage obtained in this study was $76.3 \pm 6.3\%$ compared to 95% reported previously for the dog.⁹¹ When non-specific esterase staining was used to identify mononuclear phagocytes in peripheral blood and alveolar lavage samples, there was a significant increase in their respective percentages (Tables I and II). In making differential counts, monocytes must be differentiated from immature neutrophils and large lymphocytes. When morphology is used as the sole means of identification, designation of monocytes into these categories may result in an underestimation of monocyte count. In bronchoalveolar lavage samples, large alveolar macrophages are easily recognizable using morphology. However, there appeared to be a population of small macrophages readily confused with large lymphocytes on morphological grounds. This population corresponded to the undifferentiated macrophage population reported by Rebar et al.⁹¹ in canine bronchoalveolar lavage samples. These cells could be identified as belonging to the mononuclear phagocyte family as they stained intensely with non-specific esterase. The accurate identification of these macrophages could explain the difference in the macrophage estimations obtained with morphology and non-specific esterase as criteria. The percentage of alveolar macrophages identified using esterase staining ($86.2 \pm 5.1\%$) more closely approximated that for man ($93 \pm 3\%$)¹² and other species.

Non-specific esterase staining was particularly useful in mixed cell suspensions containing a variety of cell types. In the suspensions obtained from the sequential chemical and enzymatic treatment of the canine ileum biopsy specimens, $3.5 \pm 1.2\%$ nucleated cells were esterase-positive. This compares with $10 \pm 4\%$ from suspensions prepared from human colonic biopsies using the same technique as described above,¹³ 9.5% from suspensions prepared from human colonic biopsies using mechanical techniques,²⁴ and $1-2\%$ from suspensions prepared from rabbit ileum using mechanical techniques.⁹⁹ Comparison of these values is difficult as there is variation in technique, site of biopsy and species. However, the results obtained in this study were within the range of those in current literature. The mixed cell suspensions obtained from treated canine ileum contained considerable quantities of mucus despite extensive flushing of the mucosal surface and treatment with the mucolytic D.T.T. Mucus clumps appeared to contain large numbers of esterase-positive cells. It is likely that intestinal mononuclear phagocytes become trapped in the mucus network while attempting to phagocytize the debris.

The sequence of density gradient centrifugation with Ficoll-Hypaque^R followed by adherence to plastic proved to be an effective method of concentrating monocytes from peripheral blood of the dog. However, the adherent population of monocytes was contaminated with lymphocytes and neutrophils to a greater degree than that reported by Ho et al.²⁹ whose adherent preparations contained $83.7 \pm 6.2\%$ mononuclear phagocytes. In several samples, platelet aggregation occurred during centrifugation and clumps of neutrophils and platelets were found in the mononuclear layer. The neutrophils adhere to the plastic surface of the culture plate avidly thus decreasing the surface area for attachment of monocytes. Phagocytosis of the adhered neutrophil aggregates was not observed when the plates were examined after 2 hr incubation. However,

this would be an expected complication with prolonged incubation and could result in alteration of the state of activity of the adhered monocyte. In order to minimize platelet clumping and contamination of the mononuclear suspension, adequate heparinizing of the peripheral blood sample is essential. However, it has been reported that even in heparinized blood, platelets tend to adhere to monocytes during centrifugation and disturb their ability to stick to glass and plastic.⁸⁹ An alternative to the use of heparin is defibrination of the blood sample. Pertoft et al.⁸⁹ found defibrination preferable to the inhibition of coagulation with heparin or removal of calcium by ion-exchange resins. It was suggested that results were more reproducible and the cells obtained more viable because platelets were removed concomitantly.

The efficiency of the separation of neutrophils from monocytes using Ficoll-Hypaque^R is dependent on temperature and speed of centrifugation. The separation procedure is markedly more efficient if carried out at room temperature than at 4°C.¹¹² Centrifugation in this study was carried out after the method of Boyum⁸⁴ at 400g. Ho et al.²⁹ carried out the separation of canine mononuclear cells using centrifugation at 200g. As it is considered that centrifugation at higher speed results in platelet contamination of the mononuclear layer, there may be a critical speed at which this occurs in canine blood.

The separation procedure is also dependent on the Ficoll^R concentration and density of the medium. In this study, a commercially available Ficoll-Hypaque^R mixture was used with a Ficoll^R concentration of 6.4% and density of 1.077g/ml. Ferrante et al.¹¹² report that optimum separation of human mononuclear cells occurred when the medium was adjusted to a Ficoll^R concentration of 8.2% and density of 1.114g/ml. Techniques for separation of canine mononuclear cells have been adopted from human research with little change. The

Ficoll-Hypaque^R mixture producing optimum results with canine mononuclear cells may differ from that described for human work.

Monocytes prepared by adherence tend to be contaminated with B lymphocytes as well as platelets and neutrophils, due to a property of these cells described as surface "stickiness".^{29,85} Because of the difficulty in distinguishing between canine T and B lymphocytes on the basis of surface markers, a reliable estimate of the percentage of circulating B lymphocytes is not available. However, it is possible that unlike the human, canine B lymphocytes represent a significant proportion of the lymphocytes isolated by the Ficoll-Hypaque^R technique. Consequently, these cells represent a problem in using adhered populations of monocytes; firstly, because they must be distinguished from monocytes, and secondly, because they may share properties in common with the monocyte population.

Because of the relatively high concentration of alveolar macrophages in the initial lavage samples, neither concentration techniques nor adherence to plastic resulted in a significant increase in the yield of alveolar macrophages. The adhered population was pure except for some contamination with eosinophils, mast cells and neutrophils. In this study, the Ficoll-Hypaque^R step could have been deleted without noticeably affecting the result. However, the decision to employ density centrifugation as part of the isolation process must ultimately depend on the cellular composition of the lavage sample. The role of the alveolar macrophage in various pathological states of animal lung will be studied in the future. If such a condition results in an increase in the lavage concentration of neutrophils as occurs in idiopathic pulmonary fibrosis, then density centrifugation would be an essential part of the isolation process.

The interaction between method of staining, type of mononuclear phagocyte population and isolation step was complex in this study. Although the

estimations of percent mononuclear phagocytes at each isolation step using non-specific esterase staining for identification were consistently higher than using morphology alone, there were 2 places in which esterase staining was highly efficient when compared to morphology. (Table III). The first was in the bronchoalveolar lavage samples prior to use of Ficoll-Hypaque^R. Difficulty occurred here in distinguishing small or undifferentiated macrophages from lymphocytes by morphology. The second area where esterase staining proved highly efficient was in identifying accurately the adherent monocyte population. Some adherent monocytes lose their characteristic morphology on adherence and become difficult to distinguish from large lymphocytes. The problem of adherent B lymphocytes may be partially addressed using esterase staining. These cells are esterase-negative and can thus be differentiated from monocytes.

Although the non-specific esterase stain is an extremely useful technique, there are some problems associated with its use. Firstly, it is time-consuming both to prepare and use. Several components of the stain must be freshly prepared and will deteriorate in storage.²⁰ Secondly, for optimum results, close attention to the species requirements for pH and incubation time is necessary.¹⁷ Thirdly, hexazonium pararosaniline has been reported as a cause of bladder cancer in man after prolonged exposure to the dye.¹¹³ Other dyes such as Fast Blue RR are available for use in non-specific esterase staining but the color produced is not easily distinguished.²⁵

The percentage of non-specific esterase-positive cells in the adhered populations of mononuclear phagocytes gives an estimation of the degree of contamination with adherent, non-specific esterase negative cells as well. The results of the function tests were corrected for the contamination by expressing the results as a function of the percent of non-specific esterase positive cells in the test population.

The percentage of canine monocytes expressing the receptor for the Fc portion of IgG ($78 \pm 10.4\%$) approximates that reported previously for the dog by Ho et al. ($81.5 \pm 2.4\%$).⁸⁷ There are no corresponding values for alveolar macrophages reported for the dog. However, the results reported here ($93.4 \pm 6.1\%$) agree with those reported for the pig (90% - 95%)^{31,95} and man (93%).¹² The significantly higher proportion of alveolar macrophages with Fc receptors compared to blood monocytes is consistent with the idea that alveolar macrophages are functionally more mature than the monocytes from which they are derived.¹¹⁴ It may also reflect the difference in the local tissue environment between the two types of cell.⁷⁸ The Fc receptor is evident on a percentage of promonocytes in the bone marrow but reaches its full expression in the mature macrophage. However, prolonged in vitro culture of monocyte and macrophage populations encourages the increase of receptor activities and essentially eliminates the difference in the populations with regard to Fc receptor possession.⁷⁸ Much interest exists in the fact that approximately 20% of blood monocytes and 7-10% alveolar macrophages do not exhibit EA rosette forming ability. It appears that the alveolar macrophage population, at least, exists as a heterogeneous group of cells that in response to stimuli differentiate into a population possessing functional uniformity with enhanced Fc receptor activity.⁷⁸ When considering the percentage of cells forming EA rosettes, it should be remembered that a significant proportion of B lymphocytes have this ability.²⁹

It is of interest that neither adhered monocytes nor alveolar macrophages formed spontaneous rosettes with washed unsensitized sheep red blood cells. Spontaneous rosetting of canine monocytes has been reported with human²⁹ and guinea pig red blood cells.¹¹⁵ This indicates the presence of either cytophilic antibody to human and/or guinea pig red cells or the occurrence of a

natural receptor for these cells on the monocyte.²⁹ The former explanation is at least partially responsible for this phenomenon because the percentage of monocytes spontaneously rosetting is decreased by absorption of the serum used in the suspension medium with guinea pig red cells.¹¹⁵

The percentage of alveolar macrophages phagocytizing C. albicans and latex particles was significantly higher than the percentage of monocytes phagocytizing these particles. As discussed above, the difference is consistent with pre-existing ideas on the integrated maturation of monocyte to macrophage and/or the difference in the local environment. The alveolar macrophage, by virtue of its position in the bronchoalveolar tree, is in constant contact with potential antigens. Approximately 40% of the 2 hr incubated cultures of normal adherent canine monocytes phagocytized latex or C. albicans particles over a 45 minute time span. However, ingestion of latex particles is widely used as a marker for monocytes in suspensions.^{29,50} Unless monocytes are functionally altered by culturing in vitro or by exposure to antigenic stimuli, a significant number of them will not phagocytize latex. When assessing either the ability of monocytes to phagocytize or the percentage of monocytes which contaminate a suspension, this fact should be taken into consideration.

The enhanced phagocytic ability of alveolar macrophages as compared to blood monocytes is reflected in the higher phagocytic index that alveolar macrophages have for both C. albicans and latex. Not only do a greater proportion of alveolar macrophages phagocytize particles but more particles are ingested per cell over a standard period of time. This gives support to the idea of the alveolar macrophage as the premier phagocyte of the lung.

In both populations (blood monocytes and alveolar macrophages), more latex beads are ingested than C. albicans. The most obvious explanation for this observation is the relative difference in the size of the particles, C. albicans

being approximately 20X larger than the latex particles. There may also be differences in the surface tension characteristics which allow faster ingestion of the latex beads. The tendency of the latex particles to adhere to the membrane of the cells despite vigorous washing may lead to an overestimation of the number of particles ingested.⁴⁰ The use of phase contrast microscopy⁴⁰ or the use of latex beads of larger diameter will decrease the error due to this cause.

There is an interaction between the source of mononuclear phagocytes, the type of particle and the phagocytic index. The difference between the number of latex and C. albicans ingested by alveolar macrophages is greater than for the blood monocytes. This may indicate a difference in the mechanism of phagocytosis for the 2 types of particle. Macrophages non-specifically bind latex and yeast particles but binding involves different macrophage membrane structures for each particle.³⁹ Differences in the electron microscopic patterns of bound latex and other particles have been reported.³⁹ It is conceivable that the alveolar macrophage may have a surface membrane with a higher concentration of sites for latex binding than for C. albicans. As the difference between latex and C. albicans ingestion is not as marked in the blood monocyte, this may be characteristic that develops with maturation of the alveolar macrophage. When interpreting this interaction, it is necessary to consider the possibility that the phagocytosis of C. albicans had reached saturation point. However, when establishing the experimental method, 45 minutes was found to be on the linear portion of the uptake graph with the saturation point occurring at 1-2 hours for the alveolar macrophage population. Thus, it is unlikely that the alveolar macrophage population was saturated at the time of assaying the phagocytic index.

Isolation of a population of lamina propria macrophages from canine ileum in a suspension pure enough to allow adherence was not successful.

Contamination with debris and mucus with resultant cell trapping were major problems. Mucus contamination was more pronounced in samples from well-conditioned dogs and seemed to increase with the age of the dog. 24 hour fasting did not noticeably decrease the degree of mucus contamination. In an attempt to minimize this problem, the mucosal surface of the ileum was scraped with the edge of a glass slide. This procedure removed a significant proportion of the debris and disrupted the villus structure. However, due to their depth, the crypts in the canine ileum remained intact. The deep crypts contain a large number of Goblet cells which on chemical disruption later in the treatment recontaminated the suspension with mucus.

There is no doubt that methodology for obtaining macrophages from solid tissues such as the intestinal lamina propria will improve in the future. However, at present, useful information can be gained from studying macrophages in situ within intestinal tissue. Unless they occur in large number, macrophages of the absorptive regions of the ileum are inconspicuous in ordinary histological specimens. The use of non-specific esterase staining in cryostat sections of the canine ileum proved to be a useful marker for lamina propria macrophages. Esterase-positive cells were identified in the epithelial layer, the villus tips, the villus cores and the deep lamina propria of the canine ileum. Because of the position of the interepithelial esterase-positive cells, it was difficult to recognize the distinct morphology associated with macrophages under the light microscope. In some situations, the epithelial cells appeared to stain lightly with the esterase stain. The mean number of esterase-positive cells within the epithelium was 9.6 ± 2.88 per 200 epithelial cells counted. This compares with 5.4 ± 1.4 per 200 epithelial cells count recently reported from the human colon.⁹ Although no reports on the quantitation of interepithelial macrophages in the canine ileum are available as yet,

it is of interest to compare the findings of this study with those recently described for interepithelial lymphocytes in the canine ileum. Thomas and Anderson¹¹⁶ reported an average of 13.59 ± 0.91 interepithelial lymphocytes per 100 epithelial cells counted in ileum biopsies taken from dogs over 6 weeks of age. 85% of these cells were located at or below the level of the nuclei of adjacent epithelial cells. There is a possibility that interepithelial macrophages make up a proportion of cells previously identified as interepithelial lymphocytes.

There are drawbacks to using a fixed number of epithelial cells as a reference value for expressing the number of interepithelial lymphocytes and macrophages. The epithelial cells may vary in shape, size and number under certain conditions and consequently, relative increases and decreases in the interepithelial populations occur with fluctuations in the epithelial cell distribution.¹¹⁶ However, as this method is frequently used to quantify the numbers of the interepithelial lymphocytes and as the reports of interepithelial macrophages are sparse, it appears to be useful for comparison.

There was a tendency for the esterase-positive cells in the epithelium to accumulate at the tips of the villi or at the base of the crypts. This observation has been reported previously by Bartnik et al.⁹ in the human colon.

Within the lamina propria of the canine ileum, esterase-positive cells were readily identifiable as macrophages on morphological grounds. The mean number of lamina propria macrophages within a 0.15mm^2 area was 33.3 ± 9.6 but the variation between dogs was statistically significant. Bartnik et al.⁹ reported a mean of 5.3 ± 0.8 esterase-positive cells per 0.049mm^2 in the lamina propria of the human colon. Comparisons between these values are difficult because of the significant individual variation between the dogs and the difference in the species and site of biopsy.

Within the lamina propria unit used for this study, there was a significant accumulation of esterase-positive cells at the villus tips. This fact has been previously reported in human,⁹ and in guinea pigs.²⁷ It has also been reported that macrophages appear to be migrating from the lamina propria into the intestinal epithelium at this point.¹¹⁷ This may explain the apparently higher concentration of interepithelial macrophages at the villus tips. Macrophages in the lamina propria showed close association with plasma cells and eosinophils.

It has been postulated that this histological arrangement of macrophages in the villi of the intestinal tract represents a mechanism for the shedding of accumulated waste material into the intestinal lumen.⁷ Macrophages accumulate at the villus tips, migrate into the extrusion zone and are lost with the intestinal epithelial cells. Migration of macrophages from the lamina propria into the intestinal epithelium in iota carrageenan-fed guinea pigs has been demonstrated.¹¹⁸ Astaldi et al.¹¹⁷ have shown apparent migration of iron-laden macrophages into the intestinal lumen. Sawicki et al. found macrophages containing Feulgen-positive granules (apparently degraded DNA) in the tips of guinea pig villi.¹⁷ The DNA was probably derived from the ingestion of cells but whether these were migrating epithelial cells or interepithelial lymphocytes could not be determined. Migration of latex-containing macrophages from Peyer's patches to the adjacent villi and their subsequent loss into the lumen has been postulated as one of the major mechanisms responsible for the gradual clearing of the latex from Peyer's patches.⁷ This mechanism of excretion is analogous to the migration of waste-laden macrophages within the lungs and their loss via the mucociliary elevator system.

It is not known if macrophage migration occurs in the opposite direction, i.e., from the lumen to the lamina propria. However, it is unlikely that

the macrophages in this position play a large role in antigen-sampling of the intestinal lumen. This remains the function of the Peyer's patch lymphoid cell population.

Migrating intestinal macrophages may transport immunoglobulin, a function ascribed to them elsewhere in the body. Pittard et al.¹¹⁹ described the ability of colostral macrophages from the human breast milk to bind large amounts of IgA. Intestinal macrophages could bind and transport IgA from the plasma cells with which they are closely associated in the lamina propria.¹²⁰ However, alveolar macrophages lack receptors for IgA and do not recognize IgA complexes.¹² It would seem that if IgA transport were a function of macrophages located at body surfaces alveolar macrophages would have this capacity.

The existence of anatomically discrete populations of macrophages within the epithelium and lamina propria of the canine ileum raises the problem of isolating these populations. The technique used in this study preferentially removes the epithelial layer and disrupts the villus tips. Consequently, the lamina propria macrophage population that would have been isolated if it had been successful would consist of deep lamina propria macrophages. It is also important to realize that this method results in the loss of a large proportion of the total macrophage population of the lamina propria; probably one reason for the poor results obtained in isolation. Because of the possibility of functional as well as anatomical heterogeneity in the macrophage populations in the lamina propria, the populations isolated by the various chemical and mechanical techniques need to be carefully defined.

CHAPTER VI

CONCLUSIONS

1. Esterase-positive cells were identified within the epithelial layer, the villus tip and core and in the subvillus lamina propria of the canine ileum. There was a statistically significant larger number of esterase-positive cells at the tips of the villi than in other locations within the lamina propria. Thus, non-specific esterase staining is a useful marker for identifying mononuclear phagocytes in cryostat-cut sections of formalin fixed canine ileum.
2. Ficoll-Hypaque^R density gradient centrifugation followed by adherence to plastic is an efficient method for isolating canine blood monocytes for functional studies.
3. Bronchoalveolar lavage is a simple high-yield technique for harvesting canine alveolar macrophages in relative purity. Adherent populations of these cells proved to be an efficient system for functional assay.
4. Values for percentage of cells bearing the Fc receptor for IgG, percentage of cells phagocytic for C. albicans and latex particles and phagocytic index have been obtained for populations of monocytes and alveolar macrophages isolated from normal dogs.
5. Significantly greater percentages of normal canine alveolar macrophages showed evidence of the Fc receptor for IgG than did normal canine blood monocytes.
6. Normal canine alveolar macrophages showed significantly greater phagocytic ability as measured by percentage phagocytosis and phagocytic index using C. albicans and latex as test particles, than did canine blood monocytes.

CHAPTER VII

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CHAPTER VIII

APPENDIXHematological values for the dogs used in this study1. Red Cell Parameters

Dog #	PCV%	Hb(gm/dl)	RBC $\times 10^6/\text{mm}^3$	MCV (fl)	MCHC (gm/dl)
A1	45.7	16.3	6.63	69.0	35.3
B2	46.8	17.2	7.37	63.0	36.4
C3	51.7	18.8	7.63	68.0	36.1
D4	50.3	16.7	6.92	73.0	32.9
E5	46.4	17.5	7.44	62.0	37.3
F6	45.8	16.8	7.29	63.0	36.3
G7	37.8	12.8	6.46	58.0	33.6
H8	43.1	15.4	7.02	61.0	35.4
I9	47.7	16.7	7.54	63.0	34.6
J10	48.6	17.8	7.13	68.0	36.3

Hematological values for the dogs used in this study

2. White Cell Parameters

Dog#	WBC x10 ³ /mm ³	Neutro- phils	Bands	Lympho- cytes	Mono- cytes	Eosino- phils	Baso- phils
A1	11.1	7770	111	1112	111	666	-
B2	15.3	11232	-	3276	468	627	-
C3	13.2	8052	-	3630	198	1320	-
D4	14.9	7748	74	3055	223	2459	1341
E5	8.8	5014	88	3256	88	264	-
F6	12.7	6731	-	2286	762	2921	-
G7	17.0	12750	340	2465	935	510	-
H8	12.6	6480	63	4725	819	504	-
I9	8.0	5680	80	1360	640	240	-
J10	10.8	6156	216	2700	432	1296	-

STUDIES ON GUT MACROPHAGES, BLOOD MONOCYTES
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by

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AN ABSTRACT OF A MASTER'S THESIS

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ABSTRACT

The mononuclear phagocyte system is implicated in the pathogenesis of chronic inflammatory disease in man and there exists a possibility that the canine may provide an animal model for some of these syndromes. A study of the role of mononuclear phagocytes in disease can be undertaken only after their characterization in the normal animal.

The objectives of this study were to enumerate and characterize macrophages from a tissue site; the normal canine intestine, and to define functionally and morphologically, two reference populations of canine mononuclear phagocytes; blood monocytes and alveolar macrophages.

10 clinically healthy, cross-bred, adult dogs were used in the study. Peripheral blood and bronchoalveolar lavage samples and an ileal biopsy were obtained from each dog. Tissue sections of the ileum were prepared both routinely and cryostat-cut for histological examination and non-specific esterase staining. Ficoll-Hypaque^R density centrifugation followed by adherence to a plastic surface was used to isolate canine monocytes for functional studies. Bronchoalveolar lavage proved to be a simple, high-yield technique for harvesting canine alveolar macrophages. Adherent populations of these cells were used as a system for functional assays. Values for percentage of cells bearing the Fc receptor for IgG, percentage of cells phagocytic for C. albicans and latex particles and phagocytic index are reported for populations of monocytes and alveolar macrophages isolated from normal dogs. A significantly greater percentage of normal canine alveolar macrophages showed evidence of the Fc receptor for IgG than did normal canine monocytes. Normal canine alveolar macrophages showed significantly greater phagocytic ability as measured by percentage phagocytosis and phagocytic index using C. albicans and latex as test particles, than did normal canine monocytes.

The use of the non-specific esterase stain provided a useful marker for mononuclear phagocytes in formalin-fixed, cryostat-cut sections of canine ileum. Esterase-positive cells were identified and counted within the epithelial layer, the villus core lamina propria and in the sub-villus lamina propria of the ileum. There was a significantly larger number of esterase-positive cells located at the tips of the villi than in other zones within the lamina propria. The functional significance of this finding is discussed.