ASPECTS OF EOSINOPHIL BIOLOGY IN THE AUGUST RAT INCLUDING A MODEL OF NEMATODE INFECTION WITH *NIPPOSTRONGYLUS BRASILIENSIS*

By

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ABSTRACT

ASPECTS OF EOSINOPHIL BIOLOGY IN THE AUGUST RAT INCLUDING A MODEL OF NEMATODE INFECTION WITH *NIPPOSTRONGYLUS BRASILIENSIS*

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Various novel paradigms of eosinophil involvement in the adaptive immune response have been described in the literature over the last two decades. The fundamental objective of this thesis was the elucidation of active eosinophil involvement during the primary immune response to the enteric phase of a nematode infection in rats. Initially we tested the hypothesis that the previously observed eosinophil accumulation in the peritoneal cavity of the August (AUG) strain of rat is primarily the consequence of phenotype. This characterization of the strain would also facilitate the next phase of the project, which was the comparison of eosinophil activity in the AUG rat during the intestinal phase of primary infection by the nematode parasite *Nippostrongylus brasiliensis* (*Nb*). The investigations presented here utilized primarily morphological methods, including a new photoreactive fluorescent technique using Biebrich scarlet (BS) for the detection of eosinophil specific granules.

The eosinophil accumulation in the peritoneal cavity of AUG rats was determined to be principally due to the phenotypic expression of heritable trait(s)

in this inbred strain. Consistent peritoneal lavage harvests of large numbers (6.9 ± 0.5 x 10^6/animal) of eosinophils from AUG rats of both sexes provided a ready source of these cells that is not present as a rule in other rat strains. In contrast, eosinophil numbers in the peripheral blood (113 ± 22 cells/μl) and in the jejunal mucosa (25.9 ± 1.1 cells/villus-crypt (VC) unit) of these rats were within the standard range observed in other rat strains.

A significant eosinophilic response to adult stage *Nb* occurred in the lamina propria of the jejunum (72.9 ± 5.2 cells/VC unit). These cells showed altered morphological profiles including cytoplasmic dispersion of specific granules, nuclear change and cytoplasmic vacuolization. Additionally, eosinophils were observed migrating through the basement membrane of the gut wall and found within the epithelial layer. The peritoneal cavity showed a similar reactivity with a two-fold increase in eosinophil number and increased level of leukotriene C_4. These responses occurred at time points relevant to the rejection of the worms by the gut and were thus consistent with the active involvement of eosinophils in the primary immune response to adult stage *Nb*.

To my parents, Charles and Virginia Eversole,
by their examples of love, strength and courage, taught me that all things are possible...

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KEY TO ABBREVIATIONS

ANOVA	analysis of variance
AUG	August strain
BS	Biebrich scarlet
C	complement
ECP	eosinophil cationic protein
EDN	eosinophil-derived neurotoxin
EDTA	ethylene diamine tetra-acetic acid
EPO	eosinophil peroxidase
FcR	cell receptor for antibody
FMLP	n-formyl-methionyl-leucyl-phenylalanine
GM-CSF	granulocyte-macrophage colony stimulating factor
HBSS	Hank's balanced salt solution
HEPES	hydroxyethyl-piperazine-ethane sulfonic acid
HLA	human leukocyte antigen
IACUC	institutional animal care and use committee
IFN	interferon
Ig	immunoglobulin

IHES	idiopathic hypereosinophilic syndrome
IL	interleukin
L_3	third stage *Nb* larvae
L_4	fourth stage *Nb* larvae
LFA	leukocyte functional antigen
LSCM	laser scanning confocal microscopy
LT	leukotriene
MBP	major basic protein
MHC	major histocompatibility complex
Nb	*Nippostrongylus brasiliensis*
NBF	neutral buffered formalin
PAF	platelet activating factor
PECAM	platelet endothelial cell adhesion molecule
PI	post infection
RANTES	regulated and normal T-cell expressed and secreted protein
RT	room temperature
SD	Spraque-Dawley strain
SEM	standard error of the mean
SNK	student Newman-Kuels
SOZ	serum opsonized zymosan
TEM	transmission electron microscopy

Th	thymocyte helper (cell)
TNF	tumor necrosis factor
VC	villus-crypt
VLA	very late antigen
WBC	white blood cell

Chapter 1

REVIEW OF LITERATURE

Introduction

In 1879 Paul Ehrlich discovered that the cytoplasmic granules of certain blood cells had a strong affinity for the negatively charged stain, eosin. These bright orange-red staining cells he classified as a distinct group of leukocytes and aptly named them eosinophils. Later it was shown that the proteins found in the compact specific granules of eosinophils were highly cationic thus explaining their affinity for the bright red, brominated fluorescein derivative; eosin. This histologic feature remains perhaps the most widely known characteristic of eosinophil granulocytes.

Armed with the ability to detect eosinophils in blood and tissue, Ehrlich began the first investigations linking these cells with specific pathologic changes in certain disease states of humans. He proposed that eosinophils originated in the bone marrow, demonstrated the transient nature of their levels in blood during disease and hypothesized that eosinophils "exerted their function in tissues" (Ehrlich & Lazarus, 1900). The evidence produced in these early investigations is, for the most part, still the accepted dogma in the field today. In the same time frame, physicians described the accumulation of eosinophils in tissues during

asthma (Gollasch, 1889), allergic skin diseases (Canon, 1892) and endoparasitic infections (Brown, 1898). These clinical conditions remain the primary areas of investigation in the current eosinophil-related literature.

Eosinophil Morphology

Morphology remains the single most reliable method for determining the presence and activity of eosinophils within a given tissue compartment (Owen, 1993). Mature eosinophils range in size from 8-17 μm with a bilobed nucleus in humans and a multilobed donut-shaped nucleus in rats (Hamada et al., 1992). Their characteristic specific or secondary granules allow rapid identification at the light and electron microscopic levels. As mentioned previously, the arginine-rich basic proteins in eosinophil specific granules have a high affinity for acid dyes such as eosin (Ehrlich, 1879), chromotrope 2R (Lendrum, 1944) and Biebrich scarlet (Spicer & Lillie, 1961), all of which give these granules a distinct orange-red appearance under bright-field light microscopy. The ultrastructural profile of eosinophil specific granules is a distinctly unique ellipsoid shape with a bimodal packaging scheme where one or more axial, crystalline cores are surrounded by an outer matrix, all packaged in a double-unit membrane. The cores are comprised entirely of major basic protein (MBP) and the matrix is a composite of eosinophil cationic protein (ECP), eosinophil-derived neurotoxin (EDN) and eosinophil peroxidase (EPO) in humans (Egesten et al., 1986). Rat eosinophils contain the enzymes described in human cells (Pimenta et al., 1987) and also have

peroxisomal enzymes in their crystalloid granules (Yokota et al., 1984). The relative electron-densities of these granule compartments varies with staining methodology but the profile remains relatively consistent in normal tissue eosinophils (Faller, 1966). The number of eosinophil specific granules has been shown to vary in normal eosinophils for both humans and rats with 20-40/cell being the typical range. The volume of individual specific granules varies within cells as well as total granule volume/cell (Elmalek & Hammel, 1987; Caulfield et al., 1990). The ratio of total specific granule volume to total cytoplasmic volume in tissue-dwelling eosinophils is markedly decreased compared to the majority of blood- and bone marrow-derived eosinophils (Owen, 1993). These specific granule characteristics contribute to the physical heterogeneity observed in eosinophils *in vivo* and will be discussed in detail later in this review.

A second type of granule common in eosinophils are primary granules. Primary granules are the principal type in early promyelocyte eosinophils but are also observed in tissue dwelling cells where specific granules are the predominant granule type. Primary granules vary in size, often larger than specific granules and are spherical with a homogeneous electron-dense ultrastructural profile (Zucker-Franklin, 1980). Mature eosinophils have a third population of granules which are round and much smaller in size than the specific or primary granules at 0.1-0.5 μm diameter and which possess acid phosphatase and arylsulphatase activity (Parmley & Spicer, 1974). The greater number of the small granules in tissue dwelling

eosinophils compared to blood and bone marrow eosinophils has led to the hypothesis that these small granules may be derived from the specific granules since both have acid phosphatase; this enzyme is only detectable, however, in specific granules of degranulating eosinophils which have lost their central crystalloids (Enomoto & Kitani, 1966). Additionally, the small granules have been shown to accumulate microendocytosed gold particles demonstrating involvement in heterophagic activity in peritoneal-derived eosinophils (Fukuda et al., 1985).

The eosinophil cytoplasm contains a highly developed cytoskeletal microtrabecular lattice which is capable of orchestrating rapid cellular and granular shape changes (Pryzwansky, 1987). Microvesicular and vacuolar cisternae of the smooth endoplasmic reticulum are a common feature of eosinophils and extensive membrane-bound, labyrinthine, tubulovesicular structures have been observed in peritoneal-derived eosinophils (Fukuda et al., 1985). Golgi apparatus, mitochondria and rough endoplasmic reticulum are also found in both blood and tissue eosinophils (Spry, 1988). A prominent cytoplasmic feature of tissue eosinophils involved in disease states are non-membrane bound cytoplasmic lipid bodies (Weller & Dvorak, 1985).

Cell Biology of Eosinophils

Migratory populations of immune regulatory cells, of which eosinophil leukocytes are one type, require complex signal transduction pathways and communication molecules to accomplish a vast array of functions in a multitude of

tissue microenvironments. In order to perform some of these functions, cell surface receptors are known to serve as initiation sites for many communication pathways. Some of the dynamic cell surface receptors that have been identified on eosinophils include those for soluble serum-derived mediators, cytokine mediators, immunoglobulin (Fc) receptors and adhesion receptors for both cell-cell and cell-matrix interactions (Gleich et al., 1992; Giembycz & Barnes, 1993; Wardlaw, 1994). Known complement (C) receptors include C1q, C3b/C4b (CR1), iC3b (CR3) and C5a and eosinophils will respond to C3a. Cytokine receptors have been described on eosinophils for: interleukins (IL-2,-3,-5), granulocyte, macrophage-colony stimulating factor (GM-CSF), regulated and normal T-cell expressed and secreted protein (RANTES), and are presumed for tumor necrosis factor (TNF-α) and interferon (IFN-γ). Additionally, the experimental peptide n-formyl-methionyl-leucyl-phenylalanine (FMLP) has been shown to bind to eosinophils via membrane receptors. Receptors for highly potent lipid mediators of eosinophil function include platelet activating factor (PAF) and leukotriene B_4 (LTB_4). Immunoglobulin (Ig) binding receptors for the IgG, IgA and IgE classes are expressed on the surface of eosinophils. There are three receptors for IgG: high affinity FcγRI and the lower affinity FcγRII and FcγIII. Multiple receptors for the IgE class are also known. The first discovered was the low affinity FcεRII but a recent finding by Capron and colleagues (1995) indicated that the high affinity

FcεRI is also expressed on eosinophils *in vivo*. Adhesion receptors for eosinophils include: the integrins, VLA-4, VLA-6, LFA-1 and Mac-1 (CR3); Immunoglobulin-like PECAM; the L-selectin moiety and ligands for P-selectin and E-selectin. It is important to note that VLA-4 is the only member of the integrin family known for both cell-cell adhesion and binding to the extracellular matrix molecule, fibronectin (Anwar et al., 1993; Anwar et al., 1994) suggesting a versatile cellular migration mechanism in tissues.

Eosinophils have the ability to secrete many types of mediators in the chemical web of the inflammatory response. These include relatively large stores of the highly alkaline preformed protein mediators found in the specific granules (Gleich et al., 1992). MBP with a pI of 10.9 contains 17 arginine residues contributing to its cationic status. It is synthesized and packaged as an acidic proprotein (pI 6.2) which may serve to protect the cell itself from its highly cytotoxic constituent (Barker et al., 1988). MBP has distinctive helminth-killing capability which has been shown to be inhibited by mast cell-derived or endogenous heparin by an unknown mechanism (Kierszenbaum et al., 1982). The strongly positive charge of MBP has been implicated in toxicity to respiratory epithelium at low concentrations. MBP can cause the release of histamine from mast cells and both MBP and EPO have been shown to increase arachidonic acid metabolism and granule secretion in platelets (Rohrbach et al., 1990). The activity of the heme containing protein EPO has been shown to be more potent than neutrophil

myeloperoxidase when utilizing bromine in the halide-H_2O_2 killing mechanism (Wardlaw, 1994). EPO is also a cationic toxin with a pI of 10.8 that can mediate the killing of parasite and mammalian cells. The remaining highly cytotoxic granule protein found in eosinophils is ECP which also has a pI of 10.8 and possesses some ribonuclease activity. ECP shares a strong homology to eosinophil-derived neurotoxin (EDN) but EDN has a much higher ribonuclease activity and is only weakly cytotoxic. A number of other preformed granule enzymes have been isolated from eosinophils but a clear functional role for these has yet to be established (Spry, 1988). These include: acid phosphatase (large amounts located in the small granules), lysophospholipase, alkaline phosphatase (in rats), collagenase, arylsulphatase B, histaminase, phospholipase D, catalase and non-specific esterases.

The primary lipid mediators of inflammation produced by eosinophils are eicosanoids and platelet activating factor (PAF) (Weller, 1993). Arachidonic acid metabolism in mammalian eosinophils has been shown to act primarily through the lipoxygenase pathway with 15-hydroxyeicosatetraenoic acid as a major product (Turk et al., 1983). Additionally, the late phase sulfidopeptide leukotrienes of anaphylaxis, LTC_4 and LTD_4 are produced with relatively large amounts of LTC_4 (up to 70 ng/10^6 cells) being a unique eosinophil capability (Weller et al., 1983). In fact the levels of LTC_4 generated by eosinophils in many experimental models far and away exceeds other inflammatory cell populations' capacity to produce this

sulfidopeptide leukotriene (personal communication, Dr. F. Sun). Interestingly, evidence suggests that LTC_4 concentrations are lowered in the presence of EPO as a possible self-regulatory scheme by eosinophils in hypersensitivity reactions (Goetzl, 1982). PAF also can elicit LTC_4 from eosinophils (Bruynzeel et al., 1986) which indicates another autocrine mechanism since eosinophils are also known to produce PAF. However, when eosinophils produce PAF more than 90% of it remains cell-associated (Cromwell et al., 1990).

The investigations on stimulus-response coupling in eosinophil function began with *in vitro* models of helminth parasite killing. The finding that the eosinophil leukocyte could kill the schistosomula of *Schistosoma mansoni in vitro* provided the first bioassay for eosinophil-mediated cytotoxicity (Butterworth et al., 1974). The mechanism was shown to be a multi-step process of reversible adherence to the complement and antibody opsonized schistosomula followed by the eosinophils actively degranulating, at which time, binding became irreversible and killing ensued. This mechanism was confirmed for rat and human eosinophils (Mackenzie et al., 1977; McLaren et al., 1977; Ramalho-Pinto et al., 1978). Additionally, models including trematodes, cestodes and other nematodes also demonstrated eosinophil-mediated killing mechanisms (Butterworth & Thorne, 1993). The released granule contents may serve as ligand or "glue" in this last stage (Butterworth et al., 1979) as well as their known cytotoxic activities. The opsonizing antibody was shown to be IgG and, therefore, could be inhibited by

protein A which binds the Fc portion of this antibody class (Mackenzie et al., 1977). Additionally, complement or antibody were shown to be equally effective in eliciting eosinophil adherence and degranulation but neutrophils and mast cells required antibody for adherence (Ottesen et al., 1977; Mackenzie et al., 1981). Some variations in eosinophil adherence were observed and ascribed to immunologic variation in the nematode surfaces that are either stage- or species-specific (Mackenzie et al., 1978). The *in vivo* and *in vitro* correlates of these mechanisms of eosinophil adherence were described in the killing of microfilariae in human onchocerciasis (Mackenzie et al., 1985; Williams et al., 1987).

Eosinophil binding and degranulation on antibody opsonized targets is known to be regulated by the FcγRII, which is the only constitutively expressed IgG receptor on eosinophils. This receptor regulates many other eosinophil functions including phagocytosis and the production and release of LTC_4 and PAF (Wardlaw, 1994). Recent evidence has demonstrated the upregulation of the high-affinity FcεRI on human eosinophils *in vivo* and the involvement of this receptor in eosinophil-mediated cytotoxicity *in vitro* (Gounni et al., 1994a). Prior *in vitro* evidence has linked the low affinity FcεRII to some eosinophil effector functions in rats and humans via binding assays (Gleich et al., 1992). The release of eosinophil granule proteins has been shown to be differentially controlled depending on the stimulus of a given immunoglobulin receptor class. In one study, IgG complexes

caused release of ECP but not EPO but IgE complexes induced the opposite secretory profile (Khalife et al., 1986).

The aforementioned studies clearly tie complement receptors on eosinophils to cytotoxic effector functions. A strong synergism was noted when both antibody and complement were added to the *in vitro* cytotoxicity assays (Ottesen et al., 1977; Mackenzie et al., 1981). The binding of complement receptors CR1 and CR3 has been identified as potent stimulus for eosinophil degranulation *in vitro* (Spry, 1988). These receptors are differentially up-regulated by histamine, FMLP and LTB_4 (CR1) (Anwar & Kay, 1977; Kay et al., 1979; Nagy et al., 1982) and IL-5 (CR3) (Vadas et al., 1986). Another study has linked the CR3 receptor to the respiratory burst in eosinophils stimulated with serum-opsonized-zymosan (SOZ). This phenomenon was further enhanced by PAF exposure (Koenderman et al., 1990). Possible roles for C5a and C1q have been suggested by *in vitro* investigations but definitive studies remain to be done.

Several cytokines have been shown to produce marked effects on eosinophil function (Walsh et al., 1993). These include short-term effects where response is immediate and long-term effects where protein synthesis is required such as in increased receptor expression. TNF was shown to increase eosinophil cytotoxicity to helminth and mammalian cells (Silberstein & David, 1986; Sluggard et al., 1990). IL-3, IL-5 and GM-CSF can affect mature eosinophil function in regard to enhanced cytotoxicity, prolonged survival, enhanced adhesion to vascular

endothelium and delayed apoptosis (Silberstein et al., 1986; Owen, Jr. et al., 1987; Rothenberg et al., 1987; Rothenberg et al., 1988; Tai et al., 1991; Lopez et al., 1996). Eosinophils stimulated with GM-CSF and IL-3 synthesized and expressed CD4 protein which allowed entry of the human immunodeficiency virus (Lucey et al., 1989). Recent work by Weller and colleagues (1993) has produced evidence for the role of GM-CSF stimulated eosinophils in HLA-DR dependent, MHC-restricted antigen-presentation. The discovery that pediatric dialysis patients presented with peritoneal eosinophilia and the eosinophils obtained expressed HLA-DR proteins (Roberts et al., 1990) led to further *in vitro* study. When eosinophils were co-cultured with fibroblasts and GM-CSF they produced HLA-DR proteins and IL-1α indicating plausible antigen-presentation and T-cell stimulation capability. The significance of these cytokines to eosinophil accumulation and activity has been supported by their detection *in vivo* under various eosinophilic conditions (Gleich et al., 1992).

Early studies on eosinophil chemotaxis produced an enormous list of potential chemotactic agents for eosinophils *in vitro* (Spry, 1988). Further investigations narrowed the list with the most potent chemotactic agents *in vivo* being PAF, LTB_4, C5a and RANTES. Recent evidence indicates that T-cell and platelet-derived RANTES is a long acting eosinophil-specific chemotaxin (Kameyoshi et al., 1992). The production of PAF by mast cells and LTB_4 by neutrophils may be the most relevant sources of these lipid mediators in the early stages of eosinophil-

related inflammation. While LTB_4 is known for potent eosinophil chemotaxis in guinea pigs it is weak in human systems and has not been shown to stimulate degranulation in eosinophils (Wardlaw et al., 1986). The pro-inflammatory capabilities of PAF are beyond the scope of this review, however, it is probably the most biologically relevant chemotactic and secretory stimulus for eosinophils *in vivo* (Giembycz & Barnes, 1993). PAF selectively enhances eosinophil migration over neutrophils *in vitro* (Wardlaw et al., 1986) and produces an eosinophil-rich infiltrate when given intradermally (Henocq & Vargaftig, 1988). Virtually all lipid mediators in the eosinophil repertoire are secreted in response to PAF. Helminth-killing capability is enhanced by a PAF mediated up-regulation of FcεRI and cytophilic IgE binding producing increased degranulation of both specific and small granules of eosinophils (Capron et al., 1984; Capron et al., 1985). In general, functional stimulation of eosinophils has been demonstrated by PAF both with and without degranulation occurring.

Physical Heterogeneity of Eosinophils

In the previous section the functional heterogeneity of eosinophils was demonstrated. The vast majority of these studies examined peripheral blood eosinophils of humans purified by density gradient centrifugation. Various types of discontinuous gradients and cushions were used including bovine serum albumin, Metrizamide and Percoll (Alexander & Spriggs, 1962; Day, 1970; Gleich & Loegering, 1972). These early purification protocols designed to provide

segregated eosinophils for *in vitro* bench investigations led to the observation that peripheral blood eosinophils had a wide range of functional capabilities. In an attempt to find clinical correlates for the peripheral blood eosinophilia associated with helminth infections, comparative studies of healthy and infected donors were run on density-centrifugation gradients. The observation was made that the eosinophilic donors had more prominent centrifugation bands of less-dense eosinophils than the non-eosinophilic donors (Vadas et al., 1979; Prin et al., 1983; Prin et al., 1984). Similar studies in asthma (Fukuda et al., 1985; Kauffman et al., 1987) and tryptophan-induced eosinophil myalgia syndrome (Owen, Jr. et al., 1990) produced the same prominent "hypodense" bands; whereas normal subjects usually had blood eosinophils that are 90% "normodense". These investigations clarified the concept of physical heterogeneity in blood eosinophils and demonstrated at least an increase in population of certain physical phenotypes in eosinophilic disorders.

Unfortunately, attempts were made to class all "hypodense" and "normodense" eosinophils into singular functional categories. This approach has proved to be too simplistic. It became apparent that the various gradient materials produced multiple standards of sedimentation coefficients for defining "hypodense" eosinophils. In fact, these standards proved to be overlapping with "normodense" standards depending on the eosinophilic disorder that was being investigated, the location the cells were harvested from and the gradient that was chosen for the

separation (Owen, 1993). Also, an overlap in functional capacity was demonstrated when "normodense" eosinophils from idiopathic hypereosinophilia syndrome (IHES) patients elicited a three-fold increase in LTC_4 production over healthy donors (Owen et al., 1989). Regardless of these discrepencies, it has been shown that peripheral blood eosinophils of lesser centrifugation density generally have increased capacity when assayed for antibody-dependent helminth cytotoxicity and LTC_4 generation *in vitro* (Owen, Jr. et al., 1987b).

This correlative evidence for physical and functional heterogeneity led researchers to hypothesize that certain pathogenic stimuli gave rise to the "hypodense" phenotype from the 'normodense" which was the the phenotype of the majority of eosinophils in the circulation. Multiple investigations were performed *in vitro* which demonstrated the conversion (Owen, 1993). Acute conversions of eosinophils were accomplished by PAF, F-MLP, LTB_4, calcium ionophore (A23187) and SOZ after 15 minute exposure (Kloprogge et al., 1987; Fukuda & Gleich, 1989). Long-term culture (7 days) -which can only be accomplished in the presence of GM-CSF, IL-3 and IL-5- produced the "hypodense" from the "normodense" phenotype in 90% of the cells (Owen, Jr. et al., 1987a; Rothenberg et al., 1988a; Rothenberg et al., 1989a). These investigations demonstrated *in vitro* the production of physically heterogeneous eosinophil populations by the same cytokines known for inflammatory regulation and specific eosinophilopoiesis *in vivo*.

Evidence that eosinophils obtained from human tissues (bronchial and pleural lavage) were mainly "hypodense," while there was a relative paucity of these cells in the peripheral circulation, provided evidence for the hypothesis that lower density eosinophils may be the majority tissue phenotype (Prin et al., 1984; Prin et al., 1986). This was also confirmed in rat peritoneal lavage where the eosinophil majority in control animals were "hypodense" and these populations were further increased by *T. spiralis* infection (Hamada et al., 1992). Strong *in vitro* evidence supporting this showed that co-culture of a homogeneously dense eosinophil population with 3T3 fibroblasts alone could produce the physical and functional heterogeneity described above (Rothenberg et al., 1989; Owen, Jr. et al., 1991). Further work on describing the morphological heterogeneity of tissue eosinophils in various functional and pathogenic settings *in vivo* would provide much needed information toward delineating the roles that eosinophils play in mammalian immunity.

It is of interest, that neither the *in vivo* nor *in vitro*-derived "hypodense" eosinophils possessed a singular morphologic profile consistent with the lighter densitometric profile. In fact, the *in vitro* conversion described previously (Owen, Jr. et al., 1987a) produced 90% "hypodense" eosinophils but none had the specific morphological granule changes usually seen in "hypodense" cells taken from the blood of hypereosinophilic patients. Therefore, it is not surprising that several morphometric and ultrastructural profiles have been observed in the eosinophils

taken from the less dense bands of centrifugation gradients performed on peripheral blood leukocytes in humans and rats. These included various combinations of nuclear hyper-segmentation, alterations in specific granules, increased cytoplasmic lipid content, increased cytoplasmic vacuolization and cell swelling (Owen, 1993). Increased nuclear segmentation would be consistent with long-lived fully differentiated cells which most likely occurs in eosinophilia (see Immunobiology of Eosinophilia). The increased cytoplasmic lipid stores may serve as a source of arachidonic acid metabolites (Weller & Dvorak, 1985) and would definitely affect buoyant density. Cell swelling would be consistent with the increased metabolic activity demonstrated in "hypodense" eosinophils. A variety of alterations in ultrastructure have been observed in disease states, producing a heterogeneous specific granule appearance within and among eosinophils involved in pathogenesis *in vivo* (Dvorak et al., 1982; Peters et al., 1988; Torpier et al., 1988; Caulfield et al., 1990; Colombel et al., 1992). Important correlations between hypodensity, function and ultrastructure were made utilizing peripheral blood eosinophils from human subjects with IHES (Peters et al., 1988; Caulfield et al., 1990). The relative volume of specific granules within the cytoplasm decreased in eosinophils of lower density. This was due primarily to a decrease in granule size and enhanced by cell swelling as indicated by an increase in measured cell diameter (Caulfield et al., 1990). The specific granule matrix and crystalloid cores showed heterogeneous clearing and loss of electron density. Cellular MBP

content was shown to be 41% of control cells and plasma MBP levels were eight times higher in IHES patients (Peters et al., 1988). Similar ultrastructural evidence for MBP release by eosinophils from the lamina propria of the small intestine have been described in gastroenteritis and adult coeliac disease (Torpier et al., 1988; Colombel et al., 1992). Unfortunately, the morphologic profiles of eosinophils during most eosinophil-related immune responses *in vivo* remain poorly characterized, especially for tissue-dwelling eosinophils.

Immunobiology of Eosinophilia

It is generally accepted that eosinophils are a well differentiated leukocyte population that arises from myeloid progenitor cells in the bone marrow, traverses the vasculature and lymph channels, and comes to reside predominately in submucosal tissues. In a restricted number of disease states, eosinophil production is up-regulated and these cells accumulate in certain tissues, a condition described in the literature as eosinophilia. Some specific criteria of eosinophilia have been established for blood in humans and rats, where total eosinophil counts above 0.65 x 10^9/l are considered eosinophilic (Krause & Boggs, 1987; Wolford et al., 1987). Unfortunately, blood counts cannot account for the differentially marginated pools of eosinophils likely in the vascular compartment under eosinophilic conditions (Spry, 1988). When considering blood counts as a measure of overall eosinophil activity one must keep in mind that the blood to tissue eosinophil ratio is 1/300. The major tissue compartments of eosinophil residence are bone marrow, skin and

the gastrointestinal tract (Rytomaa, 1960). The eosinophilic response in most tissues relies on cell counts from tissue sections which may underestimate eosinophil involvement through sampling error and/or the poor staining characteristics of degranulated eosinophils. Therefore, assessment of eosinophil accumulation in most tissues (blood included) remains comparative and semi-quantitative and requires careful interpretation. In contrast to this, there are models of peritoneal eosinophilia where lavage provides a relatively efficient cell harvest and a simplified quantitative assessment of resident cell populations. These models of peritoneal eosinophil accumulation will be covered later in some detail.

Interest lies in the nature of the conditions which induce eosinophilia, indicating immune system discretion of these particular disease states from other types where eosinophils are not prevalent. Eosinophil production is often strongly up-regulated in these disease states compared to other populations of immune effector cells. These features point to a mechanism of control on eosinophil production and migration that has a unique specificity. In the western world, the primary focus of pharmacological research involving pathologic conditions associated with eosinophilia has been on the pulmonary eosinophilia associated with asthma in a relatively small atopic segment of the human population. It remains evident, however, that the morbidity and mortality of asthma, though not trivial, is vastly overshadowed by the pandemic observed in helminth parasite infections in humans throughout the world. This fact, coupled with the diversity

and availability of experimental animal models of helminth-associated eosinophilia (see Animal Models of Peritoneal Eosinophilia), has produced the majority of the evidence in the literature defining the mechanisms of eosinophil production and accumulation in tissues.

Basten and Beeson (1970) produced the first definitive evidence for the role of thymus-derived lymphocytes in the mechanism of eosinophilia. This comprehensive work showed that immunologically competent T-lymphocytes are active participants in the production of eosinophils in response to *Trichinella spiralis* in the rat (Basten & Beeson, 1970; Basten et al., 1970; Boyer et al., 1971). They demonstrated a highly consistent increased intensity of the vascular eosinophilic response when live muscle-stage larvae were given intravenously as opposed to an oral challenge; this effect also could not be achieved through subcutaneous, intramuscular or intraperitoneal challenge. Additionally, only intact live muscle-stage larvae produced the eosinophilia; larval homogenates were ineffective. Thymus-dependence was clearly shown, as neonatal thymectomy, anti-lymphocytic serum, lymphotoxic irradiation and chronic thoracic duct drainage performed singularly or in combination abolished the eosinophilic response. Adoptive transfer experiments demonstrated lymphocyte memory with reconstitution of irradiated rats with lymphocytes, but not serum, from primary challenged rats, giving a characteristic secondary response in the recipient animals upon challenge with *Trichinella spiralis* for the first time. Similar work with

Trichinella spiralis in mice (Walls et al., 1971), *Ascaris suum* (Nielson et al., 1974), *Ascaris lumbricoides* (Walls, 1976), *Schistosoma mansoni* (Colley, 1972), *Taenia taeniaformis* (Davis & Hammerberg, 1990) and an *in vitro* derived T-cell line stimulated by *Mesocestoides corti* (Lammas et al., 1990) have all supported a thymus-dependent mechanism of eosinophilia.

The combinations of antigen condition and route of exposure in the eosinophilic response has led to some generally accepted understanding, as well as controversy. Work by Walls and Beeson (1972b) demonstrated the need for vascular transit and/or local tissue reactions to intact *T. spiralis* larvae for the response; a life cycle/migration pattern common to almost all helminth parasites. Yet the larval homogenates produced no eosinophilic response by either route, proving the parasite antigen alone is not enough. In contrast, certain non-parasite antigens have been shown to produce an eosinophilia, especially when the antigenic particles were of sufficient size to become lodged in the pulmonary vasculature. The intravenous injection of Sephadex beads (dextran polymers) in rats produced a distinct primary and secondary eosinophilic response whereas inert polystyrene beads of the same size did not (Walls & Beeson, 1972a). Schriber and Zucker-Franklin (1974) used latex beads adsorbed with gamma-globulin intravenously injected which also produced blood eosinophilia. A contradictory finding using the same gamma-globulin adsorbed latex beads, produced pulmonary and blood eosinophilia in athymic (nude) rats (Pritchard & Eady, 1981) when

Nippostrongylus brasiliensis (*Nb*) infection would not (Ogilvie et al., 1980). Nevertheless, in the vast majority of investigations, a central theme of large, insoluble antigenic particles traversing tissue and vascular compartments has emerged from the literature on mechanisms of eosinophilia.

Adoptive transfer experiments conducted with cell-exclusion chambers provided definitive evidence that the lymphocyte-derived mediators of eosinophilia were diffusable and did not require cell-cell contact (Basten & Beeson, 1970; Miller & McGarry, 1976). These data led to the *in vitro* search for eosinophilopoietic cytokines. The earliest eosinophil colony stimulating factor identified was GM-CSF. However, as the name implies it is not specific to eosinophil production alone (Metcalf et al., 1974). Sanderson and colleagues (1985) discovered an eosinophilia-specfic soluble factor in mouse spleen cells isolated from animals infected with *Mesocestoides corti*. This factor was later identified as IL-5 (Kinashi et al., 1986). IL-5 has been shown to induce eosinophilia *in vivo* (Kings et al., 1990) and helminth-induced eosinophilia is attenuated by administration of anti-IL-5 antibodies (Coffman et al., 1990; Sher et al., 1990). Additionally, transgenic animals with constitutive IL-5 production presented with eosinophilia (Yamaguchi et al., 1990) and patients with a clinical presentation of hypereosinophilic syndrome demonstrated measurable levels of IL-5 in serum (Owen et al., 1989). The evidence clearly shows IL-5 as the only currently defined eosinophilia-specific cytokine. Other evidence from *in vitro*

investigations has shown that Multi-CSF, now called Interleukin-3 (IL-3) works in concert with IL-5 to produce eosinophilopoiesis in bone marrow cultures (Clutterbuck & Sanderson, 1990). Endothelial cells were also found to produce GM-CSF and therefore extend eosinophil viability *in vitro* (Rothenberg et al., 1987). Macrophages also are known to produce GM-CSF in host-defense (Adams & Hamilton, 1992). The elicitation of this cytokine by these cell populations and the antigen exposure experiments described earlier provides a strong mechanistic link to eosinophil production *in vivo*.

The definition of a distinctive cytokine profile in eosinophilia has shed some light upon the unique nature of the eosinophil response in a multitude of thymus-dependent immune reactions. The discovery that $CD4^+$ T-helper (Th) lymphocyte clones have cytokine profiles with two main subclassifications and that their regulatory mechanisms are differentially coordinated, sharpened the focus on immunologic specificity in helminth infections. Distinct T-cell clones observed in a murine model of *Schistosoma mansoni* were given the designations Th1, which selectively produced interferon-γ (IFN) and IL-2, and Th2, which selectively produced IL-4 and IL-5 (Mosmann et al., 1986). Some cytokines, such as GM-CSF, IL-3 and TNF, are produced by both Th1 and Th2 subclasses. Other lymphokine profiles have also been observed indicative of additional Th subsets (Firestein et al., 1989). These specific cytokine production profiles by clonal Th subsets help explain some of the diverse immunologic phenomena associated with

eosinophilia. Generally, Th1 cells regulate local inflammatory reactions in delayed-type hypersensitivity reactions and macrophage activation, whereas Th2 cells produce IL-4 and IL-5 which regulate antibody production of the IgE and IgA classes and eosinophilia, respectively (Street & Mosmann, 1991).

Through investigations in a mouse model of *Schistosoma mansoni*, the specificity to antigen by the Th2 response was clearly defined (Grzych et al., 1991). The initial reaction to acute infection by cercariae was the Th1 response; at 8 weeks post-infection, egg-laying commenced and the Th1 response was down-regulated and then the Th2 response predominated (Pearce et al., 1991). Unisex infections (i.e., no eggs laid) did not produce a Th2 response and mice infected with irradiated cercariae (sterile) produced only Th1 responses and remained strongly immune to reinfection, implicating the Th1 response as a primary immune mechanism in murine schistosomiasis. Conversely, in mice infected with *Nb* where an intestinal eosinophilia and expulsion phenomenon are hallmarks, the Th2 subclass predominates (Street et al., 1990). Rapid nematode expulsion has been shown to actually be dependent on the Th2 response in *Trichuris muris* infection where Th2-deficient mouse strains present with no intestinal eosinophilia and slow expulsion (Else & Grencis, 1991).

The preferential production of IgE antibody often accompanies tissue eosinophilia in humans and rats infected with helminth parasites, raising serum levels as much as 25-fold in the IgE class (Ogilvie, 1964; Johansson et al., 1968).

This potentiation of IgE antibody production has been shown in *Nb* infection in the rat to be both antigen-specific and polyclonal (Jarrett & Bazin, 1974). As stated previously, the mechanism is T-cell mediated (Jarrett & Ferguson, 1974) and specifically enhanced by the Th2 subclass. Two mechanisms have been demonstrated for the selective induction of IgE class antibodies. The first involves IgE-binding factors released by T-cells stimulated in *Nb* infection which increase the production by B cells already committed to the IgE antibody class (Ishizaka, 1988). The second involves antibody class-switching promoted by the synergistic effects of IL-4 and IL-5 on plasma cell precursors (Pene et al., 1988). Capron and colleagues (1994b) have demonstrated the presence of high affinity E-class antibody receptors (FcεRI) on human eosinophils, providing impetus for antigen-specific IgE in the arming of resident tissue eosinophils, as well as mast cells in Type I hypersensitivity and cytotoxic immune reactions. Additional studies have showed that FcεRI activation led to the production and secretion of GM-CSF, IL-3, IL-4, and IL-5 by mast cells (Burd et al., 1989; Plaut et al., 1989; Wodnar-Filipowicz et al., 1989). Another study stimulated blood and tissue eosinophils with IgE-antigen complexes and detected ultrastructurally both intracellular and secreted IL-5 (Dubucquoi et al., 1994). These studies clearly implicate IgE in site-specific eosinophilia.

The development of localized tissue eosinophilia denotes a mechanism for cell-specific recruitment by the vasculature in the affected tissue. Cell adhesion in inflammation has been shown to be a multi-step process where leukocytes are first weakly bound to vascular endothelium by selectin receptors to its carbohydrate ligand. This produces a rolling of blood-borne leukocytes at the affected site. Rolling is followed by the leukocyte integrin tightly binding to the immunoglobulin-like ligand on the vascular endothelium and extravasation proceeds. Eosinophils and lymphocytes but not neutrophils are known to constitutively express the integrin VLA-4 which binds to VCAM-1 on endothelial cells (Walsh et al., 1993). IL-4 has been shown to specifically up-regulate VCAM-1 on endothelial cells (Masinovsky et al., 1990). Eosinophil adherence to endothelium is inhibited by antisera to VLA-4 in human systems (Walsh et al., 1991) and lymphocyte migration is blocked by anti-VLA-4 *in vivo* in rats (Issekutz, 1991). The combined emigration of these two types of cells was demonstrated in late-phase cutaneous reactions from atopic human patients where CD4+ lymphocytes and eosinophils were identified (Frew & Kay, 1988). These studies implicate the VLA-4/VCAM-1 adhesion mechanism as specific for eosinophil recruitment in the production of eosinophilia. A second role for VLA-4 in localized eosinophilia involves the cell-matrix interaction of this receptor and fibronectin. Recent studies indicate that VLA-4 mediated binding of fibronectin

prolongs eosinophil survival and promotes the tissue phenotype (Anwar et al., 1993; Anwar et al., 1994).

A complete understanding of the mechanisms underlying the eosinophilic response in tissues is still needed. It is evident that multiple pathways and sources exist for the release of cytokines necessary for the production and/or accumulation of eosinophils. While T-cell control remains as a primary mechanism for the development of eosinophilia, alternative mechanisms have been proposed in the literature. Reference was already given to data supporting non-thymus dependent eosinophilia in nude rats challenged with antibody-coated latex beads. A role for suppressor T-cell function in eosinophilia was implicated in cyclophosphamide-induced eosinophilia in rats (Thomson et al., 1987). Non-antigen driven T-cell involvement in an eosinophilic response to aluminum containing adjuvants (Walls, 1977) and toxin induced eosinophilia from spanish cooking oil are other examples with unknown mechanisms in the literature. In fact, an array of hypereosinophilic syndromes has been described, most with unknown mechanisms (Spry, 1988).

Animal Models of Peritoneal Eosinophilia

Many experimental models of eosinophilia have been discussed in the previous sections. An obvious preponderance of work on peripheral blood eosinophilia exists in the literature. In contrast, the peritoneal cavity of rodents has been shown to be an accumulation site for eosinophils under certain experimental conditions. While the peritoneum offers ease of access to eosinophils under the specific

immunologic conditions of the enteric tissue microenvironment, relatively few studies have exploited this condition. Eosinophil interactions with peritoneal macrophages, lymphocytes, connective tissue mast cells, extracellular matrix, etc. remain poorly characterized. The focus of this section will be the models described in the literature and their mechanistic correlates.

Over 50 years ago, eosinophilia was elicited in the guinea pig by intraperitoneal injections of insoluble antigen extracts of *Ascaris suum* (Campbell, 1942). Various other agents were also shown to elicit an eosinophilic response in the peritoneal cavity in subsequent studies. Single injections of bovine albumin, horse serum, keratin or *Ascaris* extract all produced a two- to three-fold increase in peritoneal eosinophils in mice after 48 hours (Speirs & Dreisbach, 1956). Repeated injections over several weeks of antigenic agents such as horse serum, hemocyanin and human serum albumin, as well as repeated saline lavage was shown to produce eosinophil-rich peritoneal exudates in guinea pigs (Litt, 1960; Gleich & Loegering, 1972). These agents obviously initiated an immune response from resident cell populations of mast cells, macrophages, neutrophils, lymphocytes and eosinophils through various pathways.

G.T. Archer (1973) published an experimental model of relatively rapid peritoneal eosinophilia in Long Evans rats implicating the mast cell in a regulatory role. The observation that hyperplasia and degranulation of this cell accompanied *Ascaris* antigen-induced eosinophilia (Archer & Binet, 1971) led to experiments

on mast cell activation in the peritoneal cavity. The known mast cell granule constituents of heparin and amines were hypothesized to complex *in vivo*. Therefore, protamine-heparin complexes were administered intraperitoneally as an immunologic catalyst to mimic mast cell activity. The result after 24 hours was a two-fold increase in peritoneal eosinophils (15.8×10^6 cells). This was a selective response without neutrophilia or peripheral blood eosinophilia. There was morphological evidence of phagocytosis of the protamine-heparin complexes by macrophages and eosinophils. The eosinophils were heavily degranulated and there was cytochemical and ultrastructural evidence of macrophage uptake of eosinophil granule material. A similar study was done with *Ascaris suum* or *Echinococcus granulosus* phospholipid preparations which produced peritoneal eosinophilia and mast cell hyperplasia. Eosinophils increased 10-fold (23.0×10^6 cells/animal) over levels in control rats 24 hours after injection of the parasite phospholipid alone (Archer et al., 1977). In a continuation of the mast cell hypothesis, the role of histamine in the peritoneal eosinophilic response was examined in the guinea pig model (Pincus, 1978). This work utilized an 8-week treatment with the antibiotic polymyxin B or compound 48-80, known mucosal mast cell degranulating agents, which elicited a strong eosinophilic response when injected into the peritoneum. This response could be blocked by the antihistamine, diphenhydramine, but could not be elicited by exogenous histamine. It is of interest, that the largest average number (52.0×10^6) of eosinophils was obtained

in the polymyxin B treated animals but a standard deviation of ± 32.0 x 10^6 was due to highly inconsistent response between animals. Another study demonstrated a primary antigen sensitization protocol for the production of anaphylactic antibodies (IgG_2 and IgE), which upon secondary peritoneal challenge, released histamine and peptidoleukotrienes from the resident mast cells in rats. Peritoneal eosinophilia peaked at 4.0 x 10^6 cells/ animal 24 hours later (Spicer et al., 1985).

The dependence on T-cells for the production of peritoneal eosinophilia in mice and rats has been shown in different models. Sensitization of mice to alum-precipitated tetanus toxoid with pertussis vaccine and then challenged by peritoneal injection of tetanus toxoid and alum produced a localized eosinophilia (McGarry et al., 1971). Adoptive cell transfer experiments demonstrated T-cell dependency and recipients showed a pronounced eosinophilia independent of antigen-specific antibody production. A similar T-cell dependent murine model elicited a reduced and transient eosinophilic response to alum adjuvants alone (Walls, 1977). Antigen-driven peritoneal eosinophilia in mice was elicited by various pollen extract sensitizations (Speirs & Dreisbach, 1956; Spicer et al., 1986). Antigen challenge in these models would increase peritoneal eosinophils three- to 10-fold and persist for 5 to 10 days. Athymic mice and adoptive transfer experiments were used to establish T-cell control on the eosinophilic response to the peritoneal-dwelling cestode *Mesocestoides corti* (Johnson et al., 1979). The infections are similar in rats and mice, and produce approximately 25.0 x 10^6

eosinophils/animal after 4 to 5 weeks of infection (Barton et al., 1984; Cook et al., 1987). It is important to note that the functional capacities of these cells from infected rats have been shown to be altered, with increased cytotoxic capacity (Chernin & McLaren, 1983) and reduced FcγRc (Cook et al., 1987).

Some investigations have reported how multiple mechanisms can operate to affect the eosinophilic response in the peritoneal cavity. A murine model, utilizing various preparations of *Ascaris suum* without infection, demonstrated comparative results in alternate pathways (Kano et al., 1989). This study administered saline lavage, viable and killed *Ascaris* eggs and *Ascaris* antigen to normal and athymic mice. All four stimuli produced peritoneal eosinophilia in normal mice, with viable *Ascaris* eggs eliciting the greatest response (9.3×10^6 cells/mouse) by the second day. Repeated saline lavage alone produced the same (4.4×10^5 cells/mouse) peritoneal eosinophilia by the 5th week in normal and athymic mice. Viable eggs, but not killed eggs or *Ascaris* antigen, produced localized peritoneal eosinophilia (1.6×10^6 cells/mouse) in the athymic animals. However, this response took 5 weeks to reach peak levels, still clearly demonstrating both T-cell dependent and T-cell independent mechanisms occur with live *Ascaris suum* eggs.

A report on the repeated saline lavage protocol has implicated a role for potassium ions in the eosinophilic response. Adding potassium chloride to the saline lavage prevented the peritoneal eosinophilia (Oliveira et al., 1994). A human correlate to this mechanism was reported in dialysis patients who

developed peritoneal eosinophilia from dialysate with heparin (Santoro et al., 1985). Pediatric dialysis patients have also been reported to produce 1.4 x 10^9 eosinophils/L of dialysate (Roberts et al., 1990). Both of these mechanisms probably involve cell activation through receptor mechanisms and/or ion channels but experimental data currently does not exist. A model of murine *Toxocara canis* produces a partial T-cell dependent and mast cell dependent eosinophilia in the peripheral blood and peritoneal cavity. These conclusions were drawn from experiments with mast cell deficient mice and cyclophosphamide treatment (Sugane & Oshima, 1985; Nawa et al., 1987). A unique model of enhanced peritoneal eosinophilia was devised by Sugane and Oshima (1980) utilizing *Toxocara canis* in mice. Peripheral blood eosinophilia was monitored in the course of the infection and peritoneal challenge with worm extracts was timed with maximal blood levels producing a pronounced peritoneal eosinophilia (1.0 x 10^8 eosinophils/mouse) in 48 hours. No data was given on the morphological appearance or functional status of these cells.

In every case, the aforementioned models of peritoneal eosinophil accumulation rely on inflammatory stimuli which alter the functional status of the resident peritoneal cell populations from the resting state. Capron and colleagues (1984a) have shown that peritoneal-derived cells from *Schistosoma mansoni* infected donors can kill cercariae when both are placed in the skin of naïve recipients whereas resident cells cannot. Regardless, most of these studies have

not provided information on the condition of the cells harvested and those that have showed clear evidence of functional alterations.

The ability to harvest relatively large numbers of resting state tissue eosinophils by lavage has been reported in two rat models: the Am-1(2)/Tor rat and the August rat, both of which have been reported to have spontaneously high levels of peritoneal eosinophils (Mackenzie et al., 1981; Pimenta & De Souza, 1982). Few studies using Am-1(2)/Tor rats have been published. Those available are ultrastructural investigations on the eosinophils, as well as interactions in Leishmaniasis (Pimenta et al., 1980; Pimenta et al., 1987). The August rat has been used extensively at the National Institute for Medical Research in London with emphasis on pathogenesis in *Nb* infection (see ***Nippostrongylus brasiliensis***).

Nippostrongylus brasiliensis

The study of the nematode *Nippostrongylus brasiliensis* in the laboratory rat has provided a large body of information on mammalian immunity to helminth parasites (Ogilvie & Jones, 1971). This model is relatively unique in that rats generate a strong immune response to this nematode parasite which leads to the expulsion of the adult stage and acquired immunity to reinfection (Africa, 1931; Ogilvie, 1965). This rapid expulsion phenomenon has rarely been observed in natural mammalian nematode infections where adult stage senesence or pharmacologic intervention is the usual course. It is important to note that the expulsion phenomenon was only observed in rats given large (> 2,000 L_3/animal)

single-dose primary challenge. If a "trickle" infection of a few larvae/day were given to mimic a more natural exposure, a threshold worm population was established for prolonged periods before being eliminated. Regardless, this animal model provides a useful tool for investigating primary aspects of enteric immunology and the possible pathogenesis of certain intestinal disease states in humans.

Nb cultures are relatively easily maintained in the laboratory with a short life cycle and no intermediate hosts. Humans are not susceptible to infection and the definitive hosts in nature are rats and mice. The infection has three phases in the laboratory rat, involving the skin, the lungs and the intestines (Jarrett et al., 1968). The infective L_3 larvae are injected subcutaneously (2,000-8,000/animal) where they migrate to the blood and lymph channels. The slight majority of the L_3 larvae migrate via the vasculature to the lungs within 24-72 hours where they erupt into the alveolar airways and molt to the L_4 stage. The remaining L_3 larvae are thought to be lost to lymph drainage and/or killed by immune effector cells. The L_4 larvae move through the pulmonary airways (i.e., they are coughed up and swallowed) to the esophagus reaching the proximal jejunem 3-16 days later in naïve August rats and 4-9 days later in secondary infections (Mackenzie & Spry, 1983). In the intestine they molt to the L_5 stage (adult) and begin feeding and egg production (1000-1500/female/day) by the 5th day of residence. Eggs are passed in the feces and hatch in the external environment to produce the infective L_3 larvae thus

repeating the cycle. The adult nematodes do not penetrate the mucosal wall but mechanically dislodge the host villous epithelium after secreting digestive enzymes onto the intestinal surface. The host cells are then sucked into the worm's digestive tract. This extracorporeal digestion by adult *Nb* leads to extensive local tissue destruction to the host. Worm adherence is thought to be aided by reduced host peristalsis caused by large amounts of acetylcholinesterase secreted by the adult nematodes (Sanderson, 1969). Observed host metabolic responses to large infestations include: decreased plasma protein concentrations, catabolism of skeletal muscle and stored fat, hypoglycemia and even death in some cases (Ovington, 1987).

The immunologic events which lead to the expulsion of primary *Nb* infections in the rat have been described in two steps. The first step involves severe antibody-induced damage to the cells lining the worm's alimentary tract which deleteriously affects mobility and fecundity of the adult worms. This step was shown to be irreversible, independent of complement and occurred by day 10 (Jones et al., 1970). The following expulsion step requires immunologically competent hosts and was not observed in T-cell-deprived, irradiated, young or lactating rats (Ogilvie & Love, 1974). Adult worm antigens, with particular emphasis on females, were implicated in the expulsion phase as larval stages would not elicit the response (Ogilvie, 1965). Further work identified these effector cells of expulsion as thymus-dependent, antigen-specific, immunoglobulin-

negative lymphocytes recovered from thoracic duct lymph and/or the peritoneal cavity by day 8 post-infection (Ogilvie et al., 1977).

In conjunction with these events, other aspects of the host's immune defenses have been implicated in *Nb* rejection in primary and subsequent infections. It has been well documented that monocytes, mast cells and eosinophils infiltrate all the tissue sites where *Nb* migration occurs in initial infections and more intensely in immune animals (Taliaferro & Sarles, 1939). Additionally, goblet cell hyperplasia was observed in the intestinal phase of *Nb* infection and could be adoptively transferred by immune thoracic duct lymphocytes (Miller & Nawa, 1979). Protective antibodies of the IgM and $IgG_{1,2}$ class have been detected by day 5 in primary infections and are involved in eosinophil and neutrophil adherence to all *Nb* stages ***in vitro*** (Jones et al., 1970; Mackenzie et al., 1980). The potentiation of both antigen-specific and polyclonal IgE production by *Nb*, as well as its role in arming effector cells has been previously discussed (see Immunobiology of Eosinophilia). The peak IgE titers were detected in the third week after primary *Nb* infection (Ogilvie, 1967). This IgE peak coincides with maximal eosinophil and mast cell infiltration of the proximal jejunum, all occurring after expulsion of the majority of the *Nb* adults (Taliaferro & Sarles, 1939; Wells, 1962; Hogaboam et al., 1991). There is evidence that the infiltration mechanism of mast cells may have both antigen-specific and non-specific components (MacDonald et al., 1980). Mackenzie and Spry (1983) demonstrated homing from the vasculature of

peritoneal-derived radiolabelled eosinophils from both naïve and immune rats to skin, lungs and proximal jejunum, all sites of tissue migration by the various stages of *Nb*.

Several studies have described plausible roles of the various effector cell populations but a complete understanding of the expulsion phenomenon remains elusive. Urquhart and colleagues (1965) proposed a mast cell mediated anaphylaxis in the intestinal mucosa as the primary mechanism for *Nb* expulsion. This was countered by Ogilvie and Jones (1971) citing passive protection in neonates who did not yet possess passive cutaneous anaphylactic capability and also cited the survival of "adapted" threshold populations in "trickle" infections and transfer experiments. Further work in mast cell deficient W/W^v mice demonstrated *Nb* expulsion, although it was delayed (Uber et al., 1980; Crowle & Reed, 1981). Nawa (1994) hypothesized the goblet cell and changes to the terminal sugars of the mucin produced by these cells as specific to *Nb* expulsion. This system required immunologically competent rats or the transfer of antibody "damaged" worms. In addition, the goblet cell hyperplasia and hypertrophy, as well as the "damaged" worm expulsion could be inhibited in naïve rats by dexamethasone indicating host cellular responses as necessary (Ishikawa et al., 1994). While the presence of eosinophil leukocytes has been reported, no investigations describing the levels, the timing nor the specific morphological

aspects of these cells during the intestinal phases of *Nb* infection are in the current literature.

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Chapter 2

PERITONEAL EOSINOPHILIA IN THE INBRED AUGUST RAT

Introduction

The use of inbred strains of rodents which possess distinct phenotypes has proven invaluable in the quest for answers to some of the most basic of research questions regarding biological processes. Inbred animals are homozygous at virtually all genetic loci making them ideal for immunological studies in which preventing rejection or uniformity of response is critical. Additionally, inbred animals have long-term genetic stability making strain characteristics useful for long periods of time without genetic drift (Festing, 1979). Thus, characterization of observed traits in inbred animals has provided powerful models for scientific inquiry.

The August rat sublines were first developed from unknown outbred stock by Curtis and Bullock at Columbia University in 1921. They produced five inbred lines, one of which was obtained by the Chester Beatty Institute, Pollards Woods, England. After 26 brother x sister matings the current August (AUG) inbred strain was distributed for the first time in 1951 (Wright et al., 1993). Several breeding colonies were established in Europe including one at the National Institute for Medical Research, Mill Hill, London.

Some fifteen years ago, C.D. Mackenzie and colleagues reported the isolation of eosinophil leukocytes from the peritoneal cavity of AUG rats and their use as tissue-derived eosinophils in an *in vitro* parasite adherence assay. Further studies both *in vitro* and *in vivo* went on to describe various aspects of eosinophil involvement in helminth parasite immunology utilizing the AUG rat (McLaren et al., 1977; Ogilvie et al., 1977; Mackenzie et al., 1978; Mackenzie et al., 1980; Mackenzie et al., 1981; Mackenzie & Spry, 1983). These studies established a plausible phenotypic expression of peritoneal eosinophil accumulation in the AUG rat. The present investigation will describe and quantify, utilizing morphologic criteria, the accumulation of eosinophils in the peritoneal cavity, blood and jejunal mucosa of AUG rats of both sexes at selected ages.

Methods and Materials

Experimental Design

Age-matched AUG rats of mixed parentage and sex were grouped (n = 6-11) into 2, 4, 6, 8 weeks (wk), and >1 year (yr) of age. Cell isolations were obtained from the peritoneal cavity and peripheral blood, and tissue samples were taken from the proximal jejunum. Eosinophil leukocytes were identified by morpho-histological criteria and quantified in these tissue compartments. Ultrastructural profiles of AUG rat eosinophils isolated from the peritoneal and vascular compartments also were described. A single group of 6-wk-old Sprague-Dawley (SD) rats were used for the comparisons of villus-crypt (VC) units.

Animals

Sibling breeding pairs of August (AUG) rats were obtained from Harlan Olac Limited, Bicester, England and the National Institute for Medical Research, London. Colonies of inbred progeny were established at the Biological Sciences Department, Western Michigan University, Kalamazoo, MI and the Division of Animal Health, The Upjohn Company, Kalamazoo, MI and were the source of research animals for this study. The SD rats were obtained from Harlan Sprague Dawley, Inc. (Indianapolis, IN). All animals were housed in accordance with the Institutional Animal Care and Use Committee (IACUC) of the respective institutions. The colonies were maintained under a 12hr/12hr-light/dark cycle at 21-28°C with rodent chow #5001 (Purina Mills, St. Louis, MO) and reverse osmosis purified water provided *ad libitum*.

Tissue Collections

All chemical reagents were obtained from the SIGMA Chemical Company (St. Louis, MO) unless otherwise noted. Animals were killed prior to tissue collection by lethal carbon dioxide inhalation. Peripheral blood was taken immediately after death from tail snips and smeared between microscope slides and allowed to air dry. Additionally, an aliquot of peripheral blood was drawn into a white cell pipette (Becton-Dickinson, Cockeysville, MD) and diluted with Turk's solution for total white blood cell (WBC) determination. Peritoneal lavage was performed utilizing calcium- and magnesium-free Hanks' Balanced Salt Solution (HBSS)

with 20 mM HEPES buffer at pH 7.3 kept on wet ice. This collection was accomplished by making a small (1-3 cm) incision on the lower abdomen adjacent to the ventral midline of the animal to irrigate the peritoneal cavity with 30-50 ml of the lavage solution. The resident peritoneal cells and the lavage solution were removed within 5 minutes by syringe and pelleted by centrifugation at 200 x g for 10 minutes at 4°C in a Beckman (Fullerton, CA) centrifuge. The lavage supernatant was discarded and the cell pellet brought to 5 ml with HBSS with 20 mM HEPES and held on wet ice. Following the lavage procedure, the thoracic cavity was opened and systemic blood was drawn by cardiac puncture into 7 ml blood collection tubes with 0.07 ml of 15% EDTA (Termo Medical Co., Elkton, MD) and held at room temperature (RT). Next, 8 cm of the proximal jejunum beginning at a point just distal to the duodenal loop adjacent to the pancreatic rests, was ligated into a 2-4 cm compartment. This was injected with cold (wet ice) 10% neutral buffered formalin (NBF). These jejunal segments were excised and placed in NBF for 15 minutes at RT and then the NBF was replaced with fresh fixative solution and the samples stored at RT until paraffin embedment.

Sample Preparation and Counting Methods

All cell counting procedures were performed on a Nikon (Tokyo, Japan) Microphot-FXA® research grade microscope. Air dried peripheral blood smears were fixed for 10 minutes in cold (-20°C) methanol and stained with Diff-Quick®

stain kit (Baxter Healthcare, McGraw Park, IL). Differential determinations were made upon the first 300 WBCs/animal counted (from 2 smears) and expressed as mean percents (%) of the total. The Turk's diluted peripheral blood was counted (2x) according to the method of Brown (1984) in a Neubauer hemocytometer (American Optical, Buffalo, NY) and expressed as mean WBC/μl.

The peritoneal lavage was aliquoted for counting, cytocentrifuge preparation and electron microscopy. Total cell counts were done on a Neubauer hemocytometer as stated above. Cytocentrifuge preparations were carried out with a Cytospin 3® (Shandon Scientific, Cheshire, England) and stained with the Diff-Quick® system and differential cell determinations performed as described for WBCs. The remaining peritoneal cells were again pelleted, the supernatant discarded and the cell pellet immersed in cold (wet ice) 3% glutaraldehyde in 0.1M phosphate buffer at pH 7.3 for 1 hour. The pellet was then washed (2x) in buffer and secondarily fixed in buffered 1% osmium tetroxide (Polysciences, Inc., Warrington, PA) for one hour at RT. The sample was dehydrated in a graded ethanol series and embedded in Polybed 812® (Polysciences, Inc., Warrington, PA) epoxy resin and thin sectioned for transmission electron microscopy (TEM). Sectioning was done on an LKB (Bromma, Sweden) Ultrotome Nova® and sections stained with 5% methanolic uranyl acetate and Reynold's lead citrate (Reynolds, 1963). Microscopy was performed on a Siemens (Berlin, Germany)

101 Elmiskop® TEM. The EDTA treated systemic blood samples were prepared according to the method of Dykstra (1993) for the preparation of buffy coats for TEM. Briefly, the blood samples were centrifuged in Wintrobe tubes and the buffy coats fixed *in situ*. The glass tube was then broken and the fixed buffy coat pellet was pushed out and embedded in agar to retain the cell layering of the pellet during the embedment process for TEM. The remaining preparatory steps for TEM were as described previously. The segments of proximal jejunum were trimmed into 2-4 mm rings and embedded in paraffin. Histological sections were cut (6-10 μm) and stained by Luna's (1968) for eosinophils and Lendrum's (1944) chromotrope 2R methods. Counting of the eosinophils in the VC units of the intestinal mucosa was done according to Miller (1971). Briefly, all cells located between two gland crypts of the villus and in the lamina propria and epithelium of the villus above were counted as a single VC unit. Cells were counted only in those villi that were longitudinally sectioned and a total of 10 VC units/animal counted.

Statistics

All numerical data was expressed as mean ± SEM. Equal variance was tested by Hartley's homogeneity of variance method. Statistical significance was determined by one-way or 2 x 2 factorial analysis of variance (ANOVA) with a

95% confidence limit (p = 0.05). Group-wise comparisons were made utilizing Student-Newman-Kuels *a posteriori* test (Ott, 1988).

Results

Peritoneal Cavity

The resident peritoneal cell populations in adult (>1 yr) AUG rats (Figure 2.1.) consisted of: 51.7 ± 1.2% mononuclear cells (mononuclear phagocytes and lymphocytes), 32.8 ± 1.4% eosinophils, 9.3 ± 1.0% mast cells, and 6.2 ± 0.5% small lymphocytes. It should be noted here that no morphological or ultrastructural evidence for the presence of neutrophils in the AUG rat peritoneal lavage was ever observed. Total cell harvests (Figure 2.2) are 20.9 ± 1.6 x 10^6 (range = 16.2-27.5) cells/animal. Both the total cell harvest and the percent eosinophils (Figure 2.3) increased with age in the AUG rat. The absolute number of peritoneal eosinophils/adult animal averaged 6.9 ± 0.5 x 10^6 (range = 4.7-9.6) (Figure 2.4). The peritoneal eosinophil accumulation in 8-wk and >1 yr age groups were found to be statistically significantly greater compared to the earlier time points, while no difference was seen between 8-wk and >1 yr. Additionally, no statistically significant difference was found between peritoneal eosinophil accumulation in AUG rat males or females.

Figure 2.1. Cytospin preparations (cytoprep) and ultrastructure of the resident cells obtained by peritoneal lavage of the AUG rat. They are: mononuclear cells (M), eosinophils (E), small lymphocytes (SL) and mast cells (MC). Bar = 5.0 μm.

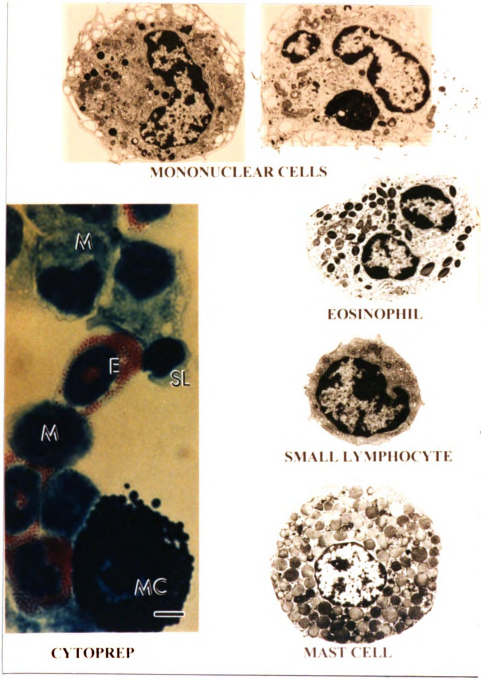

MONONUCLEAR CELLS
M
E
SL
M
MC
CYTOPREP
EOSINOPHIL
SMALL LYMPHOCYTE
MAST CELL

Figure 2.1

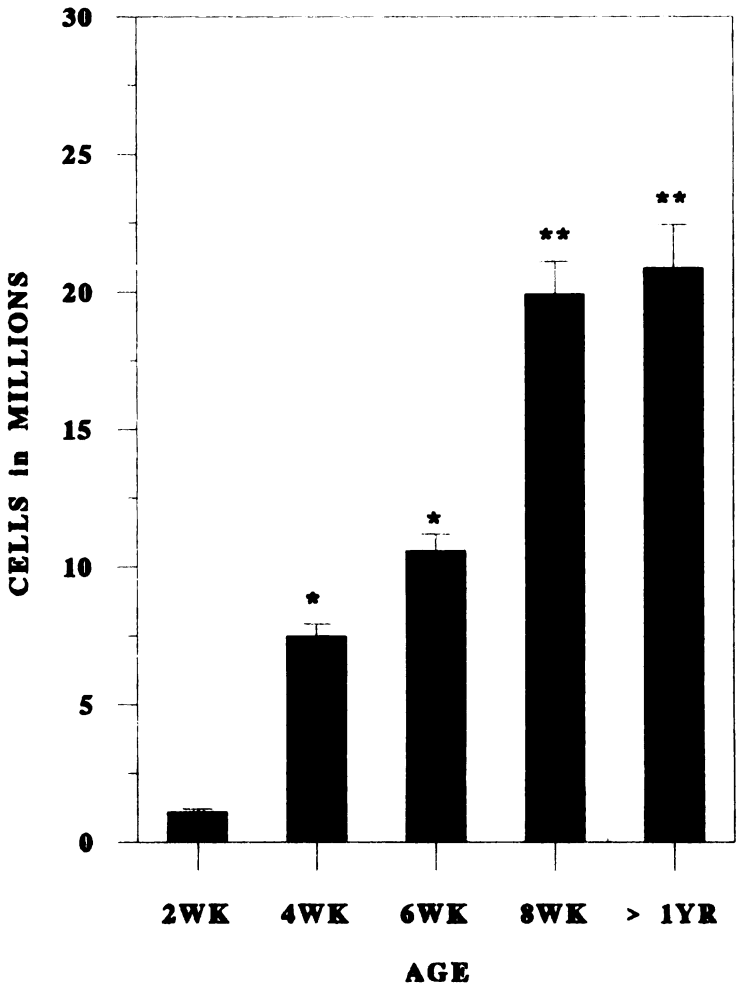


Figure 2.2. Total cell harvest from the peritoneal cavity of AUG rats. The (*) indicates significance over 2wk group and (**) indicates significance over all three younger groups.

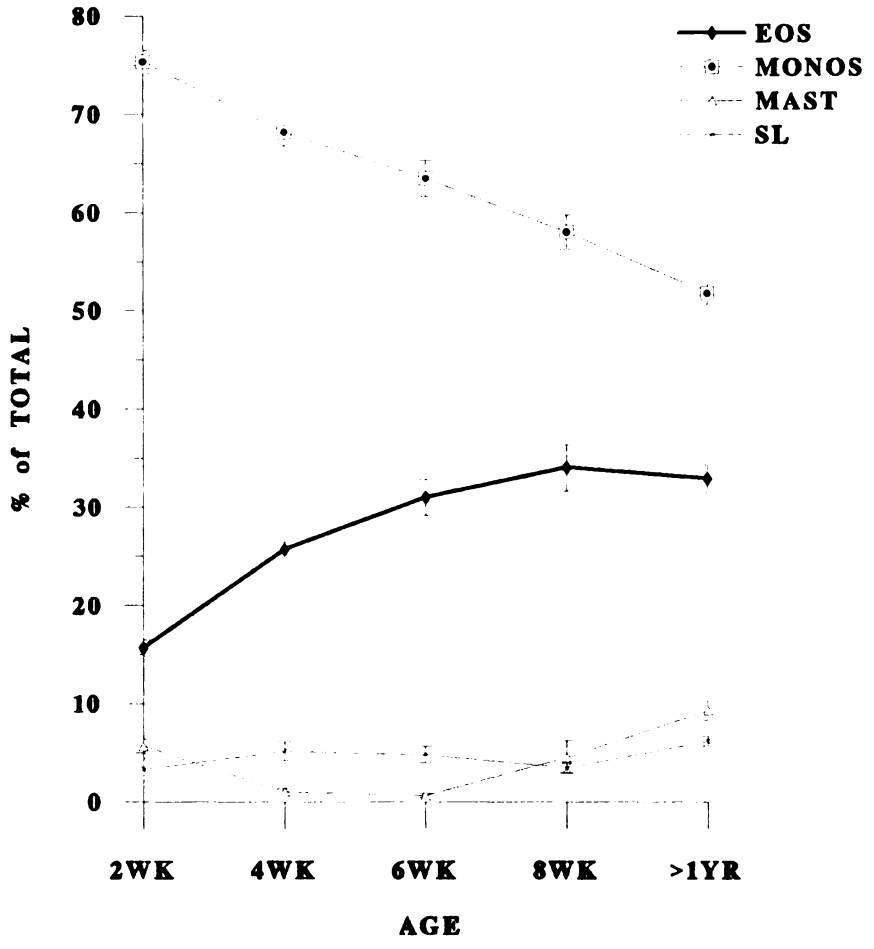


Figure 2.3. Differential count on the cells harvested from the peritoneal cavity of AUG rats.

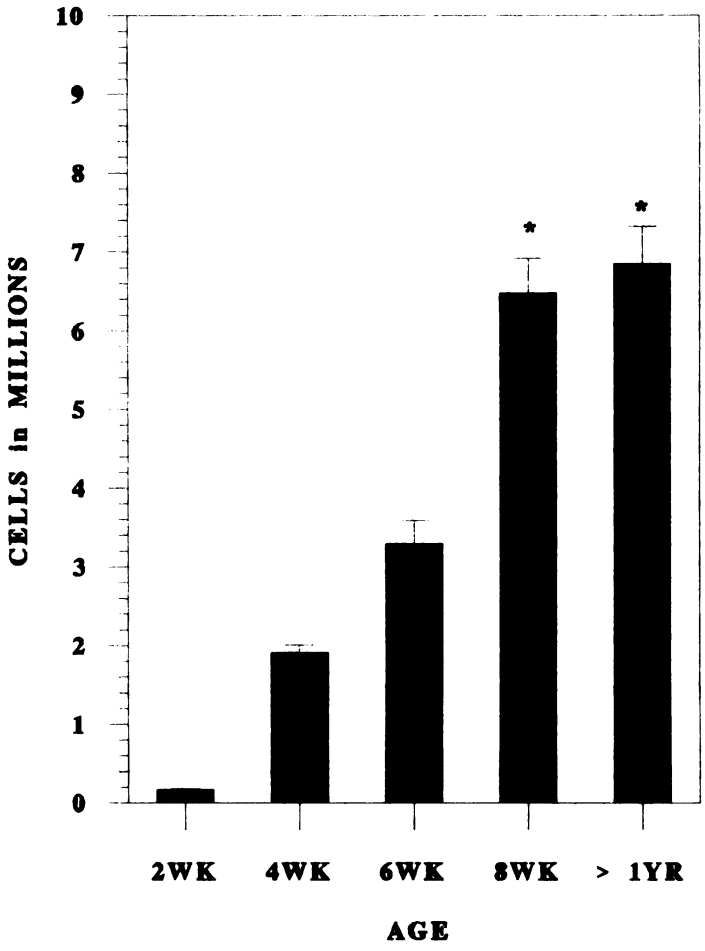


Figure 2.4. Eosinophils in the peritoneal cavity of AUG rats. The (*) indicates significant difference from younger age groups.

Peripheral Blood

Differential cell determinations from AUG rat peripheral blood were: 63.6 ± 1.2% lymphocytes, 22.3 ± 0.9% monocytes, 10.1 ± 0.9% neutrophils, 2.2 ± 0.5 eosinophils, and 1.8 ± 0.6% basophils with no significant differences between groups (Figure 2.5). Total white cell counts were 5052 ± 337 (range = 3295-6500) cells/μl with peripheral blood eosinophils being 113 ± 22 (range = 0-549) eosinophils/μl (Figure 2.6). There was no significant difference in peripheral blood eosinophil numbers between any of the age groups.

Villus-Crypt Units

Light micrographs of VC units from the 2-wk and 8-wk age groups are represented in Figure 2.7. Eosinophil numbers reached 25.9 ± 1.1 (range = 17.2-29.8)/VC unit by 8 weeks of age in the AUG rat (Figure 2.8). A significant difference was observed between the 2-wk and 8-wk old groups. but the three oldest groups (4-wk, 6-wk and 8-wk) were not significantly different. Additionally, the 6-wk old SD rats had VC unit eosinophil counts (17.6 ± 1.6, range = 14.2-21.7) that fell within the same population range as the VC counts for AUG rats at 4, 6 and 8 weeks of age.

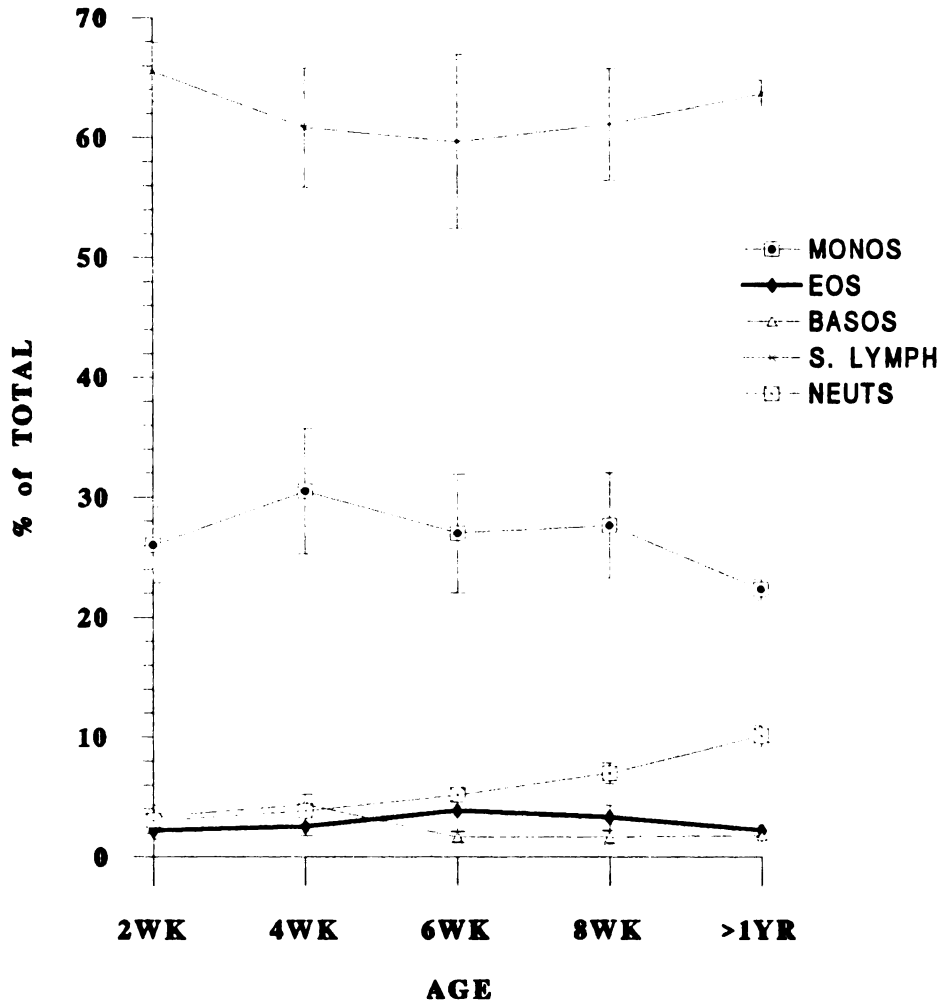


Figure 2.5. Differential white blood cell counts in the AUG rat.

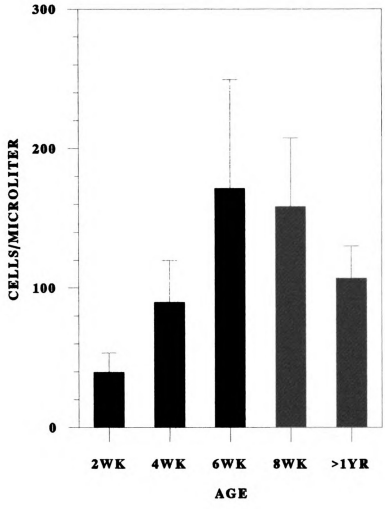


Figure 2.6. Eosinophils in the peripheral blood of AUG rats.

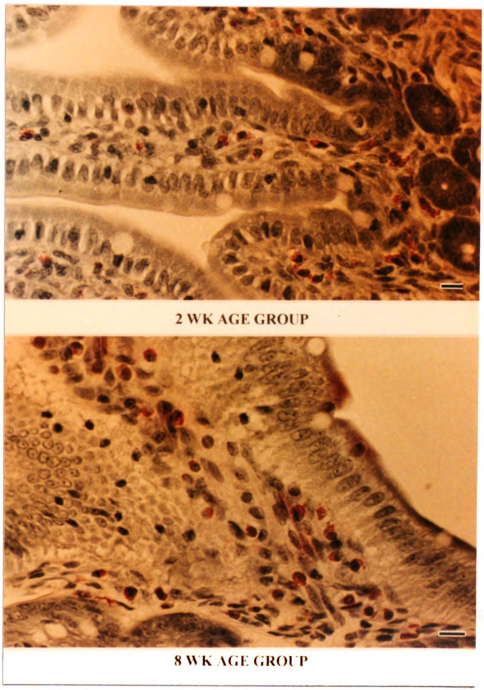


Figure 2.7. Eosinophils in the proximal jejunum of AUG rats (DIC optics). Bar = 20.0 μm.

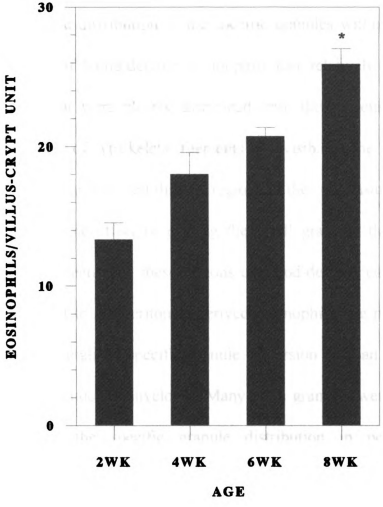
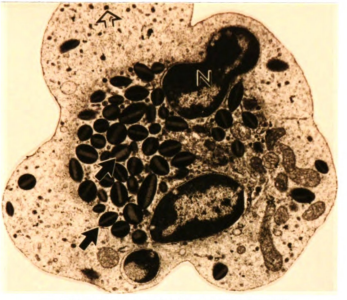


Figure 2.8. Eosinophils in the proximal jejunum of AUG rats. The (*) represents significant difference from 2wk group.

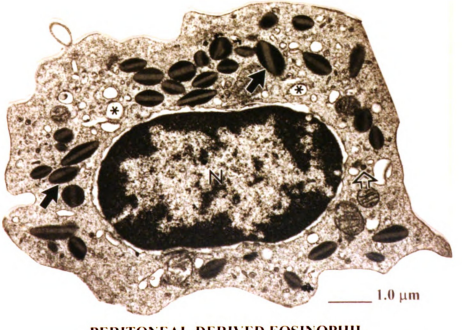
Ultrastructure

The ultrastructural profiles of blood and peritoneal-derived eosinophils in the AUG rat had distinctive qualitative differences (Figure 2.9). The most prominent distinction was in the distribution of the specific granules within the cytoplasm. The clear majority of blood-derived eosinophils had relatively densely packed specific granules that were closely associated with the nucleus. In the most compact states a halo of cytoskeletal filaments was visible at the perimeter of the granule pack. This condition left distinct regions of the cytoplasm free of specific granules which had the effect of making the small granules that are normally present a prominent feature in these regions of blood-derived eosinophils. This was in sharp contrast to the peritoneal-derived eosinophils; the majority of these cells had a more generalized specific granule dispersion with an almost granule-free zone around the nuclear envelope. Many more granules were located at the cell periphery and the specific granule distribution in peritoneal-derived eosinophils had a higher degree of separation throughout the cytoplasm. Small granules were present in the peritoneal-derived eosinophils but their apparent prominence was diminished as these cells had relatively few specific granule-free zones in their cytoplasms. An additional distinctive feature of peritoneal-derived eosinophils was the prominent microvesicular appearance of the smooth endoplasmic reticulum. Virtually all of the peritoneal-derived cells showed distension of the microtrabecular cisternae of the smooth endoplasmic reticulum.

Figure 2.9. The ultrastructural profiles of blood- and peritoneal-derived eosinophils in the AUG rat. A different dispersion profile of specific granules (➡) is evident. Small granules (⇨) are prominent in the cytoplasmic zones unoccupied by specfic granules in the blood-derived eosinophil. Note the vesicular cytoplasm (✶) common to the peritoneal-derived eosinophils.



BLOOD-DERIVED EOSINOPHIL



PERITONEAL-DERIVED EOSINOPHIL

Figure 2.9

This condition was less prominent or not evident in blood-derived eosinophils. Lastly, the plasma membrane of blood-derived eosinophils had a smooth appearance with very few projections. Plasma membrane projections which produced a ruffled cellular profile were common in peritoneal-derived AUG rat eosinophils.

Discussion

This study describes the postnatal development and consistency of an observed strain characteristic of high-level eosinophil accumulation in the peritoneal cavity of the AUG rat. This trait of 7.0×10^6 eosinophils/AUG rat is in sharp contrast to other common laboratory rats. The SD rat and the PVG hooded rat produce 2.2×10^4 and 1.5×10^5 eosinophils/animal, respectively, by peritoneal lavage (Mackenzie et al., unpublished results). The eosinophil numbers in the intestinal mucosa of the AUG rat were comparable to those found in the SD rats and the eosinophil level in the peripheral blood of the AUG rat falls within the range of normative data for all laboratory rats (Wright et al., 1993). Elevations in eosinophil numbers in one or both of these tissue compartments are well documented in rodent helminth infestations (Butterworth & Thorne, 1993; Wright et al., 1993) and other eosinophil-associated diseases (Vadas et al., 1986). The eosinophil levels observed in the blood and intestinal mucosa of the AUG rat being consistent with other rat strains and well below established criteria for eosinophilia

provides strong evidence for phenotypic control of the accumulation of eosinophils in the peritoneal cavity.

The qualitative differences in ultrastructural profiles of the blood-derived and peritoneal-derived eosinophils from the AUG rat are consistent with morphological and functional differences between blood and tissue eosinophil phenotypes reviewed by Owen (1993). While high eosinophil levels in the peripheral blood can suggest the presence of eosinophil-associated disease processes, too many studies in the past have utilized only blood-derived eosinophils for mechanistic studies. Diapedesis through the vascular wall (Walsh et al., 1993) and adhesion to the extracellular matrix of the tissue environment (Silberstein & David, 1986) are most likely to have an important impact on the functional capacities of eosinophils. This study provides further support to the concept of utilizing tissue-derived eosinophils for studies that ask questions about the functional roles of eosinophils.

As stated in the review of eosinophil literature, virtually all other animal models of peritoneal eosinophilia rely on immunologic or inflammatory stimuli to induce the accumulation needed for an eosinophil harvest of sufficient quantity for most scientific investigations. The few studies that have examined the cells derived from these models have described functionally and morphologically altered eosinophils (Chernin & McLaren, 1983; Cook et al., 1987). Additionally, an under-reported contaminant of these peritoneal models is neutrophils, a notoriously difficult cell to separate from eosinophils by centrifugation gradients alone

(Stewart, 1978). Peritoneal isolations from the AUG rat have produced neutrophil-free preparations of >90% resting-state tissue eosinophils by metrizamide gradient (Mackenzie et al., 1981). The potential use of this unique strain characteristic of the AUG rat as a research tool for studies on eosinophil biology is strong.

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Chapter 3

PRELIMINARY INVESTIGATIONS

A: Biebrich Scarlet: An Eosinophil Specific Granule Fluorochrome

Introduction

The need for markers of eosinophil specific granule proteins in rats has provided difficulties for investigators to fully utilize this species in eosinophil-related research. After screening >1000 hybridomas for monoclonal antibodies to AUG rat eosinophils it became apparent that we would not be making any contributions to the bereft pool of antibody reagents for rat leukocytes. The only known clone to rat eosinophil peroxidase (Keeping & Lyttle, 1984) died in storage and could not be regrown in our hands. In an attempt to provide stark contrast for rapid counting of eosinophils in tissues under light microscopy, Luna's stain for eosinophil granules (Luna, 1968) was chosen as a staining method in Chapter 2 of this thesis. Luna's protocol utilizes Biebrich scarlet (BS), a chromophore with high specificity to basic proteins (Spicer & Lillie, 1961). This water soluble, deep red, anionic dye is synthesized by coupling diazotized 4-amino-1, 1'-azobenzene-3, 4'-disulfonic acid to 2-naphthol (Figure 3A.1) and is also known as acid red 66.

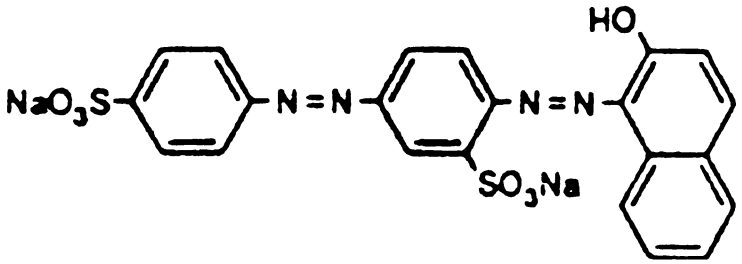

Figure 3A.1. The chemical structure of Biebrich scarlet.

It was evident upon examination of the known structure which showed 4 aromatic hydrocarbon ring structures linked by double-bond (azo) nitrogens that there was a strong capacity for electron resonance in this molecule. Available spectral absorbance data on this compound showed a broad (450 nm-575 nm) peak with a maxima at 505 nm. These data support a hypothesis that this compound might possess fluorescent capabilities.

The advantages of fluorescent probes are many. Analysis of microscopy specimens by fluorescent markers is not limited to the interference properties of transmitted light. Fluorecent markers provide point sources of narrow band emission spectra as opposed to the transmission of full spectrum light through the entire thickness of a sample under bright field microscopy. Tissue often has a high degree of variability with respect to optical properties such as refractive index and absorption. Therefore, fluorescent markers often provide better three-dimensional imaging than bright field chromophores and produce exceptional clarity for labelled objects nearest the objective. Additionally, if a given fluorophore provides

enough quantum yield at wavelengths sufficiently separate from the excitation wavelength then a brighter image on a darker field will result. This improvement of signal-to-noise can provide better resolution when imaging in thick biological specimens and/or tissue sections (Inoue, 1990). Lastly, the use of laser scanning confocal microscopy (LSCM) requires fluorescent markers. The prime virtue of this technology is high resolution optical sectioning of biological specimens. Optical sectioning eliminates the structural artifacts and invasive nature of mechanical sectioning and allows for the visualization of both living and fixed cells. The shallow depth of field (0.1-0.5 μm) of better LSCMs limits the information gathered to a small section of the whole sample. This eliminates the background and scattered fluorescence produced by the rest of the specimen and improves contrast, clarity and detection (Wright et al., 1993). This investigation identifies a new fluorochrome, Biebrich scarlet, and describes some of its properties and applications in epi-fluorescent microscopy and LSCM.

Methods and Materials

Sample Preparation

Deparaffinized histological sections (6-10 μm) of proximal jejunum from the AUG rat infected with *Nippostrongylus brasiliensis* (*Nb*) fixed in 10% neutral buffered formalin, as well as cytocentrifuge preparations of peritoneal lavage, similarly fixed and unfixed, were used. Preparations were rehydrated in distilled

H_2O at room temperature (RT) for 5 minutes. The slides were then stained in an aqueous solution (pH 6.8) of 1% BS (SIGMA Chemical Co., St. Louis, MO) for 10 seconds and cleared in running H_2O for 5 minutes. Slides were dehydrated in 100% ethanol (2x) and washed (2x) in xylene before being coverslipped with Permount® (Fisher Scientific,Fair Lawn, NJ) and allowed to dry overnight.

Fluorescence Microscopy

Microscopy was performed on a Nikon FXA epi-fluorescent research microscope (Nikon, Tokyo, Japan) with a 100-watt mercury arc lamp and a G-2A filter cassette (excitation filter EX510-590, dichroic mirror DM580, barrier filter BA590). Images were recorded on Kodak Ektachrome 50® slide film (Eastman Kodak Co., Rochester, NY) and transferred to Polaroid 59® prints (Polaroid Corp., Cambridge, MA) utilizing a Polaroid Daylab II®.

Laser Scanning Confocal Microscopy (LSCM)

The AUG rat samples were observed on the Ultima-Z-312® (Meridian Instruments Inc., Okemos, MI) LSCM with an excitation line at 514 nm and emission filters of 575 nm (short) and 605 nm (long) and on the InSight Plus® (Meridian Instruments Inc., Okemos, MI) LSCM with an excitation at 488 nm and bandpass filter of 530 nm/30 nm and longpass filter at 605 nm. These images were recorded as TIF image files on floppy discs and converted to dye sublimation prints on a digital color printer (Tektronix, Inc., Wilsonville, OR).

Results

Fluorescence Microscopy

A deep red fluorescent label was observed in eosinophil specific granules, red blood cells and the granules of large granular lymphocytes (Figure 3A.2.) within the tissues examined (fixed and unfixed). These fluorophore-tissue complexes were the source of emission spectra (>605 nm) upon photoexcitation and proved to be photoreactive, producing increased quantum emission for the first 1-10 minutes of continuous photoexcitation. This was observed qualitatively by a halving of microscope-indicated exposure times on successive micrographs from identical fields. All non-specific autofluorescence phenomena associated with these tissues were photobleached in this time frame including areas of background BS binding where photoreactive complexes were not formed (i.e., in areas of connective tissue collagen). The remaining deep red fluorescent emissions were from the various cellular constituents described above in strong contrast to the dark background.

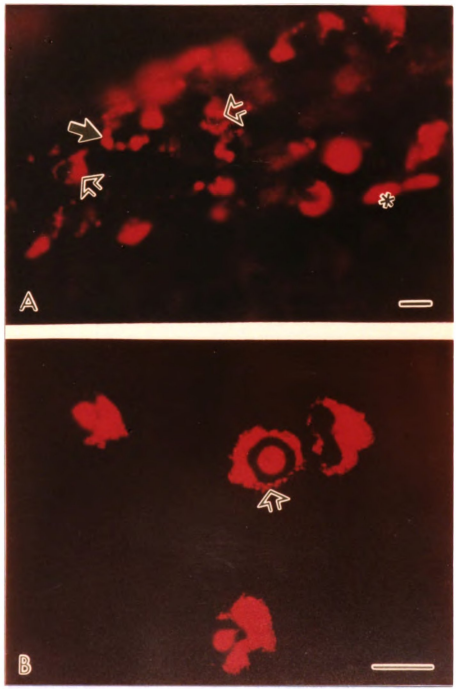

Figure 3A.2. Micrographs of the fluorescent emissions from the BS-protein complexes in NBF-fixed jejunal lamina propria (A) and unfixed cytospin preparations (B). The lablel includes: eosinophil specific granules (➪), cytoplasmic granules of LGL (➡) and red blood cells (✱). Bar = 10.0 μm.

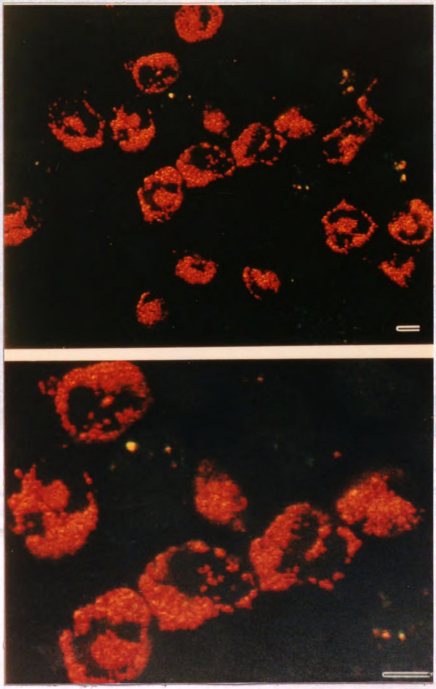

Figure 3A.3. Micrographs of a cytospin preparation showing the specific granules of eosinophils labelled with BS fluorescing under the LSCM. Bar = 5.0 μm.

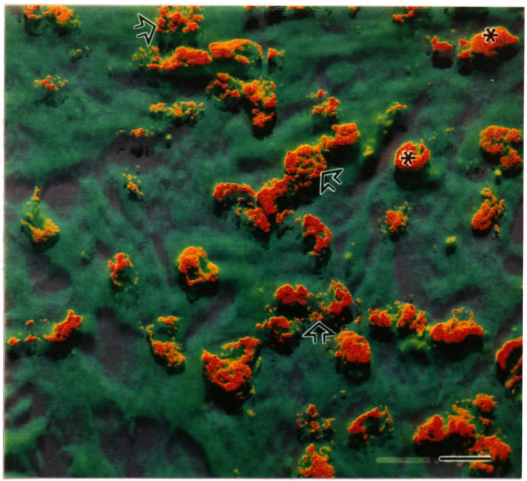

Figure 3A.4. Digital micrograph of a computer-aided 3-dimensional reconstruction of the BS-eosinophil specific granule complex fluorescence (➪) against the remaining tissues of the lamina propria as detected by LSCM. The (✱) are red blood cells. Bar = 10.0 μm.

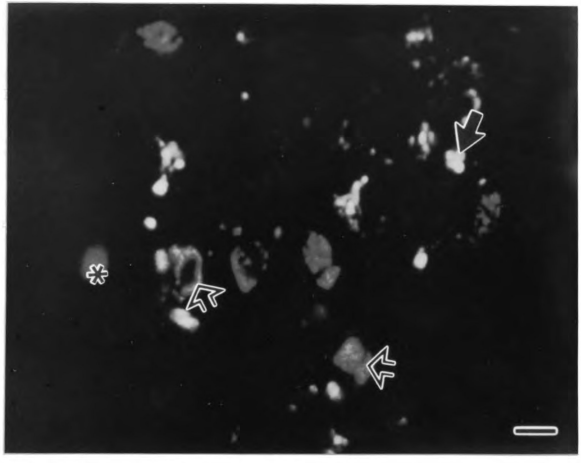

Figure 3A.5. Variation in fluorescence emission by different BS-protein complexes as detected by the LSCM. Eosinophil specific granules (➪) and LGL granules (➡) were differentiated while the red blood cells (✶) emitted at a similar wavelength as the eosiniophil granules. Bar = 10.0 μm.

Laser Scanning Confocal Microscopy

The LSCM provided exceptional resolution of individual eosinophil specific granules (Figure 3A.3) through optical sectioning the BS labelled granules were clearly visualized. Additionally, 3-dimensional reconstructions of successive optical sections resolved specific granule profiles and dispersion patterns previously unobtainable from whole eosinophils in histological sections (Figure 3A.4). The use of narrow excitation lines and emission filtration showed that the emission spectra varied in wavelength with some of the BS complexes (i.e., eosinophil specific granules vs. LGL granules) and were distinctly separable (Figure 3A.5).

Discussion

Several properties are necessary for a dye to be useful in marking cells for fluorescent detection; these include spectral properties, chemical properties and specificity (Stewart, 1978). The spectral properties of the BS complexes described here are exemplary. A high quantum yield was observed at a long wavelength minimizing interference with tissue autofluorescence common at shorter spectra. The emission was photoreactive, increasing over time; this is in sharp contrast to the vast majority of fluorochromes which tend to photobleach under continuous photoexcitation. Finally, a wide separation of excitation and emission maxima of BS, coupled with the photoreactivity, provides a brighter image against a dark field.

Biebrich scarlet has both a known structure and synthesis making it readily available in high purity. Solubility of BS in water is essential for its use in the preparation protocols for both fixed and live cells. The ability to form covalent bonds with cellular constituents prevents dyes from redistributing during tissue preparation and analysis. Biebrich scarlet has two separate sulfonated benzene ring structures. These vinyl sulfone groups bind covalently with proteins and other compounds containing amino or sulfhydryl groups. Spicer and Lillie (1961) have described the compound's affinity for basic proteins. Nevertheless, the difference in photoreactivity observed between BS bound to eosinophil specific granules and BS bound to collagen can not be a simple matter of fluorophore binding concentration. The fluorophore-granule complexes described in this study were clearly photoreactive, increasing their quantum emission with continuous photoexcitation, while background BS staining photobleached. Additionally, the complexes observed in the eosinophils were separable from the macrophages and LGL by virtue of their emission spectra under LSCM. The specific nature of these fluorescent complexes is unknown. A possible scenario may be a complex between the two vinyl sulfone groups of BS and the two amine groups of arginine, known to be in high concentration in eosinophil specific granule proteins (Yokota et al., 1984; Egesten et al., 1986; Pimenta et al., 1987). Of interest is the fact that major basic protein, a primary constituent of eosinophil specific granules, is packaged as an acidic proprotein (pI 6.2) (Barker et al., 1988). However, since

many aspects of the eosinophil specific granule packaging scheme are unknown, this may have no bearing on the availability of amine groups for BS binding. Regardless, the amount of complex formation or the saturation of binding by the BS vinyl sulfone groups may affect emission spectra from the observed cellular constituents. One relevant point is the fact that the fluorescent complexes were formed in both fixed and unfixed eosinophils. This alludes to the stability of the BS binding sites and holds promise as the first hurdle in utilizing BS in living eosinophils.

This investigation has described a new fluorophore for the study of eosinophil specific granules in individual cells and tissues. While investigations remain to fully characterize the nature of BS-tissue complexes, a careful analysis of emission spectra and binding targets may provide new data on the nature of packaging and release of these granule proteins from their cellular compartments. The need for fluorescent markers for LSCM and the properties of BS-eosinophil specific granule complexes described here demonstrate a strong potential for the use of Biebrich scarlet in future eosinophil-related research.

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B: The Eosinophilic Response to Primary Infection with *Nippostrongylus brasiliensis* in the August Rat

Introduction

A basic pattern of worm establishment and expulsion for *Nb* infection in rats was first quantitatively described by Jarrett, Jarrett and Urquhart (1968a). Primary infection was characterized by an initial loss of infecting larvae in the skin and lungs (loss phase 1), with the surviving worms maturing in the proximal jejunum (plateau phase) before the majority were expelled (loss phase 2) with a small residual population remaining (threshold phase). This basic pattern was shown to be generally retained with variation in the length of the plateau phase and rate of loss for phase 2 depending on host immune status, genotype and the number of the infective larvae given (Nawa & Miller, 1979; Rothwell, 1989). Eosinophils have been described in tissue sites around the migrating worms in the rat (Taliaferro & Sarles, 1939; Ogilvie et al., 1977; Mackenzie & Spry, 1983) implicating them as effector cells of immunity in these loss phases during *Nb* infection.

Our interests for this study were in the timing of the tissue eosinophilic response during the intestinal phase of primary *Nb* infection in the AUG rat. Presence of parasites in the small intestine has been reported between day 6-19 for the AUG rat strain during primary *Nb* infection (Mackenzie & Spry, 1983). In addition, the strain-specific characteristic of peritoneal eosinophil accumulation in

the AUG rat was described in Chapter 2, and the known reactivity of the peritoneal compartment to other helminth infections was discussed in Chapter 1. Therefore, the specific aims of this preliminary investigation were two-fold: first, to determine the eosinophilic reactivity of the peritoneal cavity in all phases of primary *Nb* infection; second, to quantitate the trends of accumulation of eosinophils in the small intestine during the same time frame. This would allow determination of the plausible time points of maximal eosinophil involvement in the immune response of the intestinal mucosa to the adult stage of *Nb*.

Methods and Materials

Experimental Design

Experiments were done in two phases. The first phase involved two groups of 6 healthy 4-week-old AUG rats. In one group, rats were injected subcutaneously into the medial aspect of the right hind leg with 3000 *Nb* infective larvae (L_3) at 6 weeks of age. Rats in the other group similarly received sterile saline and served as uninfected controls. Catheter lavage of the peritoneal cavity was performed on each animal at 4, 6, 8, 12, 16, 20, 24 and 28 weeks of age and differential cell counts performed. In the second phase there were two groups of 16 6-week-old AUG rats. One group was infected as described above and the other contained saline controls. In this phase, two animals/group were sacrificed at day 0, 4, 7, 9, 11, 15, 20 and 35 post infection (PI). Peritoneal lavage was performed to determine differential cell counts, as above. Additionally, samples of

proximal jejunum were taken for histological determination of eosinophil infiltration.

Animals

Sibling breeding pairs of August (AUG) rats were obtained from Harlan Olac Limited, Bicester, England and the National Institute for Medical Research, London. Colonies of inbred progeny were established at the Biological Sciences Department, Western Michigan University and the Division of Animal Health, The Upjohn Company, Kalamazoo, MI and were the source of research animals for this study. All animals were housed in accordance with the Institutional Animal Care and Use Committee (IACUC) of the respective institutions. The colonies were maintained under a 12h/12h-light/dark cycle at 21-28 °C with rodent chow #5001 (Purina Mills, St. Louis, MO) and reverse osmosis purified water provided *ad libitum*.

***Nb* Cultures**

The parasites were maintained in mice by methods previously described (Westcott & Todd, 1966). Fecal pellets were obtained on day 8 and 9 PI. These were macerated and mixed with activated charcoal and cultured at 19°C. The L_3 larvae were harvested from cultures between 8-20 days old in a Baermann apparatus filled with water. The concentrated larvae were counted, brought up in sterile saline and adjusted to 3000/ml for injection.

Sample Collection

All chemical reagents were obtained from the SIGMA Chemical Company (St. Louis, MO) unless otherwise noted. Peritoneal washings were performed utilizing a cold (wet ice) lavage solution of calcium- and magnesium-free Hanks' Balanced Salts (HBSS) with 20 mM HEPES buffer and 10 units/ml heparin (The Upjohn Co., Kalamazoo, MI) at pH 7.3. The resident peritoneal cells and the lavage solution were removed within 5 minutes and pelleted by centrifugation at 200 x g for 10 minutes at 4°C in a Beckman (Fullerton, CA) centrifuge. The lavage supernatant was discarded and the cell pellet brought to 5ml with HBSS containing 20mM HEPES and 1mg/ml gelatin then held on wet ice until counting. Separate collection protocols were used in the two phases to obtain resident peritoneal cells.

In the first phase repeat collections on the same animals required catheterization of the peritoneal cavity at each time point. This was accomplished by introducing 10-20 ml of the lavage solution through an 18G catheter needle (Becton-Dickinson, Cockeysville, MD) inserted adjacent to the ventral midline of the abdomen. The needle was withdrawn and after gentle massage the lavage solution recovered through the catheter sleeve into a 50 ml centrifugation tube. The catheter sleeve was then removed and the animal housed until subsequent procedures.

In the second phase the animals were killed prior to tissue collection by lethal CO_2 inhalation. The peritoneal washings were done by making a small (1-3 cm) incision on the lower abdomen adjacent to the ventral midline of the animal to irrigate the peritoneal cavity with 30-50 ml of the lavage solution. Next, the proximal jejunum, beginning at a point approximately 10 cm from the pyloric sphincter, was ligated into a 2-4 cm compartment. This was injected with cold (wet ice) 10% neutral buffered formalin (NBF). The jejunal segments were excised and placed NBF for 15 minutes at RT and then the NBF was replaced with fresh solution and the samples stored at RT until paraffin embedment.

Sample Preparation

All cell counting procedures were performed on a Nikon (Tokyo, Japan) Microphot-FXA® upright microscope. Differential cell determinations were performed on all peritoneal washings. Cytocentrifuge preparations were made on a Cytospin 3® (Shandon Scientific, Cheshire, England) and stained with the Diff-Quick® system (Baxter Healthcare, McGraw Park, IL). Determinations were made on the first 300 cells from these preparations utilizing morphologic parameters previously discussed (see Chapter 2, Sample Preparation and Counting Methods) and expressed as mean percents (%) of the total.

The segments of proximal jejunum were trimmed into 2-4 mm rings and embedded in paraffin. Histological sections were cut (6-10 μm) and stained by

Luna's (1968) method for eosinophil specific granules. The eosinophils in the villus-crypt (VC) units of the intestinal mucosa were counted according to Miller (1971). Briefly, all cells located between two gland crypts of the villus, in the lamina propria and epithelium of the villus above were counted as a single VC unit. Cells were counted only in those villi that were longitudinally sectioned with a total of 10 VC units/animal counted.

Results

The results of the first phase peritoneal catheter lavages are represented in Figure 3B.1. These data are expressed as mean % ± SEM. The *Nb* infected animals (48 ± 2.8%) had higher peritoneal eosinophil differential counts than the uninfected animals (30.7 ± 1.4%) at 8 weeks of age (2 weeks PI). This was followed by a return to similar ranges for infected and uninfected rats at 12 weeks of age (6 weeks PI), and counts remained similar for the two groups at subsequent time points.

The second phase of terminal peritoneal lavages (Figure 3B.2) also profile higher eosinophil differential counts in the infected animals compared to the uninfected animals (48% vs. 37%) at day 15 PI observed in the first phase. However, the difference between the 2 groups continued to be apparent at day 20 PI (51% vs. 37%) before counts were similar for the two groups (28-39%) on day 35 PI.

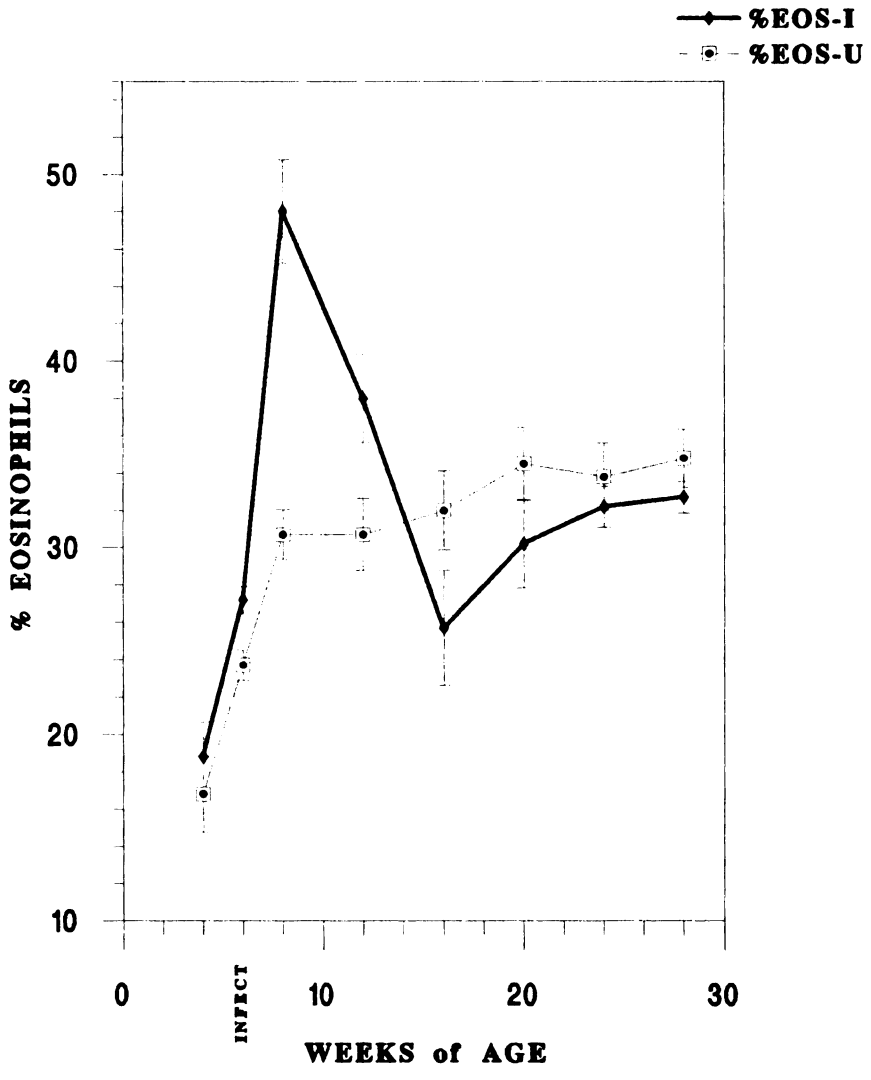

Figure 3B.1. Eosinophil differential counts of catheter lavage obtained from uninfected (U) and *Nb* infected (I) AUG rats (Phase I).

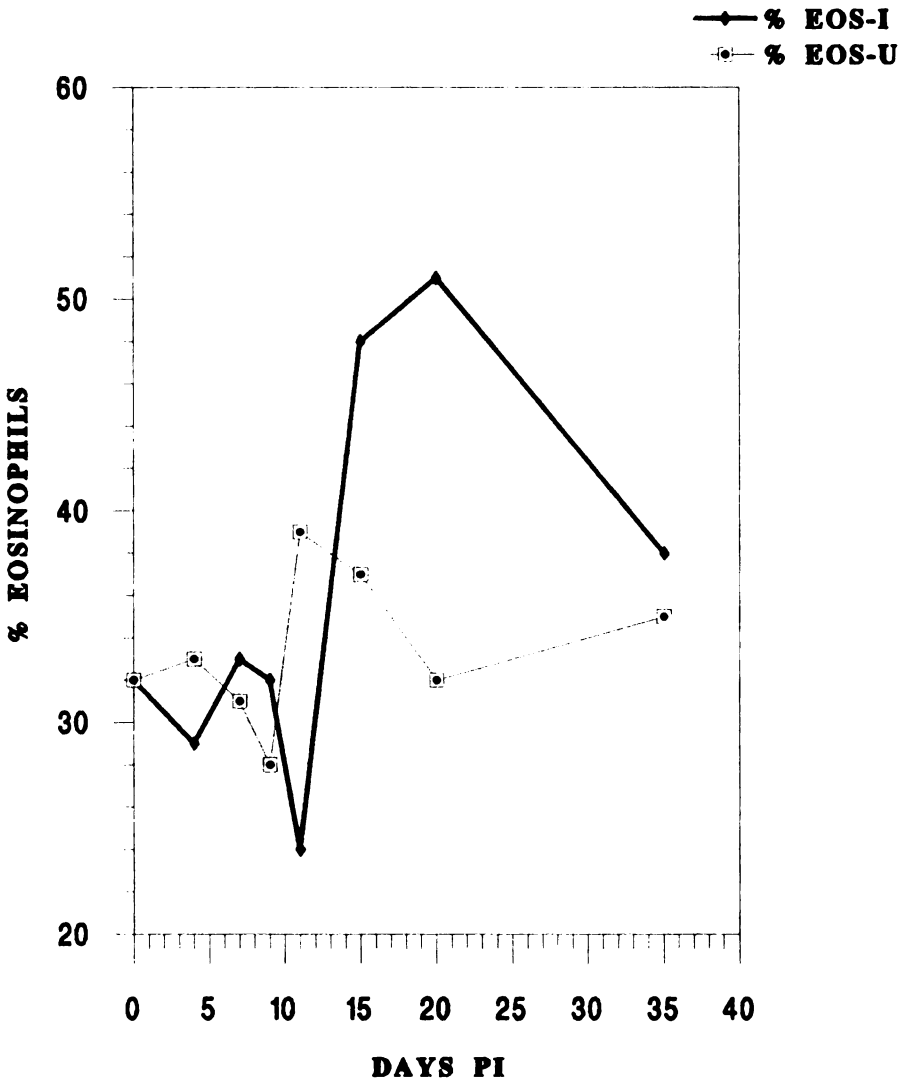

Figure 3B.2. Eosinophil differential counts of terminal lavage obtained from uninfected (U) and *Nb* infected (I) AUG rats (Phase II).

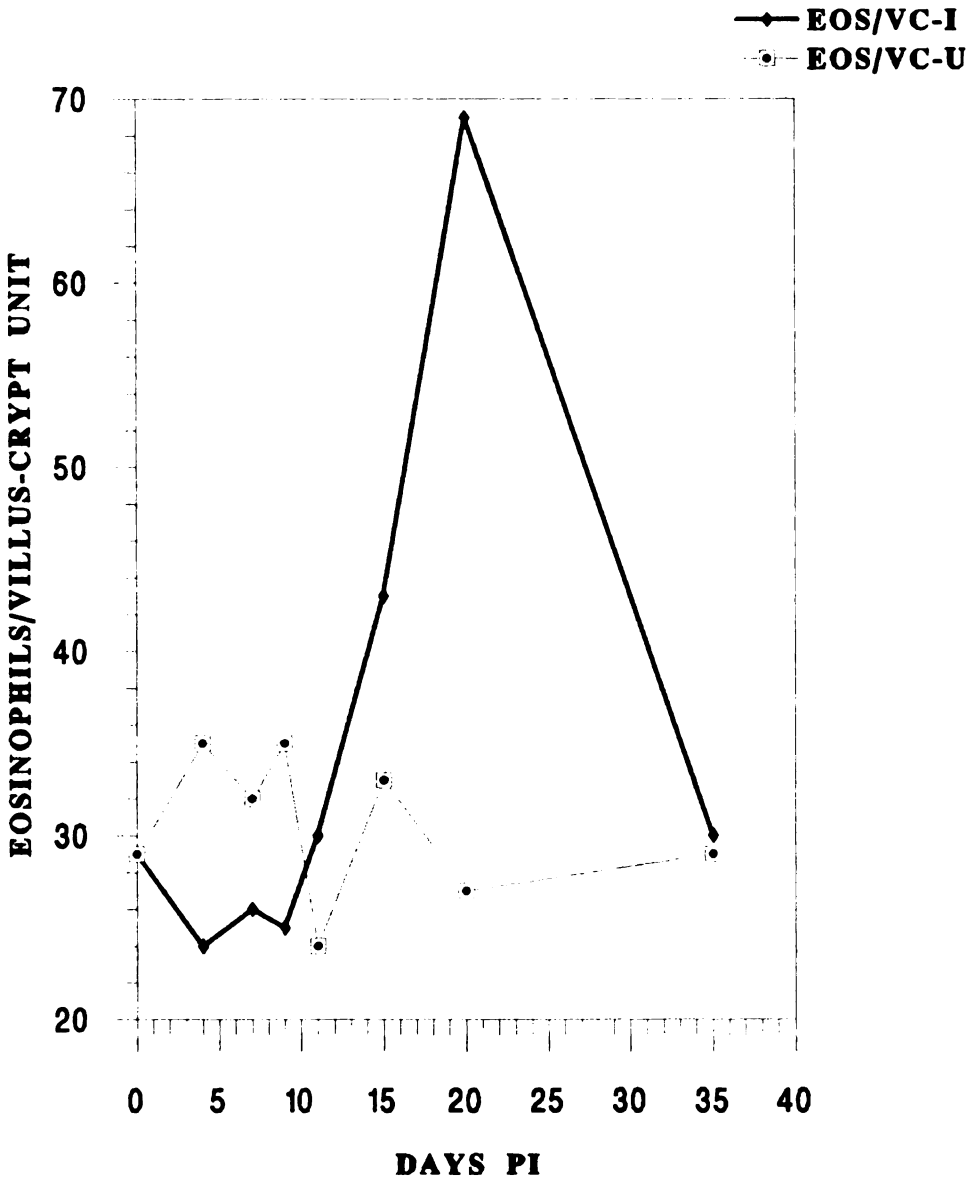

Figure 3B.3. Eosinophils in the proximal jejunum of uninfected (U) and *Nb* infected (I) AUG rats.

The eosinophil numbers determined from the VC unit counts in the segments of proximal jejunum are represented in Figure 3B.3. The eosinophils/VC unit in the infected animals remained in the uninfected range (24-35 cells/VC unit) through day 11 PI. On day 15 PI the infected group reached 43 eosinophils/VC unit and peaked at day 20 PI with 69 eosinophils/VC unit. The infected animals returned to the uninfected range by day 35 PI.

Discussion

These studies suggest a temporal association between the peritoneum and intestinal mucosa in regards to eosinophil accumulation in response to primary infection with *Nb*. This type of association in enteric helminth infections has not been well characterized. Infections with the peritoneal-dwelling cestode *Mesocestoides corti* (Barton et al., 1984; Cook et al., 1987) remain the only reported model of peritoneal eosinophilia by natural infection. Virtually all other studies of this phenomenon involve the direct injection of helminth antigen into the peritoneal cavity without infection (see Chapter 1, Animal Models of Peritoneal Eosinophilia).

With the knowledge from the literature that T-cells regulate eosinophil activity (Basten & Beeson, 1970; Basten et al., 1970; Boyer et al., 1971), it is assumed that the accumulation of eosinophils in the peritoneum observed in 6-week-old AUG rats (see Chapter 2) is an indicator of immunological activity. Previous studies of *Nb* infection in rats younger than 6 weeks of age showed

prolonged infections without rapid expulsion of the adult stage from the intestine (Jarrett et al., 1968b). We therefore chose to infect the animals at 6 weeks of age and take advantage of a responsive but relatively naïve immune system of young animals.

Antigen-specific T-cells recovered after day 8 PI from thoracic duct lymph and/or the peritoneal cavity were shown to confer immunity through expulsion when transferred to naïve hosts (Ogilvie et al., 1977). It is known that lymphocytes, monocytes, mast cells and eosinophils infiltrate the tissue sites where *Nb* migration occurs in initial infections and more intensely in immune animals (Taliaferro & Sarles, 1939). Several studies have described plausible roles for some of the various effector cell populations observed but a complete understanding of the intestinal expulsion of *Nb* remains elusive. Urquhart and colleagues (1965) proposed a mast cell mediated anaphylaxis in the intestinal mucosa as the primary mechanism for *Nb* expulsion. In contrast, work in mast cell deficient W/W^v mice demonstrated *Nb* expulsion, although it was delayed (Uber et al., 1980; Crowle & Reed, 1981). Nawa (1994) showed goblet cell hyperplasia and changes to the terminal sugars of the mucin produced by these cells to also be under T-cell control and specific to expulsion of the adult worms. Ogilvie and Jones (1971) proposed a more general theory that many cells provide a concerted effort in orchestrating the expulsion of *Nb* adults with no cell population singularly

capable. In this context, the current evidence helped define plausible roles for the eosinophil in the immune response to the intestinal phase of *Nb*.

These findings implicate days 14-21 PI as the most relevant for investigating the eosinophil accumulation profiles of the peritoneal cavity and the jejunal submucosa during *Nb* infection in the AUG rat. However, a need for studies including absolute eosinophil numbers in the peritoneal cavity and sufficient experimental group size to ascertain biological relevance was also established. The following studies (Chapters 4 and 5) will concentrate on detailed descriptions of eosinophil involvement in immunity to the establishment of adult phase *Nb*.

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Chapter 4

CHANGES IN EOSINOPHILS IN THE INTESTINAL PHASE OF THE NEMATODE INFECTION *NIPPOSTRONGYLUS BRASILIENSIS*

Introduction

The presence of eosinophil leukocytes in lesions associated with endoparasitic infections of mammalian species has been documented in the literature for nearly 100 years (Brown, 1898). Localized eosinophilic responses have been described in tissues where helminth invasion or migration has occurred. An intense and often fatal eosinophilic leukocytosis of the meninges and cerebrospinal fluid has been reported in *Angiostrongylus cantonensis* infection in both humans (Yii, 1976) and rodents (Hua et al., 1990). Pulmonary eosinophilia was noted in tropical filarial infections (Neva & Otteson, 1978) and during the migratory lung phases of many gastrointestinal nematodes (Boyer et al., 1971; Mackenzie & Spry, 1983; Kayes et al., 1987). As well, the intestinal mucosa has also been described as a site of localized eosinophilia around migrating worms in *Angiostrongylus costaricensis* infection in humans (Loria Cortes & Lobo Sanahuja, 1980), *Haemonchus contortus* infection in sheep (Gorrell et al., 1988) and *Nippostrongylus brasiliensis* infection in rats (Taliaferro & Sarles, 1939).

Distinctive patterns of eosinophilia associated with large amounts of locally released helminth antigens concomitant with host tissue destruction, as well as parasite migration, feeding and/or resulting inflammation were evident from these and other studies (Butterworth & Thorne, 1993). A plausible role for eosinophils as effector cells in parasite damage was suggested by the detailed investigations of eosinophilic responses of the skin in human onchocerciasis. Mackenzie (1980) first described increased eosinophil populations in the proximity of, and adherent to, dead microfilaria in the skin. Further study showed that some heavily infected patients had neither skin lesions nor cellular infiltrates around morphologically viable larvae. This profile of apparently "adapted" responses was in direct contrast to other indivduals who often had low filarial loads in their skin but nevertheless presented with severe skin lesions. The lesions in these reactive hosts were characterized as having many dead and damaged microfilaria surrounded by heavy eosinophil infiltrates (Mackenzie et al., 1985; Mackenzie et al., 1986). These findings support a hypothesis that eosinophils serve as direct effector cells in the immune response to limit helminth infection.

The study of *Nb* infection in the laboratory rat has provided a wealth of information about the mechanisms behind mammalian immunity to helminth parasites (Ogilvie & Jones, 1971). Rats generate a strong immune response to the infection which leads to rapid expulsion of the adult phase from the small intestine and an acquired immunity to reinfection (Africa, 1931; Ogilvie, 1965). Primary

Nb infection has been characterized by an initial loss of the infective stage (L_3) during migration in the skin and lungs. The surviving worms then home to the proximal jejunum where they mature and produce eggs. This is followed by a sharp drop in fecundity and expulsion (Jarrett et al., 1968). The expulsion of adult *Nb* is known to be T-cell mediated with both humoral and cellular components (Jones et al., 1970; Ogilvie & Love, 1974; Ogilvie et al., 1977), but many aspects of the mechanism remain poorly characterized.

The first evidence implicating eosinophils as effector cells in the immune response by rats to *Nb* was published by Taliaferro and Sarles in 1939 . In this work, mast cell, monocyte and eosinophil infiltrates were noted at all sites of *Nb* migration in initial infections and more intensely in immune animals. Other *in vitro* work showed that eosinophils, under certain conditions, would adhere and degranulate on the L_3 larvae and adult *Nb* (McLaren et al., 1977; Mackenzie et al., 1980; Mackenzie et al., 1981). Another investigation demonstrated homing of radiolabeled eosinophils from the vasculature to the small intestine of August (AUG) rats during the intestinal phase of primary *Nb* infection (Mackenzie & Spry, 1983). These studies allude to the probable involvement of eosinophils in the rejection of the adult stage of *Nb*. However, more detailed investigations are needed to provide more definitive morphological evidence of direct eosinophil involvement in the intestinal phase of adult *Nb*.

In the present study we have quantitated the eosinophil infiltrates of the intestinal mucosa at selected time points during the expulsion phase of primary *Nb* infection in rats. Moreover, we have analysed in detail the distinctive changes in the morphological profiles and behavior of eosinophils involved in the inflammatory response to adult stage *Nb* in the small intestine. This necessary detail was accomplished utilizing several morphological assessment technologies.

Methods and Materials

Experimental Design

Six-week-old August (AUG) rats of mixed parentage and sex were placed into 4 experimental groups (n = 6-11). Two of the groups were infected with 3000 *Nb* infective larvae (L_3) injected subcutaneously into the right hind leg. The remaining groups were similarly injected with sterile saline and served as age-matched uninfected controls. Tissues were harvested from one infected and one uninfected group at 2 weeks post infection (PI), and from the remaining 2 groups at 3 weeks PI. Determinations of eosinophil numbers were made from segments of the proximal jejunum and in addition, changes in the morphological, ultrastructural and behavioral profiles of these cells were described.

Nb Cultures

The parasites were maintained in mice by methods previously described (Westcott & Todd, 1966). Fecal pellets were obtained on day 8 and 9 PI. These were macerated and mixed with activated charcoal and cultured at 19°C. The L_3

larvae were harvested from cultures between 8-20 days old in a Baermann apparatus filled with water. The concentrated larvae were counted, brought up in sterile saline and adjusted to 3000/ml for injection.

Animals

Sibling breeding pairs of AUG rats were obtained from Harlan Olac Limited, Bicester, England and the National Institute for Medical Research, London. Colonies of inbred progeny were established at the Biological Sciences Department, Western Michigan University, Kalamazoo, MI and the Division of Animal Health, The Upjohn Company, Kalamazoo, MI and were the source of research animals for this study. All animals were housed in accordance with the Institutional Animal Care and Use Committee (IACUC) of the respective institutions. The colonies were maintained under a 12hr/12hr-light/dark cycle at 21-28°C with rodent chow #5001 (Purina Mills, St. Louis, MO) and reverse osmosis purified water provided *ad libitum*.

Tissue Collections

All chemical reagents were obtained from the SIGMA Chemical Company (St. Louis, MO) unless otherwise noted. Animals were killed prior to tissue collection by lethal carbon dioxide inhalation. Following this, an 8-cm segment of the proximal jejunum, beginning at a point just distal to the duodenal loop adjacent to the pancreatic rests, was ligated into two 2-4 cm compartments. One segment was injected with cold (wet ice) 10% neutral buffered formalin (NBF), excised and

placed in NBF for 15 minutes at RT with the NBF then replaced with fresh fixative solution and the samples stored at RT until paraffin embedment. The other segment was injected with cold 3% glutaraldehyde in 0.1 M phosphate buffer at pH 7.3, excised and placed in cold fixative for 1 hour prior to preparation for transmission electron microscopy (TEM).

Sample Preparation and Counting Methods

The glutaraldehyde-fixed jejunal segments were sliced into 1 x 5 mm strips. The strips were then washed (2x) in buffer and secondarily fixed in buffered 1% osmium tetroxide (Polysciences, Inc., Warrington, PA) for one hour at RT. The samples were dehydrated in a graded ethanol series and embedded in Polybed 812® (Polysciences, Inc., Warrington, PA) epoxy resin and thin sectioned for transmission electron microscopy (TEM). Sectioning was done on an LKB (Bromma, Sweden) Ultrotome Nova® and sections were stained with 5% methanolic uranyl acetate and Reynold's lead citrate (Reynolds, 1963).

The NBF-fixed segments of proximal jejunum were trimmed into 2-4 mm rings and embedded in paraffin. Histological sections were cut (6-10 μm) and stained by Luna's (1968) method for eosinophils. These preparations were used for counting of the eosinophils in the villus-crypt (VC) units of the intestinal mucosa according to the method of Miller (1971). Briefly, all cells located between two gland crypts of the villus and in the lamina propria and epithelium of

the villus above were counted as a single VC unit. Cells were counted only in those villi that were longitudinally sectioned with a total of 10 VC units/animal counted.

Similar histological sections were cut from the NBF-fixed jejunal segments for fluorescence microscopy and deparaffinized in xylene (5 minutes at RT). The sections were rehydrated in distilled H_2O at room temperature (RT) for 5 minutes. The slides were then stained in an aqueous solution (pH 6.8) of 1% Biebrich scarlet (BS) for 10 seconds and cleared in running H_2O for 5 minutes. These preparations were utilized in two ways. The first protocol, in which fluorescence observation of BS-complexes alone was desired, were dehydrated in 100% ethanol (2x) and washed (2x) in xylene before being coverslipped with Permount® (Fisher Scientific, Fair Lawn, NJ) and allowed to dry overnight. The second was secondarily labeled with the nucleic acid-specific fluorophore, Hoechst 33258 (Molecular Probes, Inc., Eugene, OR). These slides were incubated with Hoechst 33258 (1μg/ml) in 0.1 M Tris buffer (ph 7.3) for 1 hour. The preparations were cleared to desired intensity in a graded ethanol series and coverslipped in 100% glycerol.

Microscopy

All cell counting procedures and bright-field photography were performed on a Nikon Microphot-FXA® upright microscope (Nikon, Inc., Tokyo, Japan).

Fluorescence microscopy was also performed on the Nikon-FXA epi-fluorescent research microscope with a 100-watt mercury light source. A G-2A filter cassette (excitation filter EX510-590, dichroic mirror DM580, barrier filter BA590) was utilized for BS. Both the G-2A and the UV-1A filter cassette (excitation filter EX365/10, dichroic mirror DM400, barrier filter BA400) were used for the BS/Hoechst 33258 stained specimens and photographed by dual exposure. Images were recorded on Kodak Ektachrome 50® slide film (Eastman Kodak Co., Rochester, NY) and transferred to Polaroid 59® prints (Polaroid Corp., Cambridge, MA) utilizing a Polaroid Daylab II®.

Laser scanning confocal microscopy (LSCM) was carried out on a Ultima-Z-312® (Meridian Instruments Inc., Okemos, MI) LSCM with an excitation line at 514 nm and emission filters of 575 nm (short) and 605 nm (long). These images were recorded as TIF image files on floppy discs and converted to dye sublimation prints on a digital color printer (Tektronix, Inc., Wilsonville, OR).

Differential interference contrast (DIC)-Nomarski optics were utilized on a Nikon Optiphot-2® microscope.

Ultrastructural analysis was accomplished on a Siemens 101 Elmiskop® TEM (Siemens, Inc., Berlin, Germany).

Statistics

All numerical data was expressed as mean ± SEM. Equal variance was tested by Hartley's homogeneity of variance method. Statistical significance was determined by one-way analysis of variance (ANOVA) with a 95% confidence limit ($p = 0.05$). Group-wise comparisons were made utilizing Student-Newman-Kuels (SNK) *a posteriori* test (Ott, 1988).

Results

Villus-Crypt Unit Counts

Figure 4.1 represents the eosinophil counts for the VC units from each of the experimental groups. The uninfected animals had 25.9 ± 1.1 eosinophils/VC unit (range = 17.2-34.2). Eosinophil counts were significantly greater in the infected group at 2 weeks PI: 39.8 ± 2.2 eosinophils/VC unit (range = 29.5-54.6). Statistical significance over both the uninfected animals and the 2 week PI group was determined for the infected animals at 3 weeks PI; their levels were at 72.9 ± 5.2 eosinophils/VC unit (range = 49.4-87.4).

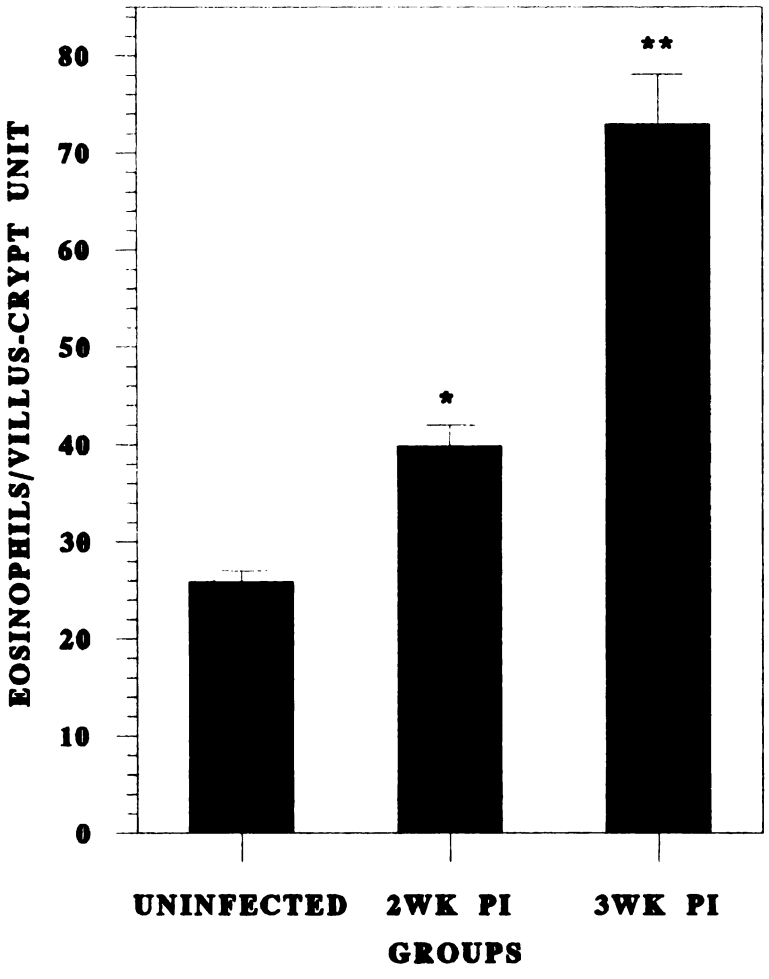

Figure 4.1. Eosinophils in the proximal jejunum of uninfected and *Nb* infected AUG rats. The (*) denotes significant difference from uninfected level and (**) as significant over both uninfected and the 2WK PI groups.

Villus-Crypt Change

The pathology of the jejunum associated with the presence of adult stage *Nb* has been described (Symons, 1957). The changes observed in both infected groups of this study were consistent with the literature. They included: distension and flabbiness of the jejunum; blunted, edematous, hyperemic villi; crypt hyperplasia, with increased numbers of mucosal epithelial cells and goblet cells; increased populations of mast cells, lymphocytes, plasma cells and eosinophils in the lamina propria, as well as the appearance of large granular lymphocytes (LGL).

Eosinophil Morphological Changes

The morphological profiles of eosinophils in the VC units of the infected animals were distinct from those of the uninfected rats. Luna's stained sections of jejunum indicated morphologic changes at both time points post-infection. It was apparent that in addition to the increased numbers of eosinophils there was a marked change in specific granule dispersion within these cells in the infected lamina propria (Figure 4.2).

The granule dispersion was so pronounced in many of these cells that their characteristic orange-red color was difficult to detect under regular bright-field microscopy. This problem was addressed in the epi-fluorescence images of the BS-stained jejunal segments. These fluorescent preparations produced striking contrast in regard to eosinophil specific granule dispersion between the uninfected and the infected VC units (Figure 4.3). The specific granules of the eosinophils in

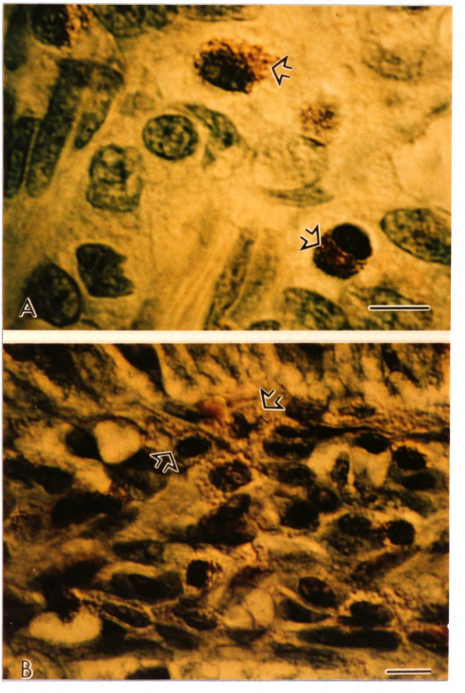

Figure 4.2. Eosinophils (⇨) stained by Luna's method in the lamina propria of villi from the proximal jejunum of uninfected (A) and *Nb* infected (B) AUG rats. Specific granule dispersion in the infected group impedes eosinophil identification (DIC optics). Bar = 10.0 μm.

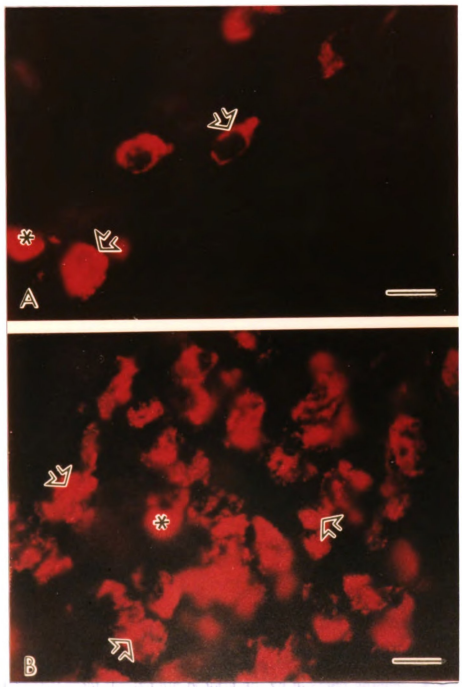

Figure 4.3. Eosinophils (➪) stained with BS in the lamina propria of villi from the proximal jejunum of uninfected (A) and *Nb* infected (B) AUG rats. Even the greatly dispersed specific granules in the infected group are easiliy identified under epi-fluorescence microscopy. Red blood cells (✱) are the only other cells visible in these micrographs. Bar = 10.0 μm.

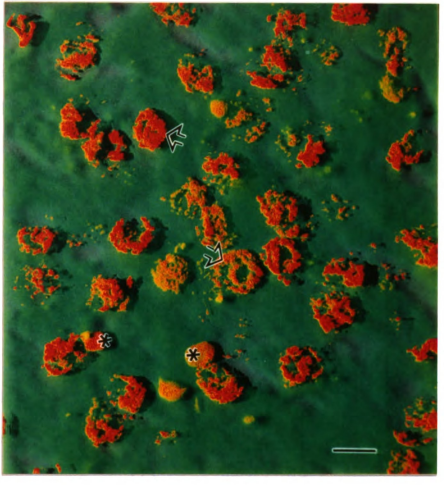

Figure 4.4. Computer reconstruction of optical sections obtained on the LSCM gives a clear 3-dimensional view of specific granule dispersion profiles in eosinophils (↮) from the lamina propria an *Nb* infected animal. ✱ = Red blood cells. Bar = 10.0 μm.

the uninfected intestines remained densely packed around the nucleus and, coupled with lower cell numbers, they occupied very little of the volume of the lamina propria. In contrast, the eosinophils in the infected intestines were more numerous, and their specific granules were greatly dispersed throughout the cytoplasm; thus, the granules occupied a greater proportion of the villus. These specific granule dispersion profiles, while always evident, varied from compact to widely dispersed in the eosinophils that populated the lamina propria of the infected animals. This was best demonstrated by 3-dimensional computer reconstruction of successive optical sections obtained on the LSCM (Figure 4.4).

Other morphologic alterations in the eosinophils from the infected groups included regions of expanded granule-free cytoplasm and changes in nuclear profiles. The DIC optics (Figure 4.5) revealed that the expanded areas of cytoplasm free of specific granules contained vacuoles; these areas were not observed in the eosinophils of the uninfected animals. The Hoechst 33258 fluorophore provided definitive imaging of the nuclear profiles of the cells in the lamina propria and the dual-label with BS allowed eosinophils to be distinguished from the other resident cells (Figure 4.6). Nuclear morphological heterogeneity was a hallmark of the eosinophils observed in the VC units in the infected animals with many more having nuclei that were donut-shaped and segmented rather than rounded and compact as was seen in the eosinophils in the uninfected groups.

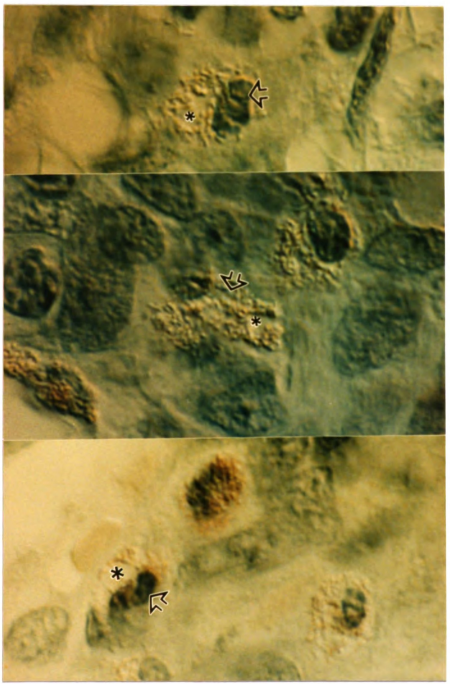

Figure 4.5. Eosinophils (⇦) in the lamina propria of the VC units from the infected animals showed cytoplasmic vacuolization (✱) not seen in cells from the uninfected group. Bar = 10.0 µm.

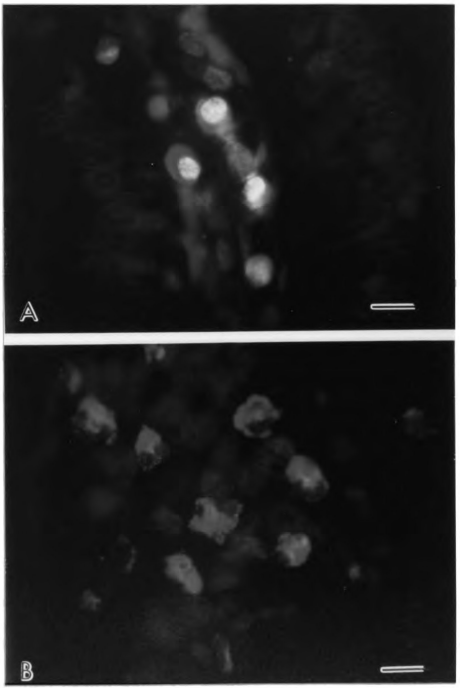

Figure 4.6. Dual fluorescence label provides positive eosinophil (BS-red) identification while showing the difference in nuclear (Hoechst 33258-blue) profiles observed between these cells in the uninfected (A) and infected (B) lamina propria. Bar = 10.0 μm.

Eosinophil Ultrastructural Changes

Ultrastructural analysis of the jejunal segments taken from the experimental animals in this study confirmed the previously described morphological findings (Figure 4.7). Eosinophil specific granule dispersion throughout the cytoplasm was observed in a clear majority of the cells from the infected groups. In contrast, the eosinophils from the uninfected lamina propria showed a relatively dense granule pack in an annular ring around the nucleus. While some ultrastructural heterogeneity in specific granules was observed, no qualitative differences could be attributed to any particular experimental group. Furthermore, no ultrastructural evidence of eosinophil degranulation within the lamina propria was detected and all intact specific granules were located within the confines of plasma membranes. In no instance were eosinophil specific granules found in the extracellular compartment of the tissues examined in this study.

Intraepithelial Eosinophil Migration

Concomitant with the eosinophil changes described in the aforementioned results, came marked distinctions in their distribution within the VC units of the different experimental groups. In the uninfected animals most eosinophils were found centrally located within the lamina propria of the VC units. This same condition held for the infected jejunum at 2 weeks PI although eosinophil numbers

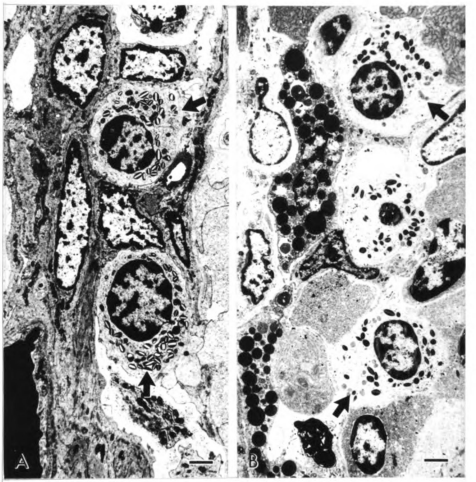

Figure 4.7. Ultrastructure (TEM) of eosinophils (➡) in the lamina propria of the VC units confirmed the morphological findings of increased specific granule dispersion within eosinophils from the infected (B) group when compared to the uninfected (A) tissue. Note that specific granules remain confined within cell plasma membranes in both groups. Bar = 2.0 μm.

had increased significantly with the majority found near the base crypts of the VC units. In addition, some eosinophils (1-4 cells/VC unit) were seen within the epithelial layer near the base crypts at 2 weeks PI, a condition rarely observed (1 cell/10 VC units) in the uninfected jejunum. At 3 weeks PI, the eosinophils appeared adherent to the basement membrane of the mucosal epithelium in large numbers and many more (2 - 10 cells/VC unit) were observed within the epithelial layer (Figure 4.8). These intraepithelial eosinophils were in the basal region between enterocytes and not found above the epithelial cell midline. The eosinophil migration through the basement membrane into the epithelium of the jejunal mucosa was confirmed by TEM in the infected animals at 3 weeks PI (Figure 4.9).

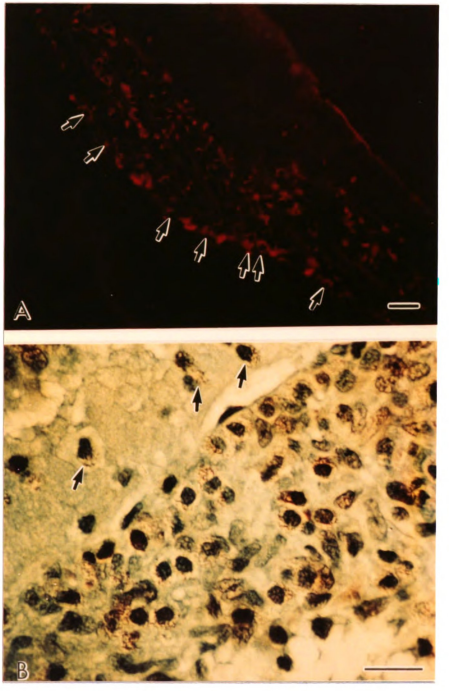

Figure 4.8. Eosinophils (➡) line the basement membrane of the mucosal epithelium by 3 weeks PI (A). In the same time frame many of these cells are found within the epithelial layer (B). Bar = 25.0 μm.

Figure 4.9. (A) Transmission electron micrograph of eosinophil (➡) migration from the lamina propria (lp) through the basement membrane (bm) and into the epithelium (e) of the jejunal mucosa at 3 weeks PI. (B) Lower magnification from the same experimental group showing an eosinophil in the epithelium wedged between enterocytes. Bar = 1.0 μm.

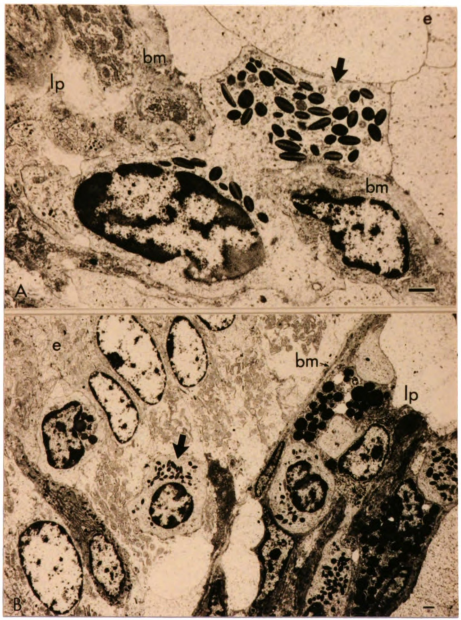

Figure 4.9

Discussion

Several theories have been advanced on the cell-mediated events associated with the expulsion of the adult stage *Nb* from the intestinal tract (Ogilvie & Jones, 1971; Miller & Nawa, 1979; Rothwell, 1989; Nawa et al., 1994). While their presence has been noted, (Taliaferro & Sarles, 1939) evidence for an active role for eosinophil leukocytes in worm damage and expulsion has been limited. In this study, we provide documentation of morphological and behavioral changes consistent with functional activity by eosinophils in the primary immune response of the jejunal mucosa to the adult stage of *Nb*. Earlier studies on mast cell involvement in nematode expulsion have been criticized for not providing evidence of morphological change along with population counts (Rothwell, 1989). Comparisons of infected and uninfected intestines in the current study show not only increased numbers of eosinophils, but also distinct morphological profiles.

The eosinophils in the infected jejunum at 2 and 3 weeks PI had widely dispersed specific granules, often making granule detection difficult. The extent of this granule dispersion was best assessed by the 3-dimensional capabilities of BS fluorescent labeling detected by the LSCM with computer reconstruction of the optical sections. This profile was consistent with the dispersion and rapid motion observed by specific granules within eosinophils when exposed to adult stage *Nb* *in vitro* (Mackenzie et al., 1981). In that study, the rapid motion and specific granule dispersion preceeded adherence and degranulation of the eosinophils on

the surface of the worm, events which required serum (and was greatly enhanced by the addition of complement factors) from immune rats. We also have observed the same specific granule activity in AUG rat peritoneal eosinophils stimulated with calcium ionophore *in vitro* (unpublished data).

The ultrastructural findings of this study clearly place eosinophil specific granules, though often widely dispersed in tissue sections, within the confines of their cell plasma membranes. The eosinophils also showed no ultrastructural evidence of extracellular degranulation within the infected lamina propria. Enteric conditions like adult celiac and Crohn's disease, for which there is direct evidence of eosinophil degranulation, are characterized by erosion of the tissue architecture of the intestinal wall (Dvorak, 1980; Hallgren et al., 1989; Colombel et al., 1992). The resident cells in the lamina propria of the infected rats in this investigation showed none of the expected effects of exposure to cytotoxic levels of eosinophil granule proteins (Gleich et al., 1992; Walsh et al., 1993).

The observed change in eosinophil nuclear profiles and appearance of cytoplasmic vacuoles in the infected lamina propria are consistent with previous findings *in vitro* as morphological indicators of exposure to cytokines. IL-3, IL-4 and IL-5 are known to specifically influence localized eosinophil accumulation and have been detected in mesenteric lymph nodes of *Nb* infected rats (Matsuda et al., 1995). These same cytokines, as well as GM-CSF have also been shown to regulate prolonged eosinophil survival and conversion to the "hypodense"

phenotype *in vitro* (Rothenberg et al., 1987; Rothenberg et al., 1988; Owen, Jr. et al., 1991). Moreover, these "hypodense" eosinophils have also demonstrated increased helminth-killing capability *in vitro* (Owen, Jr. et al., 1987). Nuclear hypersegmentation and cytoplasmic vacuolization observed in eosinophils in this study are accordant with an extended lifespan and the "hypodense" phenotype respectively (Owen, 1993).

Adult stage *Nb* feed on the host mucosal epithelium from days 6-24 PI in primary infections in the AUG rat, in which time they mate and produce eggs (Mackenzie & Spry, 1983). It is well known that worms established in the intestinal mucosa undergo many degenerative changes prior to expulsion. These changes include damage to the cells lining the alimentary tract of the worm, leading to a loss of fecundity and stunted growth. These changes are requisite for expulsion and once complete, even naïve intestines can expel passively transferred damaged worms (Ogilvie & Hockley, 1968). This damage to adult *Nb* is thought to be mediated by both humoral and cellular components of the immune response (Jones et al., 1970; Rothwell, 1989). We show eosinophil morphology in the infected lamina propria consistent with stimulation toward increased anti-helminthic capability, without evidence of cytotoxicity to the host. These observations support eosinophil involvement in potential damage to adult *Nb*, as well as a stepwise paradigm of priming prior to the delivery of their cytotoxic granule proteins to the appropriate targets.

Our demonstration of the intraepithelial migration of eosinophils in response to the presence of adult *Nb* in the jejunal lumen of rats conflicts with a previous report in which only large granular lymphocytes (LGL) were reported to exhibit this behavior (Taliaferro & Sarles, 1939). Most likely, this can be attributed to the sensitivity of the detection methodology. Regardless, this finding has many important connotations. Recent work *in vitro* has shown that eosinophils do not migrate across intestinal epithelium without prior stimulation by GM-CSF (Resnick et al., 1995). As stated previously, the morphologic profile of the eosinophils in the infected lamina propria seen in the present study is consistent with GM-CSF priming. Furthermore, we have demonstrated increased numbers of eosinophils along the basement membrane and in the mucosal epithelium in the successive time points PI. The positioning of these cells is consistent with an active role for eosinophils in the developing gut immunity to primary *Nb* infection where successive worm establishment is sharply curtailed. Finally, the intraepithelial migration of eosinophils observed in this study supports their role in direct cytotoxic damage to adult stage *Nb*, perhaps through their ingestion by these worms.

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Chapter 5

THE PERITONEAL RESPONSE TO *NIPPOSTRONGYLUS BRASILIENSIS* IN THE AUGUST RAT

Introduction

At the turn of the century Ehrlich (1900) suggested that "eosinophils exert their functions in tissues". Subsequent research has led to a general consensus among investigators that eosinophils are primarily tissue-dwelling cells. However, due to the ease of access, the changes in eosinophil numbers in the peripheral blood during disease has often been the singular diagnostic standard. Blood eosinophil counts have been found to be most accurate during steady-state flux between bone marrow and tissues (Spry, 1993). Therefore, this measure cannot account for marginated vascular pools, localized tissue accumulations or functional changes common to eosinophil-associated disease (Butterworth & Thorne, 1993). Clearly, the utility of measuring circulating cell numbers alone as a indication of overall behavior by eosinophils in a given immune response is limited.

The peritoneal cavity is the most extensive serous membrane in mammals with a surface area roughly equal to that of the skin (diZerege & Rodgers, 1992). It is well known that the serous exudate of the peritoneal cavity in man (Roberts et al.,

1990) and rodents (see Chapters 1& 2) contains a mixture of migratory cells with various immunoregulatory capabilities. The cells include: mononuclear phagocytes, lymphocytes, eosinophils, connective tissue mast cells and neutrophils. Specialized folds of the peritoneal membrane form the omenta. Within the omenta, aggregates of these migratory cells called "milky spots or omental glomeruli" have been observed and are considered to be the source of the cells in the serous exudate of the peritoneum (Cranshaw & Leak, 1990). These specialized lymphoid organs of the peritoneum have been shown to be highly reactive to various inflammatory stimuli by demonstrating antigen trapping, cellular proliferation and elicitation of inflammatory mediators (Shimotsuma et al., 1993).

Peritoneal reactivity to *Nippostrongylus brasiliensis* (*Nb*) was demonstrated through direct instillation of infective larvae into the peritoneal cavity of rats. This caused adherence of resident macrophages to the worms and trapping against the omentum which led to death of the immobilized larvae (Greenberg & Wertheim, 1973). The peritoneal immune responses to helminth parasites are not fully characterized. Furthermore, most of the literature follows the approach of direct intraperitoneal antigen exposure (see Chapter 1, Animal models of peritoneal eosinophilia) with little or no data on peritoneal reactivity to the natural course of infection.

In the present study we characterize aspects of the cellular and functional responses of the peritoneal exudate and omental membranes during the intestinal

phase of primary *Nb* infection in AUG rats. This will be accomplished by quantifying changes in cell populations and leukotriene C_4 levels in the serous exudate of the peritoneal cavity. Histological changes in the omental tissues will be described through light microscopy. In addition, the eosinophilic response of the peripheral blood to *Nb* infection will be assessed and compared to the accumulation of eosinophils in the peritoneal cavity at specific time points in relation to the host immune response.

Methods and Materials

Experimental Design

Six-week-old August (AUG) rats of mixed parentage and sex were placed into 4 experimental groups (n = 6-11); two groups were infected with 3000 *Nb* infective larvae (L_3) injected subcutaneously into the right hind leg. The remaining groups were similarly injected with sterile saline and served as age-matched uninfected controls. Sample harvests were performed on one infected and one uninfected group at 2 weeks post infection (PI) followed by the remaining groups at 3 weeks PI.

Reagents

All chemical reagents were obtained from the SIGMA Chemical Company (St. Louis, MO) unless otherwise noted.

Nb Cultures

The parasites were maintained in mice by methods previously described (Westcott & Todd, 1966). Fecal pellets were obtained on day 8 and 9 PI. These were macerated and mixed with activated charcoal and cultured at 19°C. The L_3 larvae were harvested from cultures between 8-20 days old in a Baermann apparatus filled with water. The concentrated larvae were counted, brought up in sterile saline and adjusted to 3000/ml for injection.

Animals

Sibling breeding pairs of August (AUG) rats were obtained from Harlan Olac Limited, Bicester, England and the National Institute for Medical Research, London. Colonies of inbred progeny were established at the Biological Sciences Department, Western Michigan University, Kalamazoo, MI and the Division of Animal Health, The Upjohn Company, Kalamazoo, MI and were the source of research animals for this study. All animals were housed in accordance with the Institutional Animal Care and Use Committee (IACUC) of the respective institutions. The colonies were maintained under a 12hr/12hr-light/dark cycle at 21-28°C with rodent chow #5001 (Purina Mills, St. Louis, MO) and reverse osmosis purified water provided *ad libitum*. Animals were killed prior to tissue collection by lethal carbon dioxide inhalation.

Peripheral Blood Samples

Peripheral blood was taken from the rats immediately after euthanasia from tail snips, and was smeared between microscope slides and allowed to air dry. Additionally, an aliquot of peripheral blood was drawn into a white cell pipette (Becton-Dickinson, Cockeysville,MD) and diluted with Turk's solution for total white blood cell (WBC) determination.

Air dried peripheral blood smears were fixed for 10 minutes in cold (-20°C) methanol and stained with Diff-Quick® stain kit (Baxter Healthcare, McGraw Park, IL). Differential determinations were made upon the first 300 WBCs/animal counted (from 2 smears) and expressed as mean percents (%) of the total. The Turk's diluted peripheral blood was counted (2x) according to the method of Brown (1984) in a Neubauer hemocytometer (American Optical, Buffalo, NY) and expressed as mean WBC/μl. All cell counting procedures were performed on a Nikon (Tokyo, Japan) Microphot-FXA® research grade microscope.

Peritoneal Lavage

Peritoneal lavage was performed utilizing calcium- and magnesium-free Hanks' Balanced Salt Solution (HBSS) with 20 mM HEPES buffer at pH 7.3 kept on wet ice. This collection was accomplished by making a small (1-3 cm) incision on the lower abdomen adjacent to the ventral midline of the animal to irrigate the peritoneal cavity with 30-50 ml of the lavage solution. The resident peritoneal

cells and the lavage solution were removed within 5 minutes by syringe and pelleted by centrifugation at 200 x g for 10 minutes at 4°C in a Beckman (Fullerton, CA) centrifuge. The lavage supernatant was decanted and the cell pellet brought to 5 ml with HBSS with 20 mM HEPES and held on wet ice. Total cell counts were done on a Neubauer hemocytometer as stated above. Cytocentrifuge preparations were carried out with a Cytospin 3® (Shandon Scientific, Cheshire, England) and stained with the Diff-Quick® system and differential cell determinations performed as described for WBCs. The lavage supernatants were diluted (3x) with ice-cold methanol and stored at -70°C until LTC_4 determinations were performed.

Lavage LTC_4 content was determined by an acetylcholinesterase (AChE) enzyme immunoassay kit (Caymen Chemical, Ann Arbor, MI) developed by a previously described method (Pradelles et al., 1985). Prior to determination, 4 ml samples were thawed and centrifuged (1500 x g for 10 minutes at 4°C) to remove precipitates and cell debris. The resulting supernatants were dried in a heated water bath (50°C) under a continuous stream of nitrogen gas. Dried samples were then reconstituted to 1 ml in assay buffer and stored overnight at 4°C before LTC_4 determinations were done in duplicate. Results were interpreted from standard curves, generated with each assay, of percent displacement of bound LTC_4 AChE tracer versus LTC_4 concentration (pg/ml).

Omental Samples

Portions of the greater omentum (gastro-splenic section) were excised and placed in 10% neutral buffered formalin (NBF) for 15 minutes at RT and then the NBF was replaced with fresh fixative and the samples stored at RT until paraffin embedment. After embedment, histological sections were cut (6-10 μm) and stained by Luna's (1968) method for eosinophils.

Statistics

All numerical data is expressed as mean ± SEM. Equal variance was tested by Hartley's homogeneity of variance method. Statistical significance was determined by one-way analysis of variance (ANOVA) with a 95% confidence limit ($p = 0.05$). Groupwise comparisons were made utilizing Student-Newman-Kuels (SNK) *a posteriori* test (Ott, 1988).

Results

Peripheral Blood Counts

Absolute blood eosinophil numbers increased significantly in the infected animals at 2 weeks PI (Figure 5.1). Peripheral blood eosinophil counts in uninfected animals were 158 ± 49 (range = 0-492) cells/μl compared to 827 ± 170 (range = 51-1,746) cells/μl for the 2 weeks PI experimental group. However, by 3 weeks PI, the peripheral blood eosinophil count had returned to uninfected levels, at 197 ± 93 (range = 0-734) cells/μl.

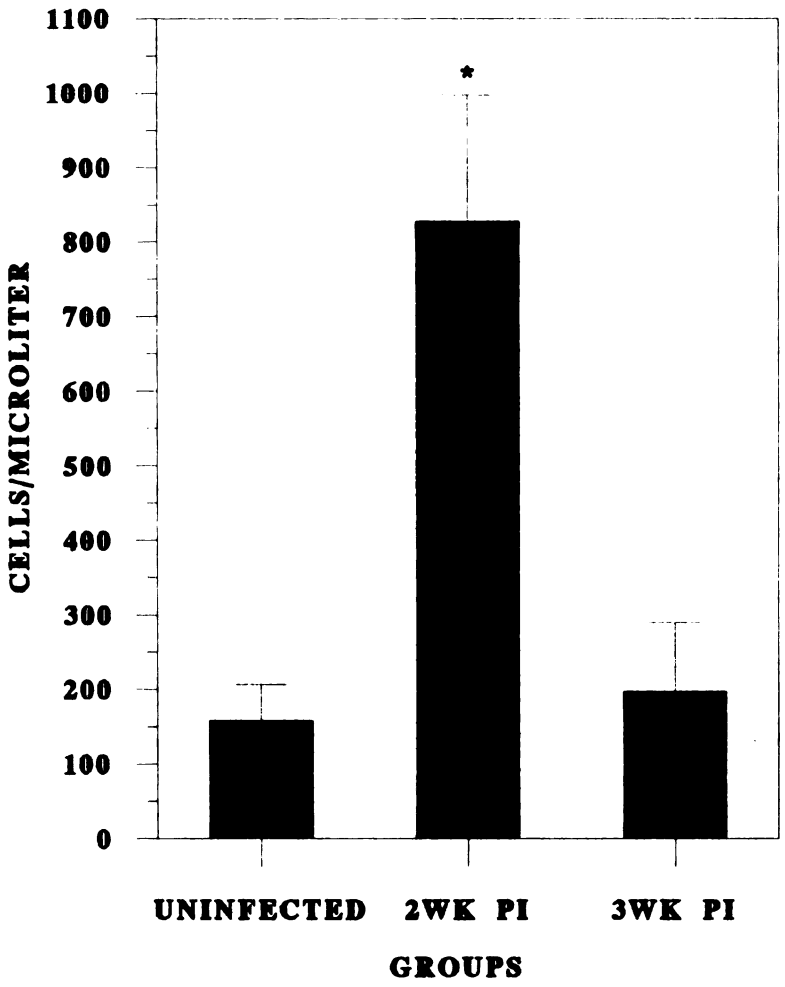

Figure 5.1. Eosinophils in the peripheral blood of *Nb* infected and uninfected AUG rats. A significant (*) increase was observed in the 2wk PI animals but returned to levels observed in the uninfected animals by the 3 wk PI time point.

Peritoneal Eosinophilia

Significant increases in the total cell harvest by peritoneal lavage were observed in both the 2-week and 3-week PI groups compared to the uninfected animals (Figure 5.2). The peritoneal exudate population in the uninfected group was 19.9 ± 1.2 x 10^6 cells/animal (range = 15.0-26.9). This number increased to 35.8 ± 2.2 x 10^6 cells/animal (range = 23.5-44.3) by 2 weeks PI. The accumulation continued in the 3 week PI group with peritoneal cell harvests reaching 40.8 ± 1.9 x 10^6 cells/animal (range = 33.5-47.8).

As described in Chapter 2, the cells found in these lavages include: mononuclear cells (macrophages and large lymphocytes), eosinophils, mast cells and small lymphocytes. This cellular constitution did not change between groups in this study but a trend (not significant) of increased percentages of eosinophils was noted in the infected groups. This trend, coupled with the overall increase in cellularity, led to significant absolute eosinophil accumulation in the infected peritoneal cavities (Figure 5.3). Uninfected levels were 6.6 ± 0.4 x 10^6 eosinophils/animal (range = 4.0-8.9). Eosinophil numbers rose to 15.6 ± 1.3 x 10^6 (range = 9.2-22.6) and 18.0 ± 1.9 x 10^6 (range = 11.6-24.4) cells/animal by 2 and 3 weeks PI, respectively.

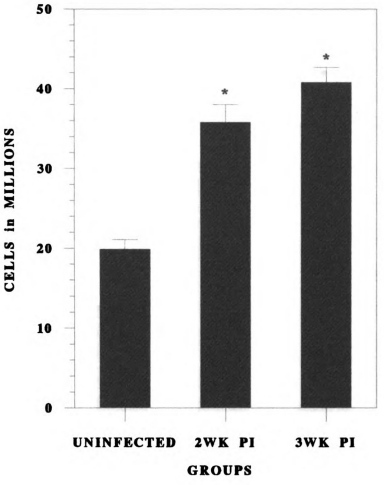

Figure 5.2. Total cell harvest from the peritoneal cavities of *Nb* infected and uninfected AUG rats. Total cells harvested in both infected groups were significantly (*) increased over the uninfected levels.

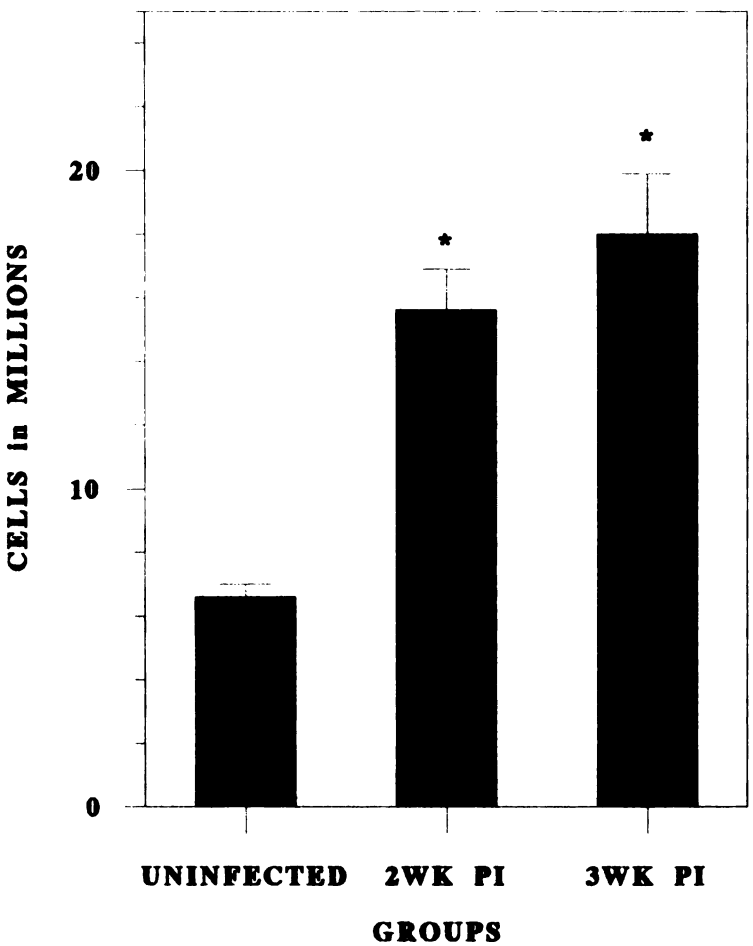

Figure 5.3. Eosinophils in the peritoneal cavities of *Nb* infected and uninfected AUG rats. The infected animals had significantly (*) higher numbers of eosinophils than the uninfected animals.

Peritoneal LTC_4 Levels

LTC_4 levels in the peritoneal lavage from the experimental groups are represented in Figure 5.4. The LTC_4 concentration was found to be 14.5 ± 2.0 pg/ml (range = 6.2-26.0) in the lavage from uninfected animals. This level was mirrored (14.9 ± 1.6 pg/ml, range = 9.6-21.8) by the 2 week PI group. However, a 3-fold increase in LTC_4 was measured in the 3-week PI group, reaching 48.1 ± 5.9 pg/ml in the peritoneal lavage. This level in the 3-week PI group was found to be a statistically significant increase, compared to the other groups.

Omental Change

As a specialized fold of the mesentery, the greater omentum lies attached to the greater curvature of the stomach, extends from the spleen and drapes over the anterior portion of the small intestine. Changes were evident in the greater omentum of the *Nb* infected groups including hyperemia and increased cellularity of the milky spots. These conditions produced an overall increase in the surface area of the omentum, roughly doubling in size in the infected groups. Histological examination revealed increased populations of mononuclear cells (macrophages and lymphocytes) and eosinophils within the layers of omental connective tissue (Figure 5.5).

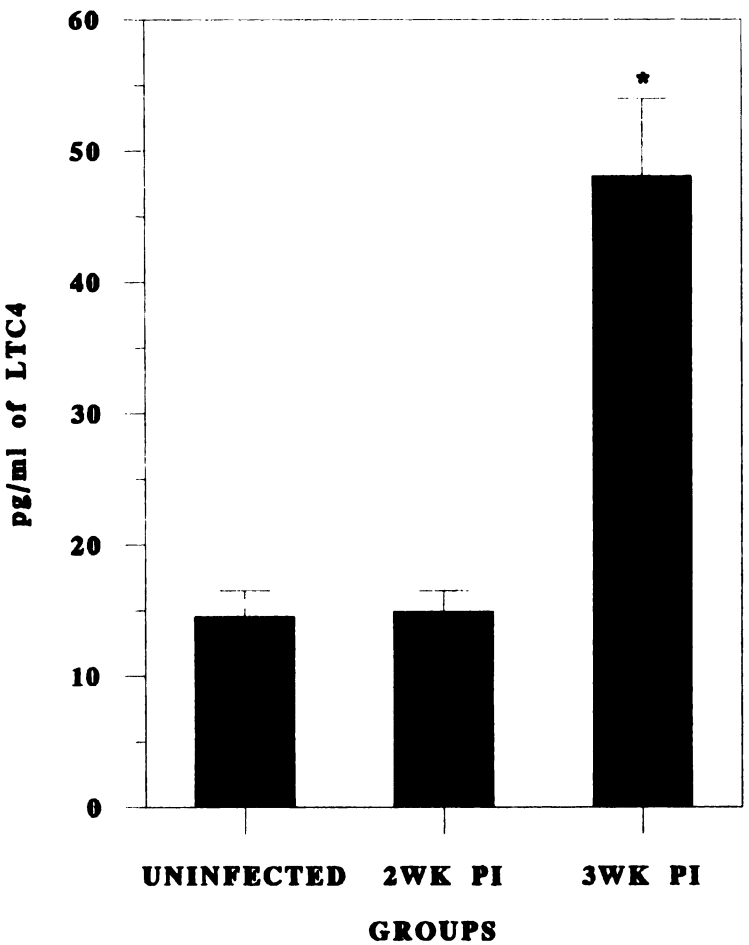

Figure 5.4. Leukotriene C_4 levels in the peritoneal exudate of *Nb* infected and uninfected AUG rats. Only the 3 week PI animals had significantly (*) increased LTC_4 in their peritoneal cavities when compared to the uninfected group.

Figure 5.5. Aggregates of cells around the "milky spot" of the omentum (as seen in the uninfected (A) animals), showed an increased cellularity which included eosinophils (➡) in the *Nb* infected (B) animals. Bar = 15.0 μm.

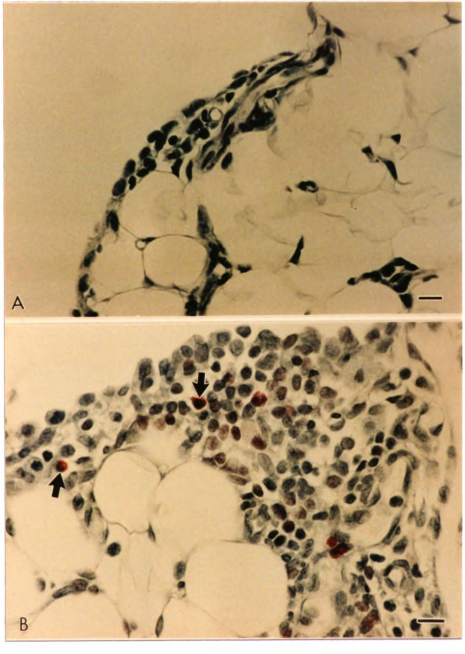

Figure 5.5

Discussion

The peripheral blood data indicates a significant rise in eosinophils only in the 2-week PI group. Peripheral blood eosinophil count in the 3 week PI group returned to uninfected levels and this blood profile is consistent with a similar profile in bone marrow eosinophil counts from another study (Ogilvie et al., 1977b). However, these data are not consistent with the behavior of the tissue eosinophils in response to *Nb* infection in this and our previous study (see Chapter 4). In that work, eosinophils in the jejunal mucosa continued to rise through the 3 week PI time point and this was paralleled in the peritoneal cavity in the present study. Additonally, the ranges observed in peripheral blood eosinophil counts showed more variability between the infected animals than the peritoneal counts. Overall, eosinophil counts of peritoneal lavage provides a higher degree of accuracy for tracking the enteric phases of eosinophilia than peripheral blood in this model.

Currently, we have provided evidence demonstrating reactivity of the peritoneal cavity to the intestinal phase of *Nb* infection. Previously we demonstrated the consistency of resident peritoneal cell populations in the uninfected AUG rat in Chapter 2. In this study, this consistency was altered in the course of primary *Nb* infection by an almost 2-fold increase in the total cellular complement of the peritoneal cavity which included a 3-fold increase in eosinophils. Additionally, the morphologic alterations in the omental milky spots

were consistent with work in mice which showed these specialized lymphoid organs as a source of peritoneal cells in immunologically stimulated (Cranshaw & Leak, 1990) and *Schistosoma mansoni* infected (Weinberg et al., 1992) animals.

One mechanism of cellular traffic in the peritoneum is by the exit of these migratory cells through the lymphatic portals of the diaphragm (Leak & Rahil, 1978). This trafficking of cells through the peritoneal cavity provides a paradigm within the enteric microenvironment where soluble and cellular components of the primary immune response to *Nb* infection can interact to produce immunocompetent effector cells for systemic redistribution. Current theory suggests that the control over which arm of the acquired immune response that predominates (Th1 vs Th2) requires specialized instructional roles by antigen-presenting cells in microenvironmental interactions with naïve T-cells (Fearon & Locksley, 1996). Weller and colleagues (1993) have demonstrated antigen-presentation capabilities in cytokine-stimulated eosinophils. Moreover, peritoneal-derived eosinophils in rat helminthiases have been shown to have increased cytotoxic responses *in vitro* (Cook et al., 1987) and can confer protective immunity to infection when passively transferred to naïve recipients (Ogilvie et al., 1977a; Ogilvie et al., 1977a; Capron et al., 1984a). These experiments include conveyance of the ability to expel adult *Nb* from naïve intestines after the passive transfer of peritoneal cells taken after day 12 PI from infected donor rats and put into the peritoneal cavity of the recipients (Ogilvie et al., 1977a).

Previous investigations have demonstrated the presence of LTC_4 (ng/ml levels) in the intestinal mucosa and gut lumen during *Nb* infection (Moqbel et al., 1986; Perdue et al., 1989). These studies concentrated on the acute phase of the anaphylactic shock response in the small intestine to massive secondary challenge and implicated eosinophils and mucosal mast cells as the LTC_4 sources. Our findings during primary *Nb* infection show peak peritoneal levels (pg/ml) of LTC_4 at 3 weeks PI. It is difficult to ascribe a similar biological significance to these findings when compared with the previous work where LTC_4 levels were found to be several hundred times higher. However, blocking of the lipoxygenase pathway in resident peritoneal cells causes an inhibition of saline-induced eosinophil accumulation in the peritoneal cavity (Oliveira et al., 1994). thus demonstrating the importance of eicosinoid metabolism by these cells in peritoneal eosinophilia. Minimally, the change in LTC_4 observed in the current study is an indication of an immunologic stimulus with potential regulatory effects on all of the resident peritoneal cells during the intestinal phase of *Nb* infection.

In conclusion, we have demonstrated peritoneal reactivity in the intestinal phase of primary *Nb* infection in rats. The observed eosinophilic response of the peritoneal cavity reflects a more accurate indication than blood of the worm-specific eosinophil response in the intestinal mucosa without losing the practically useful ease of access. Further work must be done to ascertain the interactions that

occur within the peritoneal cavity which help produce competent effector cells in the immune response to helminth parasites.

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Chapter 6

CONCLUSIONS

This thesis has centered around investigating eosinophil function and behavior *in vivo* with the development of tools toward this end; most of this work has relied on morphological and ultrastructural assessment methods. Despite the advanced state of molecular biology today many of the cell-cell interactions that occur in the pathophysiological processes between specific immunoregulatory cells and tissues still remain unattainable due, in part, to a scarcity of *in situ* probes and well characterized experimental models. Morphology based assessment has historically proved invaluable for developing new lines of evidence to broaden the knowledge of eosinophil function *in vivo,* and is again utilized in this current work.

The data presented in Chapter 2 supports the hypothesis that the eosinophil accumulation observed in the peritoneal cavities of AUG rats is the result of phenotypic expression of a heritable trait or traits in this inbred strain. We showed that the expression of peritoneal eosinophilia develops with time reaching maximal numbers by the 8th week of life. No differences were found between males and females with regard to peritoneal eosinophil accumulation either in timing of appearance or extent of accumulation. Further analyses of the eosinophil component of peripheral blood and the intestinal mucosa showed AUG rats to be

within the normal range of other rat strains in this respect. These findings help negate pathophysiological mechanisms as the primary cause in the peritoneal accumulation of eosinophils observed in these rats. Thus, this animal model provides a readily available source of tissue-derived eosinophils in an immunologically resting state. In addition, the genetic stability of this rat through its inbred nature has provided for very consistent expression of this trait throughout all of the studies which have encompassed many generations of these animals from our own breeding colony. The characterization of this trait in the August strain of rats provided a solid base for the subsequent research of this thesis.

With a paucity of eosinophil-specific antisera, the most dependable means of identifying eosinophils still revolves around the chemical staining properties of the proteins found in eosinophil specific granules. However, difficulties can arise when specific granule packaging is altered during pathophysiological responses by the cell. Histological dyes that rely solely on the property of interference of light often lose their intensity when the density or relative position of the specific granules within eosinophils are changed, such as in inflamed tissues. This can lead to under-representation of eosinophil involvement in a given condition. We addressed this problem in part, by the development of a new photoreactive fluorescent method utilizing Biebrich scarlet for the detection of eosinophil specific granules (see Chapter 3). The granule protein-Biebrich scarlet complexes

gave deep red fluorescent emissions which provided superior microscope imaging of eosinophil involvement in tissue even when specific granules were widely dispersed within the cells. This method provided exemplary results and holds much promise for future work with technologies that require fluorescent probes, such as laser scanning confocal microscopy, to assist in delineating activities in eosinophil specific granules.

The infection of rats with the nematode *Nippostrongylus brasiliensis* has been used to characterized many aspects of host-parasite relationships. While the presence of eosinophil leukocytes has been described in the responses to the intestinal phase of *Nb*, no specific action has been ascribed to these cells. Operating under the hypothesis that eosinophil leukocytes play an active role in the intestinal immune response to the adult stage of *Nb* we proceeded with the investigations described in Chapter 3 and 4 of this thesis.

Our results indicated a clear difference in eosinophil accumulation in the jejunal villus-crypt units between uninfected and *Nb* infected animals. Initial work showed a time point between 2 week and 3 week PI to be most appropriate to study eosinophil accumulation in the intestinal wall. These results were consistent with previous work in primary *Nb* infection describing the arrival of the worms in the proximal jejunum to occur between days 6-19 PI in AUG rats. With primary expulsion expected between day 12-14 PI, the timing of eosinophil accumulation

was compatible with these cells' participation in the rejection of existing *Nb* adults and in possibly preventing the establishment further incoming worms.

More detailed work, as described in Chapter 4, confirmed these preliminary findings on eosinophil accumulation in the intestinal wall. Moreover, the morphological profiles of the eosinophils that had collected in the intestinal wall of the infected rats were distinctly different from those found in uninfected animals. We carefully detailed these eosinophil changes through Nomarski (DIC) optics, as well as histological, fluorescence and ultrastructural technologies. The changes seen in eosinophils observed in *Nb* infected VC units included dispersion of specific granules within their cytoplasm, nuclear changes and emigration of the cells themselves from the lamina propria into the intestinal epithelium. These changes were concordant with eosinophil involvement in immune rejection of the intestinal phase of *Nb* from rats. Furthermore, the observed intraepithelial migration of eosinophils provides support for their role in direct cytotoxic damage to adult stage *Nb*. This strategic location of these cells in the epithelium allows for their ingestion by feeding *Nb* and the concerted or involuntary delivery of eosinophil cytotoxic proteins to the worm's gut.

The results presented in Chapter 5 demonstrate peritoneal reactivity to primary *Nb* infection in the AUG rat model. As discussed in the review of literature (see Chapter 1), studies abound that demonstrate peripheral blood eosinophilia in response to infection by helminth parasites while ignoring the important tissue

phases. We compared eosinophil accumulation in the blood and the peritoneal cavity during the intestinal phase of *Nb* in rats and found the peritoneal profile to be a more accurate reflection of eosinophil involvement in the intestinal tissue. The highly variable measure of peripheral blood eosinophilia is at best an indication of bone marrow release and transit of these cells usually falling to control levels at the time that tissue activity crested. In contrast, the peritoneal accumulation of eosinophils reaches a peak in concert with eosinophil levels in the jejunal VC units. We also found an increased cellularity (including eosinophils) in the omental "milky spots" lending support for these sites as portals for eosinophil traffic in the peritoneal cavity.

Our results on LTC_4 levels in the peritoneal exudate are somewhat different from the studies of others. Past work involved massive secondary challenges with *Nb* and showed high levels (ng/ml in gut and plasma) of LTC_4 in support of anaphylaxis as a mechanism of expulsion. The findings of our primary *Nb* challenge did not show increased LTC_4 in the peritoneal exudate over uninfected animals at the 2 week PI time interval, which would correspond to the time of worm expulsion. We did, however, measure significant increases at pg/ml levels in the 3 week PI group coinciding with the maximal eosinophil accumulation in the peritoneal cavity and the intestinal wall. This level of LTC_4 exposure (and plausible release) by the cells in the peritoneal cavity of infected rats may indicate a functional priming of cells and/or the development of an immune response. At

the very least, this evidence supports the concept of an altered status of the peritoneal exudate in response to primary infection by *Nb*.

In common with all scientific inquiry, this work has produced many new questions that are ripe for investigation. Of primary importance is an understanding of the mechanisms surrounding the intraepithelial migration of eosinophils in response to adult *Nb* in the gut lumen. In addition, the elucidation of the potential cytotoxic role that these cells play in damaging enteric parasites *in vivo* may provide valuable insights into the roles that eosinophils play as a first line of immune defense from the extracorporeal environment. Toward this end, further development of Biebrich scarlet as an eosinophil fluorophore should be productive. The utilization of this method with living eosinophils has yet to be explored but it's potential is strong for providing new information on specific granule activity in this important group of migratory cells of the immune system.

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