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EXPRESSION OF α4β1 INTEGRIN ON TISSUE EOSINOPHILS IN THE AUGUST RAT

by

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As with any endeavor in science, this study is founded on the wealth of experience for which human beings have been risking their necks over the ages. The pedigree runs from the first brave individual who discovered that fire could be tamed to serve our will, and those who defied the authorities of their day to create a secular-humanist world view in which empiricism, not theology, determines what will be considered truth, all the way to the less-imperiled modern minds who simply refuse to give up on pure science, when they could have an easier life doing just about anything else. Without those who have tested the unknown and then shared their knowledge, each one of us would still be trying to figure out which berries we could eat.

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EXPRESSION OF $\alpha_4\beta_1$ INTEGRIN ON TISSUE EOSINOPHILS IN THE AUGUST RAT

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Western Michigan University, 1996

The integrin $\alpha_4\beta_1$ is expressed on a wide variety of peripheral leukocytes, and functions in recruitment of these cells to sites of inflammaiton. We infected august (AUG) rats with the helminth parasite *Nippostrongylus brasiliensis* and later used leukocytes from the peritoneum of the sensitized animals and antibodies against $\alpha_4\beta_1$ integrin to localize that adhesion molecule on the cell surfaces after antigen challenge. We found that after antigen challenge, eosinophils, monocytes, and small lymphocytes, but not mast cells, expressed $\alpha_4\beta_1$ integrin if the animals had been previously infected, but cells from uninfected animals did not show expression of this molecule. Our study describes a novel protocol for immunohistochemical analysis of rat eosinophils.

TABLE OF CONTENTS

Table of Contents-continued

LIST OF TABLES

LIST OF FIGURES

List of Figures--Continued

INTRODUCTION

Adhesion Molecules

Background

In 1889, Julius Cohnheim reported an important observation whose details are only now becoming understood in a meaningful way. He noted that in sites of allergic inflammation, white corpuscles separated themselves from red corpuscles, and attached themselves to the postcapillary venules, in a process we now refer to as margination. He further suggested that the method of attachment was some type of "molecular glue".

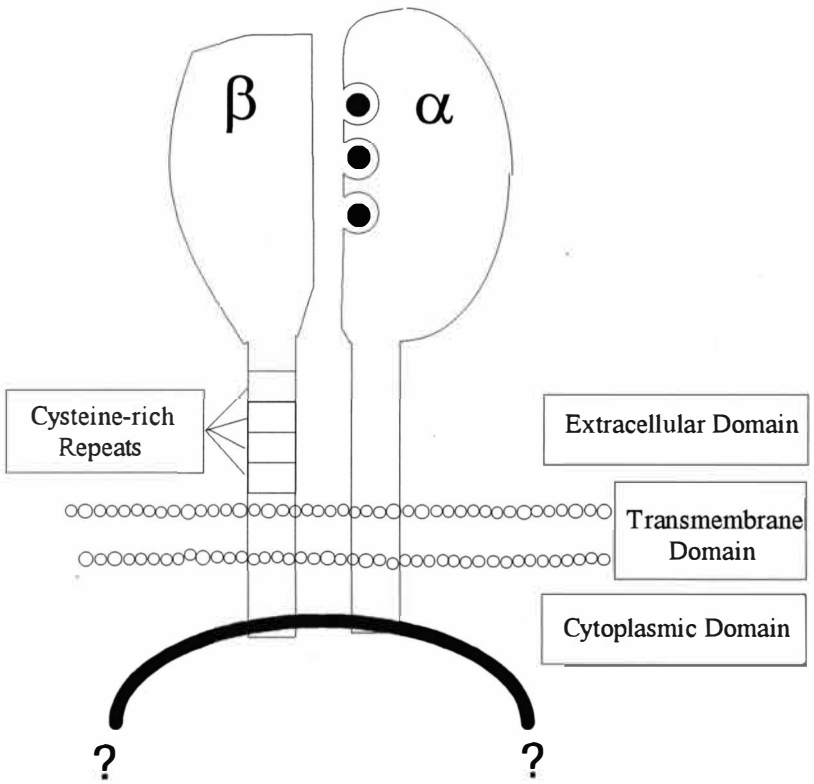
In recent decades this molecular glue has been studied and grouped into several families of "adhesion molecules", including: selectins, immunoglobulin-like molecules and integrins. Adhesion molecules are generally proteins or glycoproteins expressed on the surface of a wide variety of cells including migratory cells of the immune system as well as endothelial cells (EC). Interactions between adhesion molecules and their ligands, which are frequently other adhesion molecules or extracellular matrix molecules, allows the selective recruitment of cells from the vasculature to the site of allergic reaction, infection, or other inflammatory state. Adhesion molecules also regulate trafficking of immune effector cells to various sites in the body, such as lymphoid tissue. This adhesion is crucial to immune system function as well as non-inflammatory systems, such as development and differentiation of tissues.

$\alpha_4\beta_1$ a Multifunctional Integrin

One large family of cell adhesion molecules, widespread in the body, is the integrins. Integrins are heterodimeric proteins (Figure 1), composed of varying α and β subunits in a noncovalent association (reviewed in Hynes, 1992). This organizational scheme has led to the now-preferred system of nomenclature in which integrins are named according to their α and β subunits. Thus VLA-4 is now referred to as $\alpha_4\beta_1$. The older designation Very Late Activation Antigen (VLA) was based on experiments (Hemler, 1990) in which the expression of both VLA-1 and VLA-2 was dramatically increased on cultured lymphocytes after 4 weeks. Current knowledge indicates, however, that most cells in the body express at least some integrins constitutively, and thus the VLA designation is gradually falling into disuse.

The β subunits, which vary in size from about 90kD to 110kD, will generally associate with more than one α subunit, leading to subfamilies of integrins grouped according to the β subunit. For instance, all the integrins which had been termed "VLA" antigens are from the β_1 subfamily, while the β_2 subfamily contains the species Leukocyte Function-associated Antigen (LFA)-1 and Mac-1 (C3bi receptor). The β_2 subfamily are the only integrins still referred to commonly by their CD numbers: CD11x/CD18. The three major β subunits in humans: β1, β2 and β3, have been completely sequenced (reviewed in Hemler, 1990) and show 44-47% homology. In particular, four repeating regions rich in cysteine are highly conserved in the C-terminal half of the molecule. These repeats appear to lie in the extracellular region (see Figure 1).

The α subunits are somewhat larger at 120-180kD (Hynes, 1992) and generally contain binding sties for divalent cations, which have been shown to be necessary for the function of some integrins (Gailit & Rouslahti, 1988). The various α



Legend ● indicates cation binding domain. **?** indicates unknown cytoskeletal components, or second messengers.

Figure1. Generalized Structure of Integrin Heterodimer. (Adapted from Hynes, 1992, Hemler, 1990; Muñoz, Serrador, Sanchez-Madrid and Teixidó 1996.)

subunits are slightly less similar to each other than the β subunits, at 20-30% homology. The association region for the two molecules is unknown, although studies of altered subunits without transmembrane domains did appear to form normal heterodimers (Bodary, Lipari, Muir, Napier, Pitti & McLean, 1991). Most α subunits appear to selectively associate with only one β chain, however there are notable exceptions. One of the most interesting is the heterodimer $\alpha_4\beta_7$, which some studies have found to bind to the same ligand binding sites as $\alpha_4\beta_1$ (Ruegg, Postigo, Sikorski,

Butcher, Pytela & Erle, 1992). In another study (Chan, Elices, Murphy & Helmer, 1992), the affinity of the rarely-found $\alpha_4\beta_7$ for the ligands of $\alpha_4\beta_1$, was found to be considerably lower, and dependent on some form of activation. This suggests the possibility that $\alpha_4\beta_1$/ligand specificity may be largely determined by the α subunit. Supporting that notion is the work of Muñoz, Serrador, Sanchez-Madrid and Teixidó (1996), in which changes in the amino acid residues Arg^{89}-Asp^{90} in the α_4 subunit reduced both aggregation of transfected K562 cells and their adhesion to fibronectin, while substitutions at the nearby Gln^{101}, Pro^{102} and Ile^{108} had little or no effect. Notably, adhesion to VCAM-1 was not affected by these mutations.

The $\alpha_4\beta_1$ integrin is expressed on many leukocyte types including: eosinophils, peripheral blood lymphocytes, monocytes, B and T cell lines and NK cells, among others. Significantly, it is absent on neutrophils (Pigott, Power, 1993; Wardlaw, Symon & Walsh, 1994). Because $\alpha_4\beta_1$ is expressed by eosinophils but not neutrophils, it appears to function as a system for preferentially recruiting eosinophils into tissues.

As stated previously $\alpha_4\beta_1$ is multifunctional in that it facilitates both adhesion to extracellular matrix (ECM) and cell-cell adhesion via different ligands. Intercellular binding is accomplished through the ligand Vascular Cell Adhesion Molecule-1 (VCAM-1) which is expressed on vascular endothelial cells (EC). In human *in vitro* cell systems, eosinophils have been shown to adhere to cultured endothelial cells via the $\alpha_4\beta_1$/VCAM-1 binding system (Dobrina, Menegazzi, Carlos, Nardon, Cramer, Zacchi, Harlan & Patriarca, 1991, and Weller, Rand, Goelz, Chi-Rosso & Lobb, 1991). Both studies found that binding was abrogated in the presence of either anti-VLA-4 or anti-ICAM-1 monoclonal antibodies (mAb), but not in the presence of unrelated control antibodies. *In vivo* video microscopy studies in rabbit have shown

that $\alpha_4\beta_1$ contributes to eosinophil rolling in venules (Sriramarao, von Andrian, Butcher, Bourdon & Broide, 1994). Once again, mAbs against $\alpha_4\beta_1$ inhibited the phenomenon, while control (anti-CD18) mAbs had no effect. Finally, TA-2, a monoclonal antibody against $\alpha_4\beta_1$, was found to inhibit the *in vivo* migration of small lymphocytes to inflamed or cytokine-treated skin in a rat model (Issekutz, 1991).

Fibronectin, a glycoprotein which is a major component of ECM, is the other ligand of $\alpha_4\beta_1$. While VCAM-1 has a greater affinity for $\alpha_4\beta_1$ than does fibronectin (Chan & Aruffo, 1993), it has been shown that the integrin/fibronectin interaction has profound physiological effects on eosinophils (Anwar, Moqbel, Walsh, Kay & Wardlaw, 1993; Anwar, Walsh, Cromwell, Kay & Wardlaw, 1994). The classic binding portion of fibronectin is the peptide sequence arginine-glycine-aspartic acid (RGD) which is present in several ECM proteins including fibronectin and vitronectin (Hynes, 1992; Anwar, Walsh, Cromwell, Kay & Wardlaw, 1994). VLA-5($\alpha_5\beta_1$), which is a familiar fibronectin binding integrin, binds to the RGD sequence, as does $\alpha_{IIb}\beta_3$, and all of the $\alpha_v\beta$ (Hynes, 1992). However, the $\alpha_4\beta_1$ integrin appears to bind to fibronectin at a unique region which contains several distinct binding sites including: an incompletely characterized portion of the heparin II domain, and two sites in the 111CS region which have been designated CS-1 and CS-2 (Hemler, 1990). The multiplicity of binding sites for the $\alpha_4\beta_1$/fibronectin associations suggests that this is an important interaction for the cell.

Can $\alpha_4\beta_1$ /Ligand Binding Initiate Intracellular Signal Transduction?

It may be too simplistic to suppose that adhesion molecules act simply as a molecular glue, holding cells to each other or to ECM. On a strictly molecular basis, several observations suggest integrins may have functional roles in addition to their

extracellular binding capability. As mentioned earlier, there is approximately 20-30 percent homology between the different classes of α subunits within a species (reviewed in Hemler, 1990). When only the cytoplasmic domains are examined, however, there is very little homology. Yet the cytoplasmic domain of a given α subunit is highly conserved between species. For example, the cytoplasmic domain of human α_3 shares 31 of 36 amino acids with its chicken counterpart (Hemler, 1990). Taken together, these observations suggest that integrins may each have specific functionalities within the cytoplasm, which are highly conserved between species. These functionalities may include carrying a signal to a second messenger inside the cell, or alternately it could indicate that integrins simply bind to different components of the cytoskeleton.

In a definitive study using human eosinophils, $\alpha_4\beta_1$/fibronectin binding was shown to enhance the cells' survival *in vitro* (Anwar, Moqbel, Walsh, Kay & Wardlaw, 1993). This experiment examined the survival rate of eosinophils cultured in the fibronectin-coated, bovine serum albumin-coated and uncoated wells over several days and found that virtually all the eosinophils cultured without fibronectin had died after 96 hours, while 60% of the eosinophils in the fibronectin-coated wells were alive as measured by trypan blue exclusion. Antibodies against fibronectin and $\alpha_4\beta_1$ integrin inhibited this survival. When the medium was analyzed, it was found to contain granulocyte/macrophage colony-stimulating factor (GM-CSF), and interleukin-3 (IL-3) in those wells which had contained fibronectin, but not in the other wells. Additionally, *in situ* hybridization examination of the eosinophils from the fibronectin-coated wells showed mRNA for GM-CSF. These results indicate that $\alpha_4\beta_1$ /fibronectin binding can initiate signal transduction, producing physiological responses by the cell. In another study, binding to fibronectin caused cultured eosinophils to

increase their production of leukotriene C_4 (LTC_4) over cells which were cultured without fibronectin (Anwar, Walsh, Cromwell, Kay & Wardlaw, 1994). Finally, in the work of Kassner, Alon, Springer and Hemler (1995), chimeric forms of $\alpha_4\beta_1$, deficient in the cytoplasmic domain of the $\alpha 4$ subunit were shown to alter some aspects of cell movement such as spreading.

An additional level of complexity is added by the post-expression regulation of integrin binding affinity. Although this process has been more fully characterized in other integrins such as $\alpha_{IIb}\beta_3$, studies exist which have described a similar regulation of $\alpha_4\beta_1$. In one recent study using human peripheral blood eosinophils, (Kuijpers, Mul, Blom, Kovach, Gaeta, Tollefson, Elices & Harlan, 1993), an "activating" anti β_1 mAb (8A2) is described which increased the level of eosinophil binding to fibronectin-coated filters. This binding was blocked by the addition of the CS-1 fragment of fibronectin, indicating that the binding was mediated by $\alpha_4\beta_1$ and not $\alpha_3\beta_1$, or $\alpha_5\beta_1$, both of which bind to the RGD sequence. What makes this study particularly interesting, is that while $\alpha_4\beta_1$ integrin was locked in the higher-affinity binding state by the 8A2, the eosinophils were completely blocked from migrating through the fibronectin-coated filter. In contrast, eosinophils which had not been exposed to 8A2 were able to migrate through the filters coated with fibronectin. One could speculate then, that $\alpha_4\beta_1$/ECM interactions *in vivo* may depend on a modulation of the integrin between high and low affinity states. Reasoning further, if the integrin could not be controlled in this way, the cell might be locked in one position, and thus would not be able to "crawl" through tissues to sites of inflammation. Other recently published data (Yednock, Cannon, Vandevert, Goldmach, Shaw, Ellis, Liaw, Fritz & Tanner, 1995) indicate the possibility that changes in $\alpha_4\beta_1$ conformation may be induced by the ligands themselves. Using leukocytic cell lines, these researchers describe an anti-$\alpha_4\beta$

$_1$ mAb which only recognizes its epitope when the integrin is in the high-affinity state. The study found that nanomolar concentrations of VCAM-1 or micromolar concentrations of the $\alpha_4\beta_1$ binding portion of fibronectin could induce the mAb to bind, indicating a conformational change to the higher affinity state. Of course, much remains to be learned about the intriguing field of integrin regulation.

Eosinophils and the August Rat

The Eosinophil

Eosinophils are bone-marrow-derived granular leukocytes, which have been shown to be major participants in allergic inflammation, asthma, and host defenses in parasitism (reviewed in Smith & Cook, Ed., 1993). Generally, eosinophils are distinguished from other leukocytes on the basis of their morphology and characteristic staining properties. In particular, a subset of the cytoplasmic granules referred to as the secondary or specific granules, has a high affinity for acid stains. Rat eosinophils are particularly easy to identify on the basis of their unique, donut-shaped nucleus (Figure s. 7,9).

It is well established that eosinophils will respond to several chemotactic factors including platelet activating factor (PAF), C5a and others (reviewed in Giembycz & Barnes, 1993), and that upon stimulation with the appropriate factors, they can migrate across vascular endothelium as well as epithelium (Resnick, Colgan, Parkos, Delp-Archer, McGuirk, Weller & Madara, 1995). As discussed earlier, this movement is referred to as trafficking when it involves the normal migration of immune cells into and out of lymphoid tissues, or recruitment in the case of response to an inflammatory stimulus, and is common among many types of immune cells.

Much of the eosinophil research in recent decades has revolved around their participation in asthma. Although this is plainly a pathological condition and therefore likely distinct from eosinophils' protective role in immunity, much knowledge has been gained which can illuminate the functions of the eosinophil as a member of the healthy immune system. Eosinophils have been associated with the late phase of asthmatic response, and have been found to migrate in predictable patterns in asthma models. Furthermore, studies using blocking antibodies to various adhesion molecules have been shown to change that migration pattern, illustrating that linkage between adhesion molecule expression and cell migration in asthma. In one particularly interesting study using sheep as the animal model, mAbs against α_4 were given before *ascaris* antigen challenge in the airways (Abraham, Sielscak, Ahmed, Cortes, Lauredo, Kim, Pepinsky, Benjamin, Leone, Lobb & Weller, 1994), and were found to decrease late-phase airway hyperresponsiveness by 75%. Upon histological examination, however, it was found that eosinophil numbers in the mAb-treated animals were not significantly different than in the control group. Moving to an *in vitro* test system, these same researchers found that their mAb was able to inhibit PAF-stimulated eosinophil peroxidase release, leading them to conclude that the mAb they had been using elicited its effects by altering eosinophil function rather than recruitment.

This points out the complex layers of function in immune response, and a distinction between recruitment of an eosinophil, and activation of that same cell. It has been shown in the literature that eosinophils are multifunctional; or stated in other words, that depending on the signals it receives from its environment, an eosinophil can exhibit varying responses. This is a movement away from the more classic view of eosinophils as cells which exist solely to release granule contents when functionally "activated".

One traditional parameter for describing an eosinophil's functional state is its density. Many researchers purify eosinophils from blood by means of a density gradient using metrizamide, Percoll or other compounds, utilizing the fact that, in healthy individuals, eosinophils generally have the greatest buoyant density of all leukocytes found in the peripheral blood (Owen, 1993). Additionally, eosinophils from the blood of individuals who had experienced helminth infection were found to be less dense than those of individuals who had not (De Simone, Donelli, Meli, Rosati & Sorice, 1982), thus linking density profile with functional state. The different morphological status of the two groups has also been associated with the location of the eosinophils; summing up this physical heterogeneity in his 1993 review, Owen noted "normodense eosinophils become hypodense in the peripheral circulation and migrate preferentially to the tissue microenvironment, . . . hypodense eosinophils are the phenotype which resides in the tissue microenvironment" (page 61).

It has been observed that outside the body, normodense eosinophils do not remain viable for long periods of time, however their survival can be significantly prolonged by co-culture with fibroblasts, or by addition of cytokines such as GM-CSF, IL-3 or IL-5 (Owen, 1993). This increased survival is accompanied by a transformation from the normodense to the hypodense morphology. These hypodense eosinophils share many of the alterations in functional capability displayed by the hypodense eosinophils from patients with eosinophilia (see Owen 1993).

All of this information leads to the conclusion that eosinophils are differentially stimulated *in vivo* for certain physical and chemical functions, such as increased helminth killing capability or prolonged survival (Owen, Rothenberg, Silberstein, Gasson, Stevens, Austin & Soberman, 1987), and that without a precise sequence of

stimulatory events, the cells will not display those functions. Thus specific functional stimulation can only be approximated *in vitro* if the appropriate stimuli are known.

The August Rat as a Model of Eosinophilia

The August (AUG) rat is unusual among animal models in that it has high numbers of eosinophils in the peritoneal cavity, independently of external stimulus (Mackenzie, Jungery, Taylor & Ogilvie, 1981). The only other rat in which this phenotypic trait has been described is the Am-1(2)/Tor rat (Pimenta & De Sousa, 1982). In the AUG rat, these cells are part of a mixed-cell population containing eosinophils, mast cells, mononuclear phagocytes and small lymphocytes. In an adult male AUG rat this population numbers on the order of $3x10^7$ cells per animal. Notably, no neutrophils are detectable in the peritoneal lavage of the AUG rat. Because these cells are easily removed with a simple peritoneal lavage using a buffered isotonic medium, eosinophils may be isolated without immunological stimulation or elaborate isolation procedures. Most eosinophil studies procure cells by an inhaled antigen challenge followed by a pulmonary lavage, or by collecting large quantities of blood and removing the eosinophils with negative immunological selection or an automated cell sorter. Both of these methodologies involve significant insult to the cells, and are further removed from the natural *in vivo* condition. Our study used the mixed population as it was found in the animal, without segregating the eosinophils.

Nippostrongylus brasiliensis Infection in the Rat

Since the earliest days of eosinophil research in the late 19th century, scientists have been studying the relation of these cells to helminth parasite infections (reviewed

in Spry, 1988). This research has continued up to the present, and has been a major contributor to our understanding of eosinophil biology, not only as it relates to parasitism, but also in terms of the fundamental mechanisms of eosinophil structure and function. Helminth infection has been associated with a localized eosinophilia at the site of the parasite in a wide variety of models (reviewed in Butterworth & Thorne, 1993), often preceded by a peripheral blood eosinophilia. The eosinophilia has been found to be concurrent with elevated serum levels of immunoglobulin (Ig)E, which may be relevant to the localized eosinophilia (Butterworth & Thorne, 1993).

Nippostrongylus brasiliensis (*Nb*) is an rodent enteric parasite whose definitive host is the rat. Infective larvae (L_3) enter the animal through the skin, burrowing through tissue until encountering a blood vessel. Entering the vasculature, they migrate to the lungs, where they cross the pulmonary epithelium and molt. The resulting L_4 enter the digestive system via the esophagus, and eventually arrive in the small intestine. By 3-16 days after the initial infection, the worms molt again, thus enter the adult stage (L_5), during which they mate and lay eggs. The eggs are passed out of the animal in the feces starting around day 8-9 post-infection (Eversole, 1996).

As in other animal models, a localized eosinophilia has been reported at the site of migrating *Nb* larvae in rats (Mackenzie & Spry, 1983). *Nb* infection has also been associated with eosinophilia in the blood as well as in other tissues, and in the AUG rat this tissue eosinophilia has recently been described (Eversole, 1996). Eosinophil numbers in the peritoneal cavity were shown to increase rapidly from 11-20 days after a primary infection with *Nb*.

Purpose of This Experiment

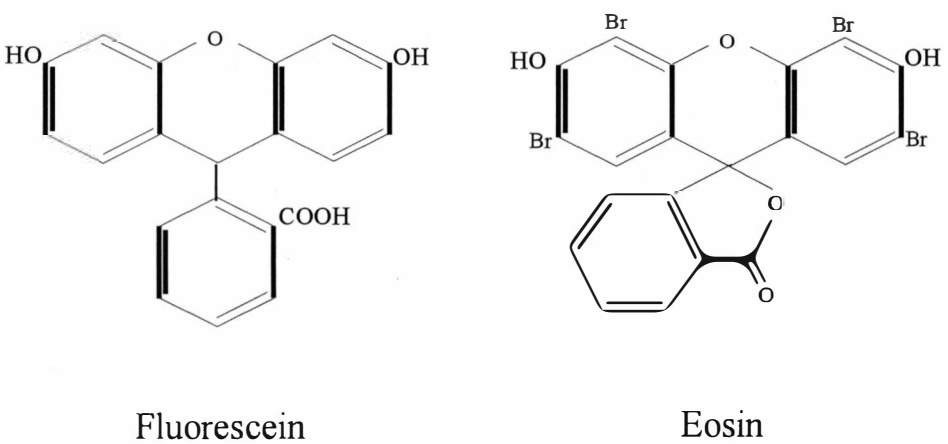
Operating under the hypothesis that sensitized eosinophils from *Nb*-infected animals may be differentially stimulated to produce $\alpha_4\beta_1$ integrin, we attempted to localize this molecule on the eosinophils after an *in vitro* treatment with antigen from *Nb*.

To do this, we challenged cells from rats which had been infected with *Nb* and a control group which had not, with *Nb* antigen. After treatments, the cells were examined using fluorescent immunohistochemical methods. We selected a time point for cell collection based on the period in which eosinophils were rapidly entering the peritoneum, reasoning that $\alpha_4\beta_1$ might be expressed at that time.

ASSAY DEVELOPMENT

Difficulties in Immunolabeling Rat Eosinophils

Although obtaining healthy, resting-state eosinophils is facilitated by the existence of the AUG rat, there are inherent difficulties in using the standard conjugated antibody markers with eosinophils in general. First, the highly basic granules of the eosinophils bind fluorescein molecules, resulting in unacceptable background levels (eosin, for which eosinophils are named, is in fact a brominated fluorescein derivative developed by the dye industry. See Figure 2). Second,



Fluorescein Eosin

Figure 2. Molecular Structures of Fluorescein and Eosin.

eosinophils autofluoresce under UV-excitation, obscuring less intense stains such as rhodamine, rendering these stains unsatisfactory as well. Third, peroxidase-linked assays are unsuitable because of the well-known endogenous peroxidase activity of the eosinophil (Spry, 1988). Fourth, rat eosinophils possess endogenous alkaline phosphatase activity, making that enzyme-linked marker system unsuitable for use in

this model. Finally, some unknown factor associated with the cells in this cell population interfered with color development in systems using glucose oxidase, although the nature of this interference remains undetermined.

We have overcome the difficulties associated with the fluorescein label, however, by treating the cells with a compound described in the 1940's (Lendrum, 1944). Lendrum's chromotrope appears to bind to the specific granule contents more effectively than does the fluorescein, essentially blocking that interaction. This compound has the added benefit of rendering the eosinophils easily visible without use of any other staining agents.

Reagents

Except where noted, reagents were the same as those listed in **Materials and Methods**. The glucose oxidase-linked assay was carried out using the ABC-GO kit (Vector Laboratories, Burlingame, CA). The alkaline phosphatase-linked assay was also performed with a Vector Laboratories kit, ABC-AP, and the levamisole solution was also from Vector Laboratories. At various times, phosphate buffered saline (PBS) with tween-20, PBS without tween-20, and reverse osmosis (RO) purified water were assessed as washing agents. Fixation was done at various temperatures with methanol, acetone, 10% neutral buffered formalin and air drying, in order to determine the most effective fixation method for this assay.

Preliminary Research

Our first attempts at labeling these cells focused on systems using fluorescein-conjugated secondary mAb's. High background obscured any labeling that may have

existed on the slides, and occurred even on the slides which had not been treated with the primary antibody; the so-called negative control slides. This suggested that fluorescein would not be useful as the secondary marker in this cell population. To confirm non-specific staining of rat eosinophils with fluorescein-containing reagents, we ran a demonstration slide of non-stimulated AUG rat peritoneal leukocytes (obtained and prepared as described in **Materials and Methods**). with one hour gelatin blocking (to block nonspecific protein-protein interactions), followed by a wash and fluorescein-conjugated anti mouse-IgG (the same secondary antisera described in **Materials and Methods**). The results are visible in Figure 3. Since the antibody is not significantly cross-reactive with any known epitopes in this cell preparation, the staining that is visible in the micrograph is assumed to be the non-specific interaction of the fluorescein with some intracellular component, possibly granular.

The autofluorescence which makes low-level stains such as rhodamine problematic is visible in a preparation called a "sham", in which cells are treated with no additional reagents after fixation and a preliminary wash, showing only their own inherent color properties under UV-excitation. An example of a sham prepared with AUG rat peritoneal leukocytes is shown in Figure 4.

We decided against attempting to adapt a peroxidase-based assay system, because of previous difficulties associated with quenching the endogenous enzyme activity in eosinophils, particularly those from the AUG rat (Mackenzie, unpublished observations).

Next, we explored the possibility of using a commercially available alkaline phosphatase-based system, which required quenching the endogenous enzymatic activity of the eosinophils. One compound which has been formulated for this purpose is levamisole, which was recommended and supplied by the same company as the

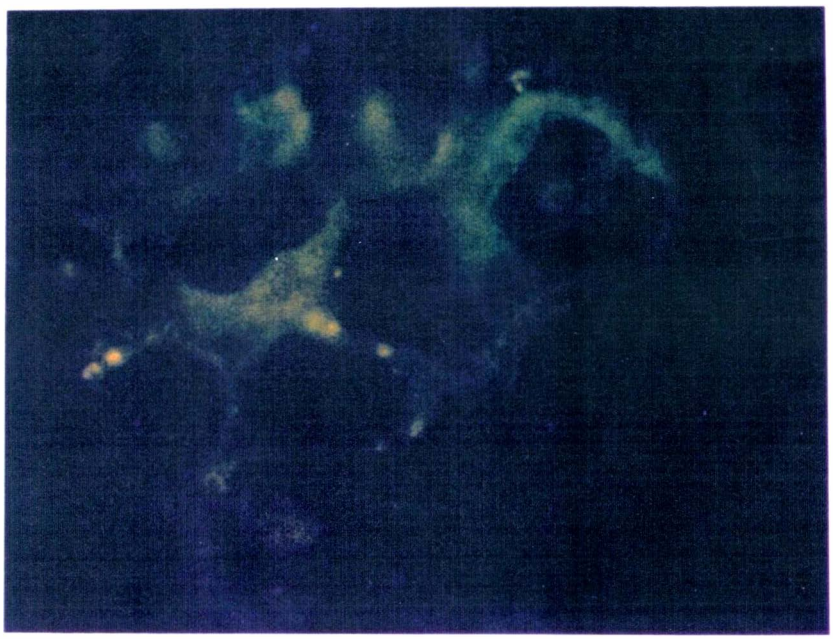


Figure 3. Epifluorescent Micrograph of AUG Rat Peritoneal Leukocytes Showing Non-specific Fluorescein Deposition.

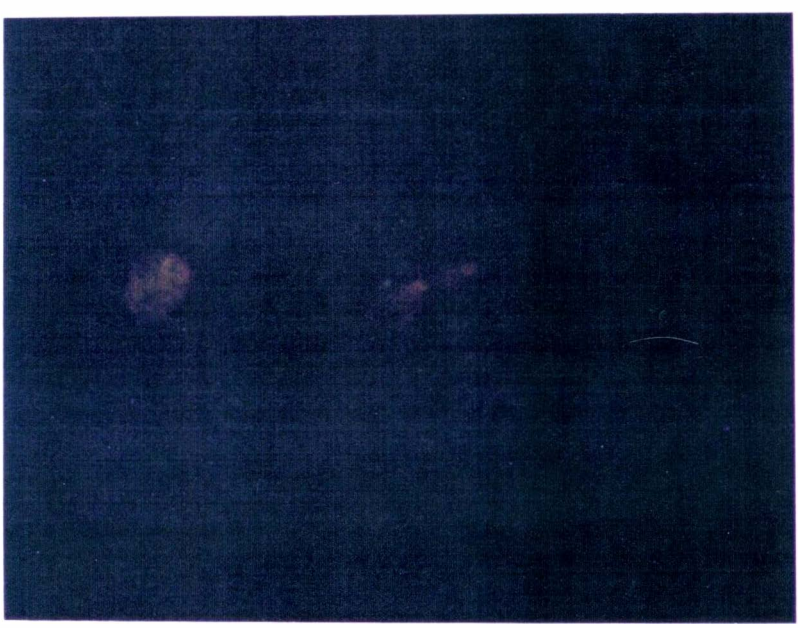


Figure 4. Epifluorescent Micrograph Showing Cellular Autofluorescence.

labeling kit itself. This kit employed a substrate system which yielded an intense red precipitate upon catalysis. Cells stained using this system still had an unacceptably high level of background, primarily in the cytoplasm. Once again, the negative controls, which should have shown no labeling, were stained with the red precipitate. To confirm that the problem was in the endogenous alkaline phosphatase activity of the eosinophils, and not simply in a cross-reactive secondary antibody, the substrate portion of the kit was used alone on one of our cell preparations with no antibodies present. The cells showed the same distinct cytoplasmic labeling as before, even after treatment with levamisole. We decided to forgo further attempts to overcome the problems associated with the ABC-AP kit, which for reasons to be described, was a fortuitous decision in that the system had another characteristic which would have made it inappropriate for use in this cell population.

Glucose oxidase activity is not generally present in mammalian cells, and so this enzyme has become widely used in biological sciences as a labeling reagent. The kit which we used coupled the oxidation of glucose with the reduction of tetrazolium salts to colored, insoluble precipitates. When we applied the kit to our cell preparation, however, no color developed under a variety of staining and washing conditions, even on the small lymphocytes which we used as the internal positive control. We then repeated the experiment using a primary mAb against a different adhesion molecule, LFA-1, which is known to be present on eosinophils (Walsh, Wardlaw & Kay, 1993), as well as other cells in the population under study (Pigott & Power, 1993). Still, no color developed. Suspecting problems with the reagents, we tested each of the individual components of the kit (kindly resupplied by the manufacturer), which all appeared to function properly. In a final side-by-side comparison, several slides bearing the cell preparation were run along with others that

had no cells, only the secondary Ab air-dried to the slide. The result of this experiment was that the non-cell slide stained strongly, while the cell-containing slides remained devoid of color. We concluded that some unknown factor in the cell preparation was interfering with the production of the colored precipitate, and rather than attempt an in-depth analysis of the problem, we would take another approach.

In returning to an examination of the feasibility of using fluorescein, we availed ourselves of the work of Johnson & Bienenstock (1974), who found that a stain which had been described in 1944 (Lendrum, 1944), had the additional beneficial property of binding to the cytoplasmic granules of eosinophils with a higher affinity than fluorescein. In our laboratory, using Lendrum's chromotrope at any point before the application of fluorescein-conjugated antibodies removed cytoplasmic accumulation of the fluorescein compounds, and resulted in the development of a deep orange-red color under UV-excitation, which contrasts well with the green of the fluorescein. Our assay was still one step from being complete.

The kits we had previously tested were designated "ABC" kits by the manufacturer, because they employ an avidin-biotin complex to enhance the intensity of labeling. In this system, the secondary antibody is conjugated to biotin rather than to an enzyme or fluorochrome. The marker portion of the system is conjugated to avidin, a glycoprotein of approximately 68 kD, which binds to biotin (Hsu, Raine & Fanger, 1981) with an extremely high affinity (the manufacturer's catalog states that the affinity is over one million times the affinity of most antibodies for their antigen). Multiple biotinylation of the secondary antibody increases the marker deposited at the site of the antigen under study, thus significantly amplifying the label. In our case, however, we found labeling on the negative control slides, which had not been treated with primary antisera. This suggested that there was some biotin-like epitope present

on cells from this preparation, and that the avidin-biotin system would have to be abandoned.

Even without the benefits of an avidin-biotin system, we did have a protocol that circumvented the problems associated with fluorescein labeling, and felt that the assay would be functional if the molecule under study were expressed at sufficient levels. Many variations were evaluated to find optimum blocking, washing, labeling and mounting conditions. Finally with the assay in hand, we were prepared to address biological questions concerning expression of adhesion molecules on the surface of eosinophils from the AUG rat peritoneal cavity.

MATERIALS AND METHODS

Experimental Design

Cells were obtained from animals 14 days post-infection with the helminth parasite *Nippostrongylus brasiliensis*, and from uninfected control animals. The cells were then cultured with immune serum from previously infected animals and *Nb* antigen or with immune serum alone. Cells were treated for 0 minutes or 20 minutes. TA-2, an mAb against rat $\alpha_4\beta_1$ integrin, was used as a marker to test for the expression of the adhesion molecule in this mixed cell population.

Reagents

Except where noted, reagents were obtained from Sigma Chemical Co. (St. Louis, MO). Ca^{++}/Mg^{++}-free Hanks' Balanced Salt Solution (HBSS) was buffered with 20mM N-[2-hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid] (HEPES) at pH 7.3. Phosphate Buffered Saline (PBS) was 10 mM, pH 7.4. *N. brasiliensis* culture was the kind gift of Sandra Johnson of Pharmacia and Upjohn, and the working worm preparation for in vitro exposure contained *Nb* larvae (L_3) which had been maintained in 60°C, reverse osmosis (RO) purified water until dead (about 14 days). Acetone was placed in -20°C freezer before the experiment, to ensure the proper temperature for fixation. Lendrum's Chromotrope (LC) was prepared as previously described (Johnson & Bienenstock, 1974): 0.1 g Chromotrope 2R was dissolved in 1.0 g melted phenol crystals, and brought to 100 ml with the addition of glass-distilled water. LC was stored in a light-tight glass container. Blocking solution was 0.1% EIA grade

gelatin (Biorad, Richmond, CA) in PBS. The first batch of antisera (the mouse mAb TA-2) was the kind gift of Anthony Manning, of Pharmacia and Upjohn; additional antisera was purchased from Endogen (Cambridge, MA). The antisera was used at a dilution of 1:50 in PBS. The secondary antibody was fluorescein-conjugated anti-mouse IgG (Vector Laboratories, Burlingame, CA) and was used at a dilution of 1:100 in PBS. Labeled slides were permanently mounted in Vectasheild® (Vector Laboratories, Burlingame, CA). Differential staining was with DiffQuick® (Baxter Healthcare Corporation, McGaw Park, IL), and total cell counts were performed using a Neubauer Hemacytometer (American Optical, Buffalo, NY).

Animals

Six male August (AUG) rats (Harlan/Olac, UK) aged over 12 months were used in this experiment, and were housed in accordance with Western Michigan University Institutional Animal Care and Use Committee (IACUC) guidelines. Animals were maintained with Rodent Chow® No. 5001 (Purina Mills, St. Louis, MO) and RO purified water *ad libitum*.

The first group of 4 AUG rats were given a dose of approximately 3000 infective (L_3) *Nb* larvae in 0.3 ml RO purified water by subcutaneous injection into the proximal ventral surface of the right leg. The remaining two rats were given no injection and served as the control group.

Cell Isolation

On day 14 post-infection, animals were killed by CO_2 inhalation and peritoneal cells were isolated as previously described (Eversole, 1996). Briefly, after creating a

small ventral incision in the abdomen, 30 ml cold (wet ice) Ca^{++}/Mg^{++}-free HBSS, was placed in the animal's peritoneal cavity. After gently massaging the animal for one minute, the HBSS and suspended cells were removed with a disposable syringe. Cells were then centrifuged at 200 x *g* for 10 minutes at 4°C and the supernatant discarded. The resulting pellet was resuspended in 5 ml HBSS and returned to wet ice. Blood was also removed by cardiac puncture, allowed to coagulate on wet ice for 15 minutes. After coagulation was complete, this blood was centrifuged and the resulting serum was kept on wet ice until needed.

Cell Treatment

Aliquots of 1.0 x 10^6 cells from the peritoneal lavages were added to 15 ml centrifuge tubes containing 0.9 ml HBSS and 0.1 ml fresh immune serum, as well as to tubes containing 0.9 ml HBSS, 0.1 ml fresh serum and 0.1 ml of the working *Nb* preparation. Since each tube received 1.0 x 10^6 cells, the volume of cell suspension added varied from animal to animal, but was always between 111-145 µl. Samples of the cell suspension were removed either immediately (0 min) or after 20 min incubation at 37°C, and placed on wet ice.

VLA-4 Assay

Slides were made from these samples using a Cytospin3® centrifuge (Shandon Inc., Pittsburgh, PA) . After centrifugation, a circle was drawn around each spot of cells with a hydrophobic PAP® pen (Kiyota International, Elk Grove Village, IL) to prevent loss of reagent during the assay, and the barrier was allowed to air dry. Cells were fixed in acetone for 10 minutes at -20°C, and washed 2 x 5 minutes in PBS.

After the post-fixation washing, cells were exposed to gelatin blocking solution in excess. Blocking was allowed to proceed overnight (10-12 hours) at 4°C. Slides were washed 2 x 5 minutes in PBS. Chromotrope was then added to all slides, covering the cell area, and allowed to stand at room temperature for 30 minutes. Slides were again washed 2 x 5 minutes in PBS. At this point, slides were divided into a positive group (those receiving 100 µl of the mAb TA-2) and a negative control group (which received additional blocking solution). Both groups were left to stand at room temperature for 4 hours. Slides were again washed 2 x 5 minutes in PBS. The secondary marker (a fluorescein-conjugated anti-mouse IgG) was added to all slides, and allowed to stand for 30 minutes in the dark at room temperature. Slides were again washed 2 x 5 minutes in PBS, Vectashield® was added, and the slides were coverslipped and sealed.

Epifluorescent microscopy was performed using a Microphot-FXA (Nikon, Tokyo, Japan), and Micrographs were taken using Kodak (Rochester, NY) Elite II 400 ISO color slide film.

Computer-assisted Image Analysis

In order to more objectively determine if those cells which were deemed to be labeled were different than those which were not and thus support the subjective assessment of labeling, measurement of the cell brightness was carried out using a Javelin JE462RGB black and white video camera (Javelin Electronics Corp., Tokyo, Japan) mounted to a Nikon SMZ-U microscope, feeding images into a computer running the Image-1 (Universal Imaging Corp., West Chester, PA) image-analysis software system. To avoid the inevitable photobleaching that would occur with prolonged exposure of the cells themselves, the micrographs, which were on slide film,

were analyzed under low magnification with backlighting. Measurements of cell brightness consisted of 5 separate average area brightness measurements of each cell, taken exclusively from the nuclear region, except in the case of the mast cells, which did not always present a clearly distinguishable nucleus under UV-excitation. The nuclear area was chosen because it was the darkest area of the cell, with the minimum autofluorescence

Subjective examination had suggested that eosinophils in particular exhibited two very different labeling profiles, and that the proportions of eosinophils in these groups varied with treatment (see **Results**). Since it was felt that this observation was biologically relevant, we examined images representing 40 labeled eosinophils divided equally between the higher and lower brightness levels. Figure 5 represents the distribution of cells with respect to brightness. It is important to recognize that these 40 cells do not represent a single treatment or infection state, but are selected to represent the range of brightness observed subjectively. Clearly, the cells segregate into two distinct groups on the basis of brightness, and while measurement of additional cells would undoubtedly have smoothed the curves shown and perhaps even partially filled the gap between the two groups, this is undoubtedly a case of two distinct profiles. These cells were the basis for our measurements of eosinophil brightness, and the distinction made later between high and low-level labeling. Additionally, eosinophils which were subjectively considered non-labeling were measured for comparison.

The same measurement procedure was carried out for mononuclear cells and mast cells, and 20 cells were counted for each of these cell types in both labeled and non-labeled states, except in the case of mast cells whose low numbers in the micrographs only allowed consideration of 13 cells, and which showed no labeling (see

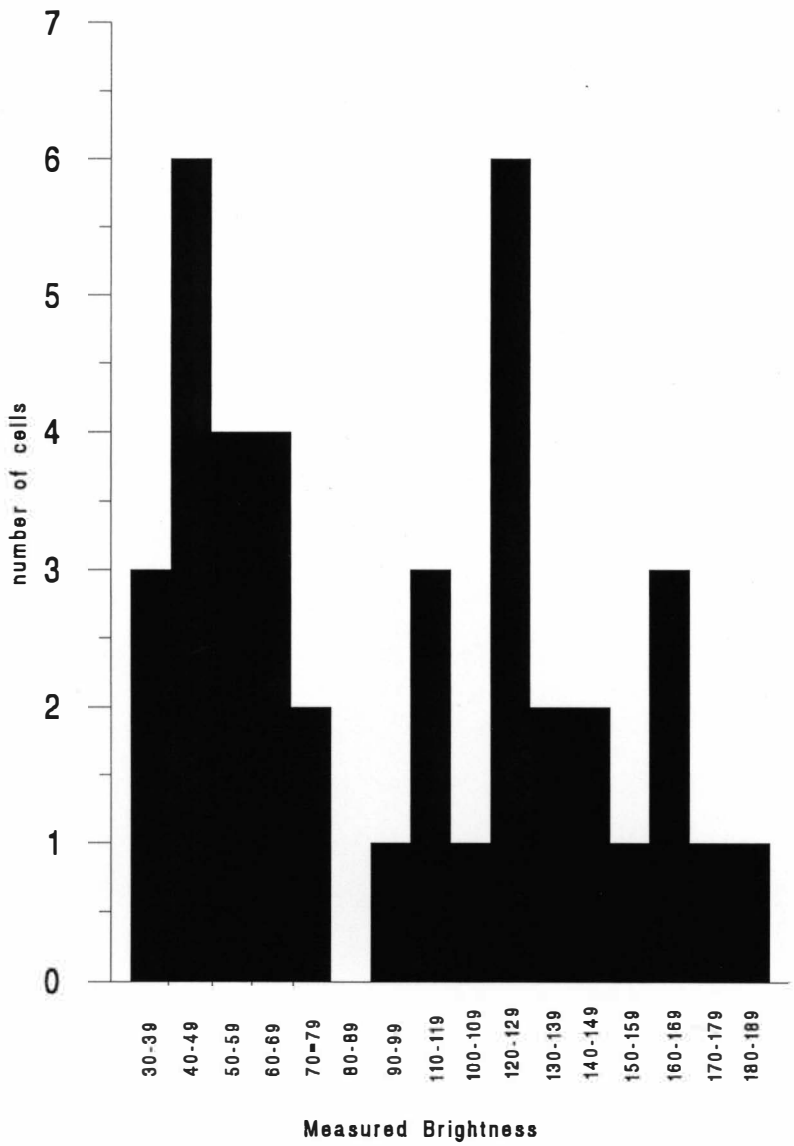


Figure 5. Distribution of Eosinophils With Respect to Brightness.

Results). Because all mast cells appeared non-labeled (see **Results**), additional measurements were taken from cell-free regions of these micrograph for brightness comparison.

There are several relevant points to consider regarding the image analysis portion of this study. It is important note that the brightness was measured as a unitless value, and not calibrated with a standard. Repeating these measurements with another microscope or even the same microscope on different settings would likely yield different values for brightness. Background (non-cell) brightness varied rather consistently between all groups from less than 1 to approximately 20, compared to a total study range of 1 to 180. Several factors could influence these brightness measurements, particularly length of exposure of the micrograph and small variations of the intensity of the backlighting over different areas.

Also, because each cell population has different inherent brightness under UV-excitation, comparisons should not be drawn between the different cell types; for example the highly labeled eosinophils were not necessarily twice as bright as the labeling mononuclear cells, even though the data might appear to indicate that this was the case.

RESULTS

Total numbers of cells in the peritoneal lavages of the infected group were elevated approximately 59% above the levels in the uninfected group (see Table 1 and Figure 6). Total cell numbers and proportions of the individual cell types were consistent with previous research (Eversole, 1996).

Morphological indications of the functional state of the cells were given by examination of DiffQuick®-stained cells with light microscopy (an example is shown in Figure 7). After 20 minutes of antigen-treatment, morphological changes were plainly visible in eosinophils and mast cells, which were consistent with degranulation, indicating a highly activated state. It was also possible to visualize granules from the eosinophils and mast cells within mononuclear cells, indicating phagocytosis. In all cases, eosinophils showed a marked morphological heterogeneity within each animal.

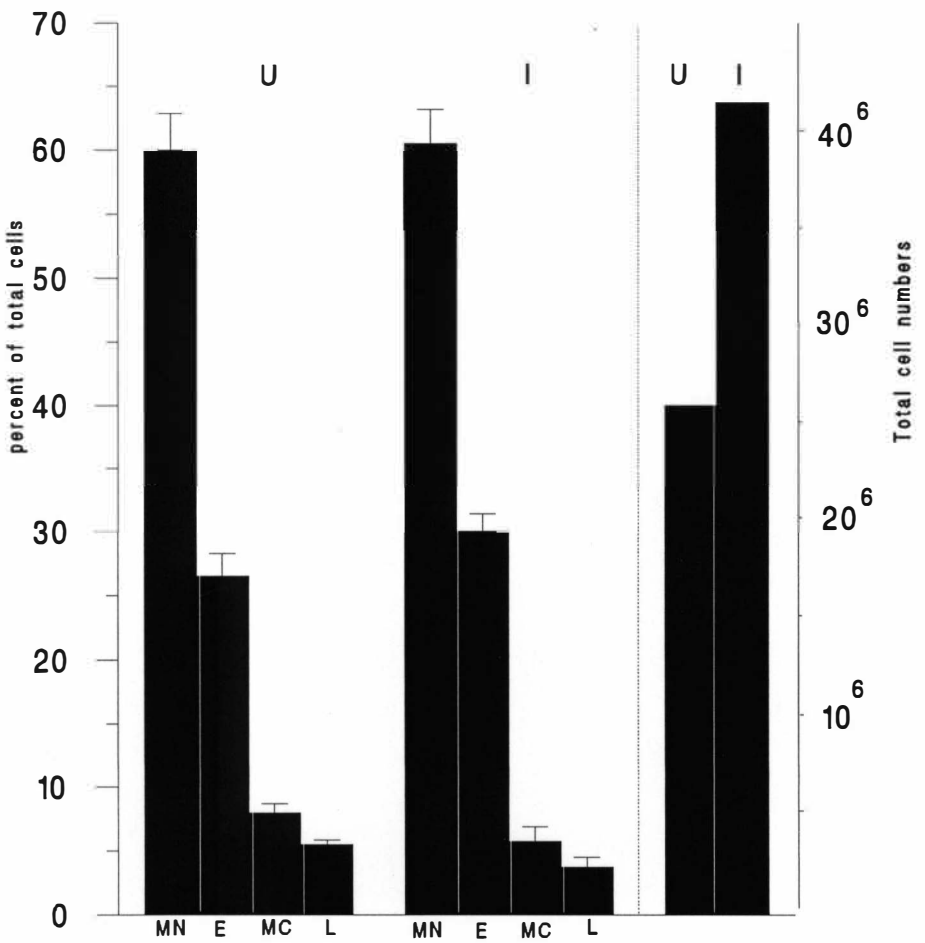
As with any antibody labeling procedure, there was some non-specific deposition of the fluorescent marker. Particularly in eosinophils, this non-specific staining occurred within cytoplasmic vacuoles. As these vacuoles increased during activation, the non-specific deposition of marker increased as well. True labeling was taken as a fluorescence over the entire surface of the cell, particularly covering the dark area of the nucleus. This label, when visualized by focusing through the depth of the cell, had a three-dimensional character which was not exhibited by the non-specific deposition.

Eosinophils from the non-infected animals did not express $\alpha_4\beta_1$ when initially removed from the animal, nor did large numbers of cells express this molecule after 20 minutes of incubation with antigen at 37°C (Figure 8, Table 2). Figure 9 shows a

Table 1

Total Cell Numbers and Differential Counts

	Uninfected (n=2)			Infected (n=4)		
	Mean	Standard Dev.	Range	Mean	Standard Dev.	Range
Monocytes	60	4	56-64	60.5	4.33	53-63
Eosinophils	26.5	2.5	24-29	30	2.74	26-33
Mast Cells	8	1	7-9	5.75	2.78	3-8
Small Lymphocytes	5.5	.5	5-6	3.75	1.48	2-6
Total Cells (in millions)	25.75	1.75	24-27.5	41	5.04	34.63-45.13



Legend MN=Mononuclear Lymphocyte, E=Eosinophil, MC=Mast Cell, L=Small Lymphocyte, U=Uninfected Animal, I=Infected Animal.

Figure 6. Numbers of Cells in the Peritoneal Cavities of Test Animals.

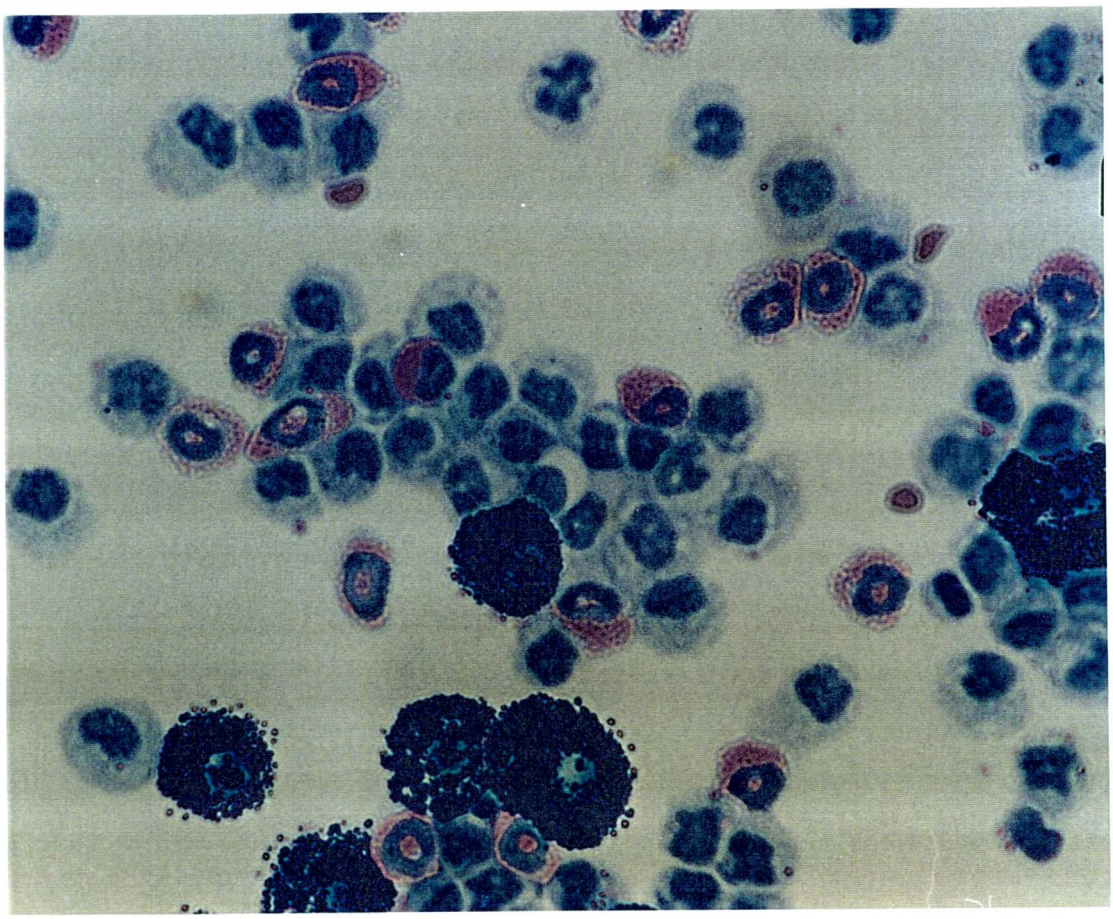


Figure 7. Differentially Stained Peritoneal Lavage From Uninfected AUG Rat.

negative control, which had no primary antisera, and for comparison, Figures 10 & 11 show a slide which was run through the entire assay but is considered to be non-labeling. Although not causing dramatic expression of $\alpha_4\beta_1$ integrin, addition of *Nb* antigen to the cell preparation did have an effect on overall eosinophil morphology and apparent activation profile.

Eosinophils which had come from infected animals, however, showed significant amounts of $\alpha_4\beta_1$ upon removal from the animal, and that amount increased after 20 minutes of incubation at 37°C (Figures 8, 12-17). Incubation with antigen increased the percentage of cells labeling to a greater extent than incubation without

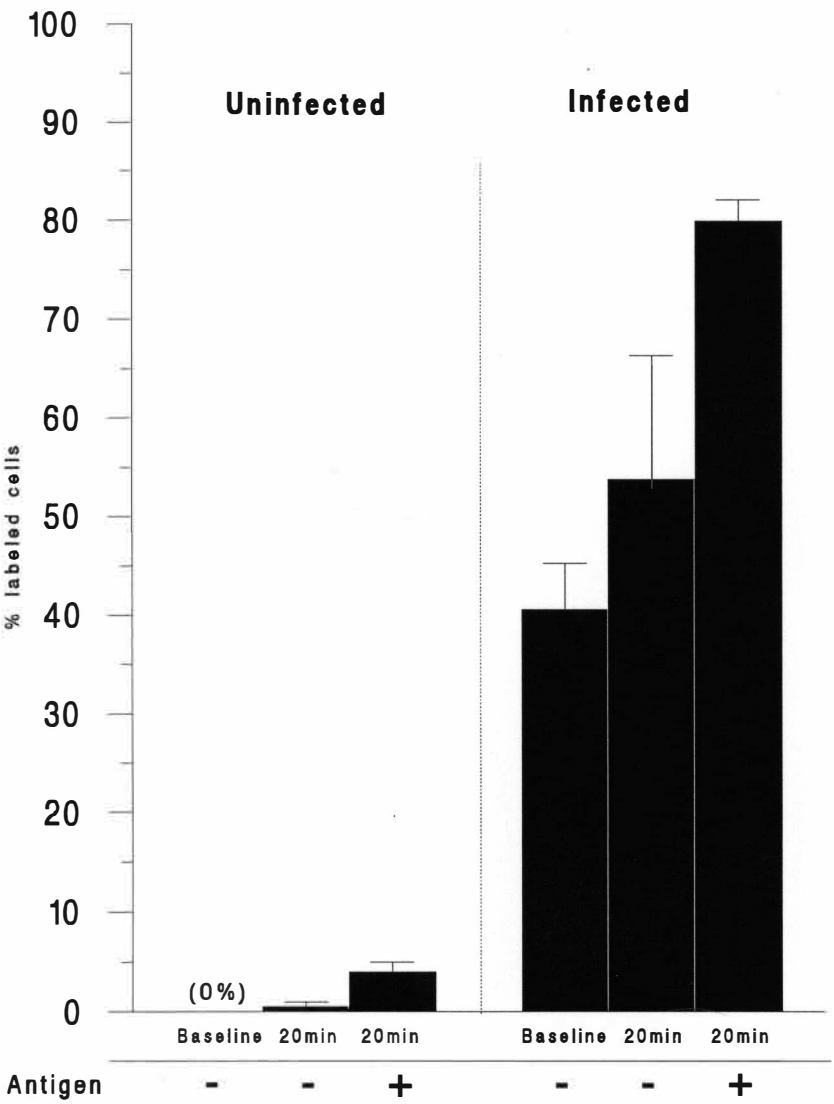


Figure 8. Percentage of Eosinophils Labeled Under Various Conditions.

Table 2

Percentage of Cells Showing Label

	Uninfected Baseline (n=2)			Infected Baseline (n=4)		
	Mean	Standard Dev.	Range	Mean	Standard Dev.	Range
Monocytes	1.5	0.5	1-2	21.25	7.12	12-30
Eosinophils	0	-	-	40.5	8.14	31-52
Mast Cells	0	-	-	0	-	-
	Uninfected 20 min incubation (n=2)			Infected 20 min incubation (n=3)		
	Mean	Standard Dev.	Range	Mean	Standard Dev.	Range
Monocytes	17	3.0	14-20	49	14.35	29-62
Eosinophils	0.5	0.5	0-1	53.67	17.75	34-77
Mast Cells	0	-	-	0	-	-
	Uninfected 20 min with antigen (n=2)			Infected 20 min with antigen (n=4)		
	Mean	Standard Dev.	Range	Mean	Standard Dev.	Range
Monocytes	22	1.0	21-23	60.25	9.42	49-71
Eosinophils	4	1.0	3-5	79.75	1.92	77-82
Mast Cells	0	-	-	0	-	-

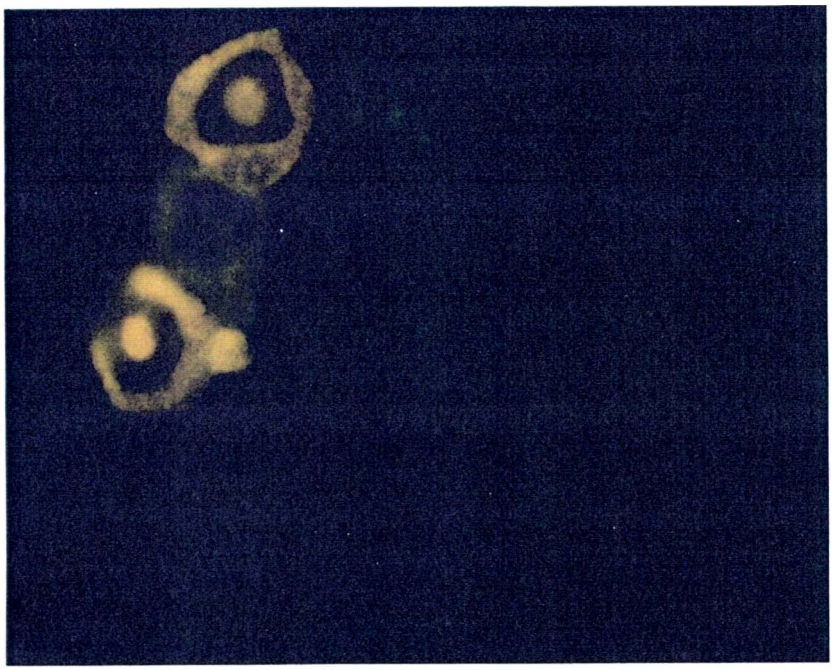


Figure 9. Epifluorescent Micrograph of Negative Control (no primary antisera).

antigen (Figure 8).

Image analysis confirmed that the cells which were considered labeled had a higher brightness over the nucleus than those which had been designated non-labeled (see Figure 18 and Table 2). It should be noted that two distinct groups of labeled eosinophils were observed during data collection. The first group had a lower level of fluorescent intensity with a mean measurement of approximately 55, than the second, more intense group, which had a mean of approximately 137 (see Table 3 and Figure 5). The few labeling eosinophils from non-infected animals were all of the low-label variety. In cells from the infected animals, less than 10% of the labeling eosinophils were initially of the high-label variety, whereas after incubation with antigen, greater than 50% of the labeling eosinophils were in the high-label condition (data not shown).

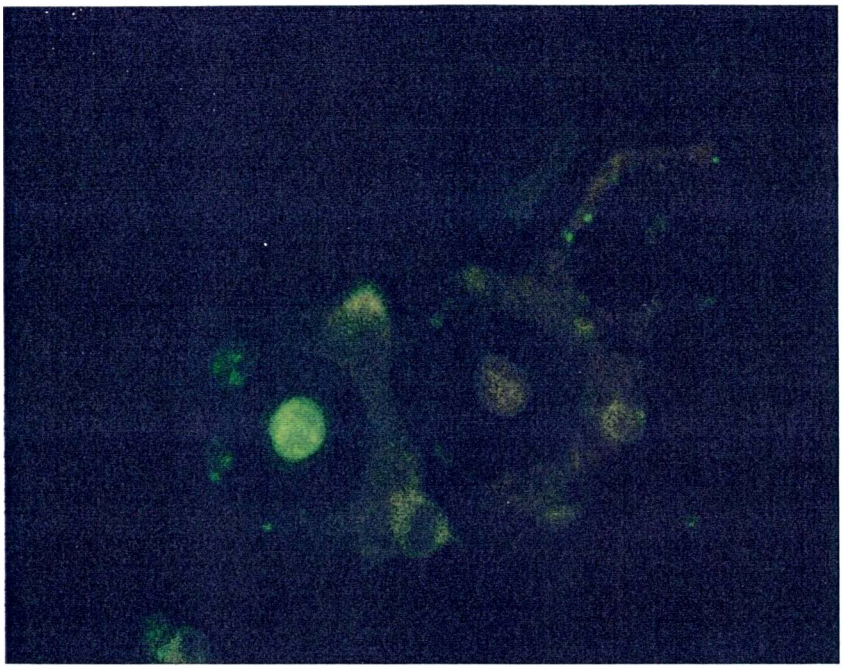


Figure 10. Epifluorescent Micrograph of Non-labeling Cells.
This micrograph shows cells from an uninfected animal which have been incubated with *Nb* antigen for 20 minutes at 37°C. While there is non-specific deposition of marker, it is clearly distinguishable from the labeling which is shown in subsequent micrographs.

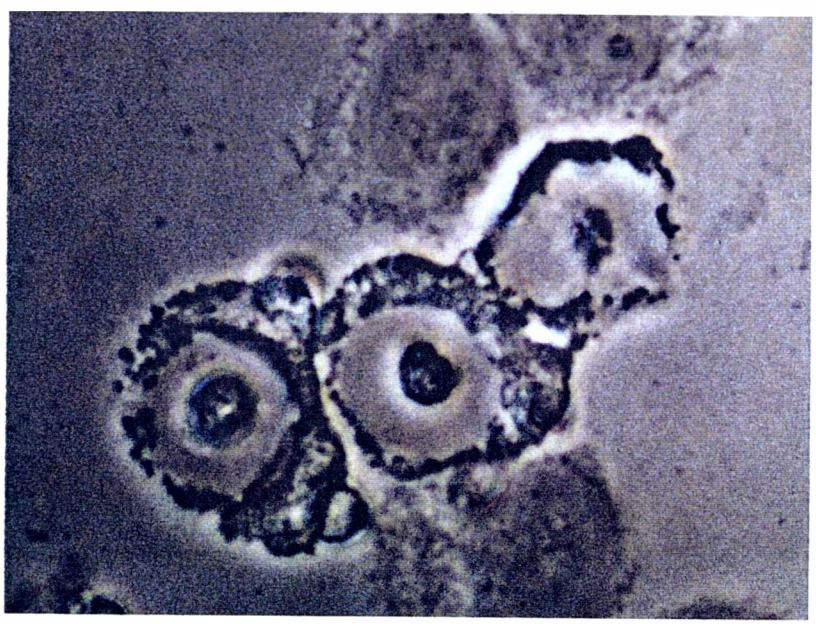


Figure 11. Phase-contrast Micrograph of Same Field-of-view Shown in Figure 10.

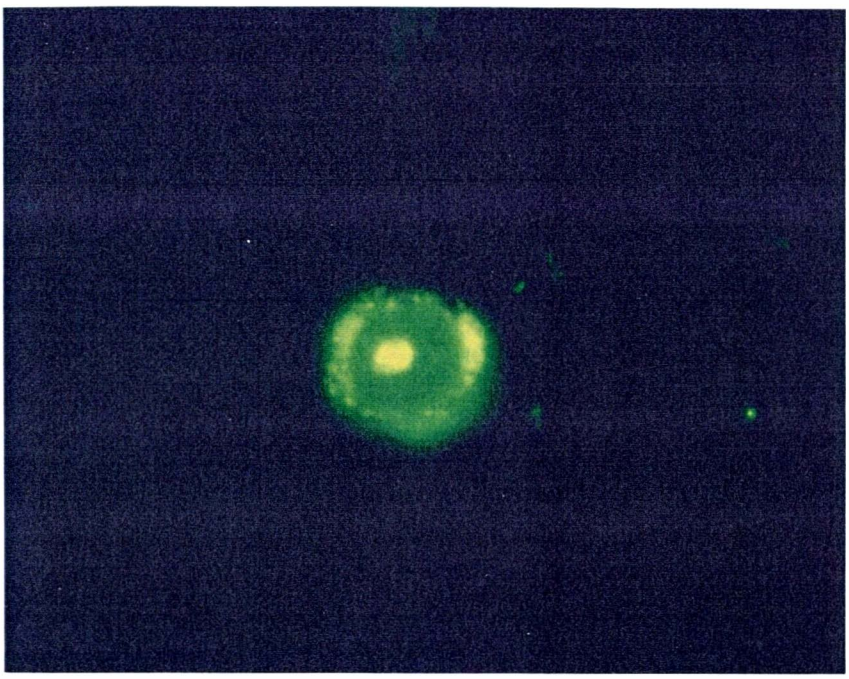


Figure 12. Epifluorescent Micrograph of High-label Eosinphil.
This micrograph shows an eosinophil from an infected animal which had been exposed to antigen for 20 minutes at 37°C. Note how the label is spread evenly over the surface of the cell, including the nucleus.

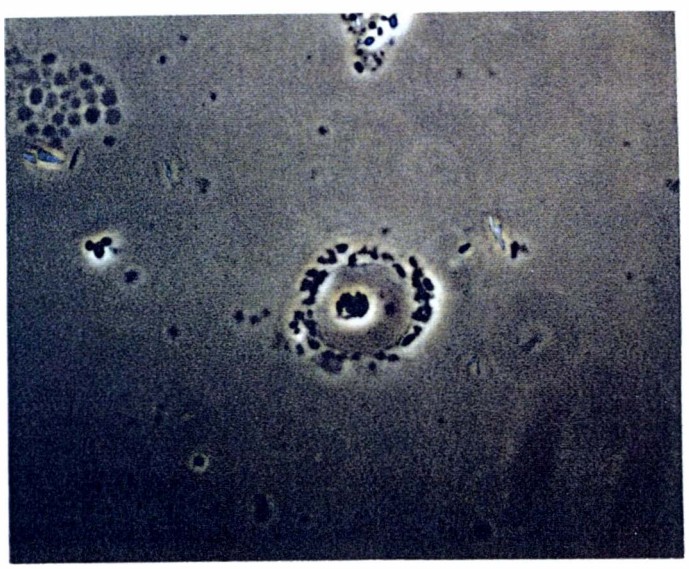


Figure 13. Phase Contrast Micrograph of Same Field-of-view Shown in Figure 12.

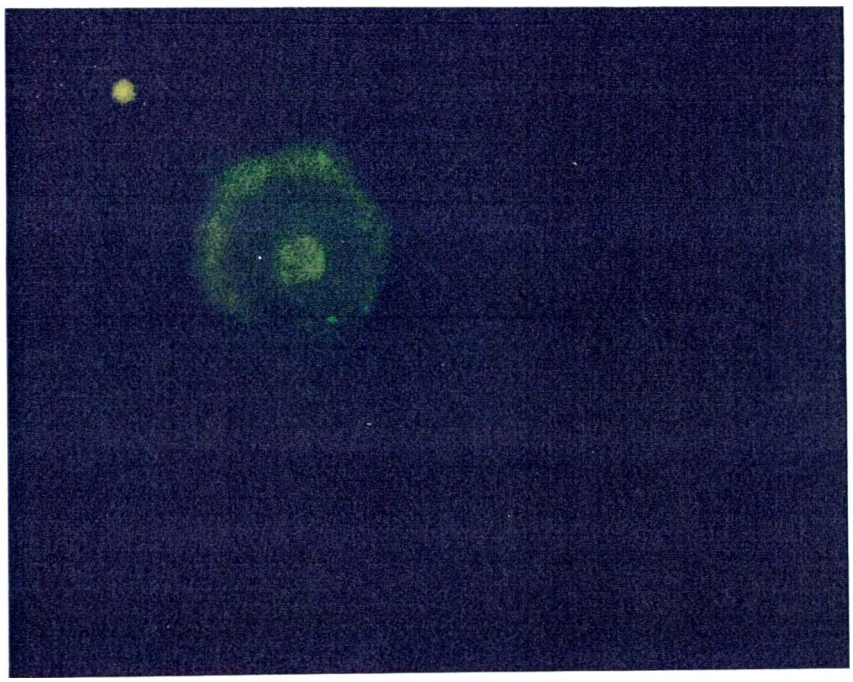


Figure 14. Epifluorescent Micrograph of Low-label Eosinophils.
This micrograph is included to show the appearance of low-label eosinophils. This micrograph shows an eosinophil from an infected animal which had been exposed to Nb antigen for 20 minutes at 37°C.

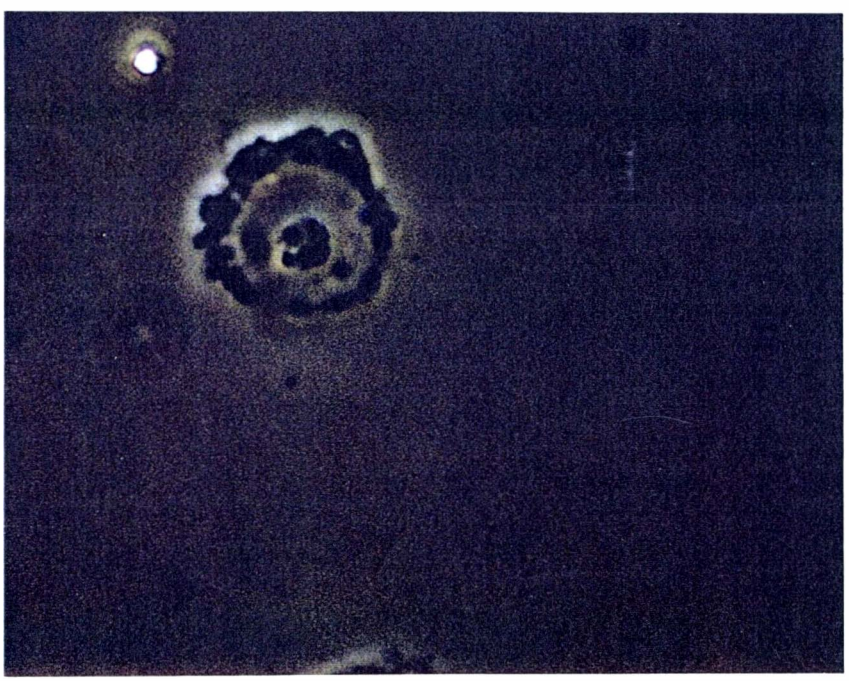


Figure 15. Phase-contrast Micrograph of Same Field-of-view Shown in Figure 14.

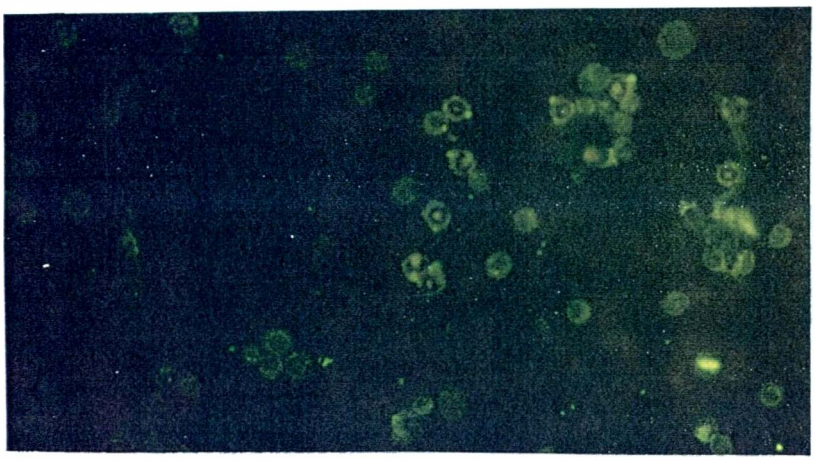


Figure 16. Low Magnification Epifluorescent Micrograph of Labeled Eosinophils Before Antigen Challenge.

This micrograph shows cells from an infected animal with no additional treatment. There are labeled cells in this micrograph, however the low magnification makes visualization of their low label difficult.

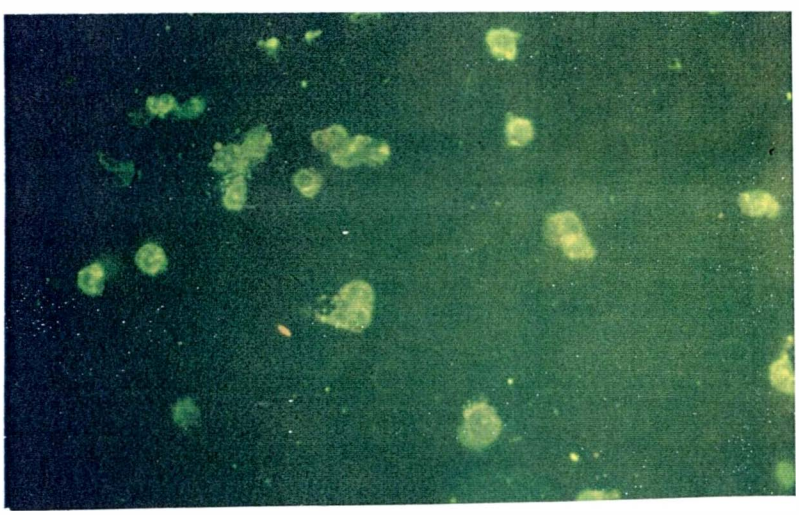


Figure 17. Low Magnification Epifluorescent Micrograph of Labeled Eosinophils After Antigen Challenge.

This micrograph shows cells from an infected animal after 20 minute expoure to Nb antigen at 37°C. Despite the low magnification, the highly labeled cells are plainly visable.

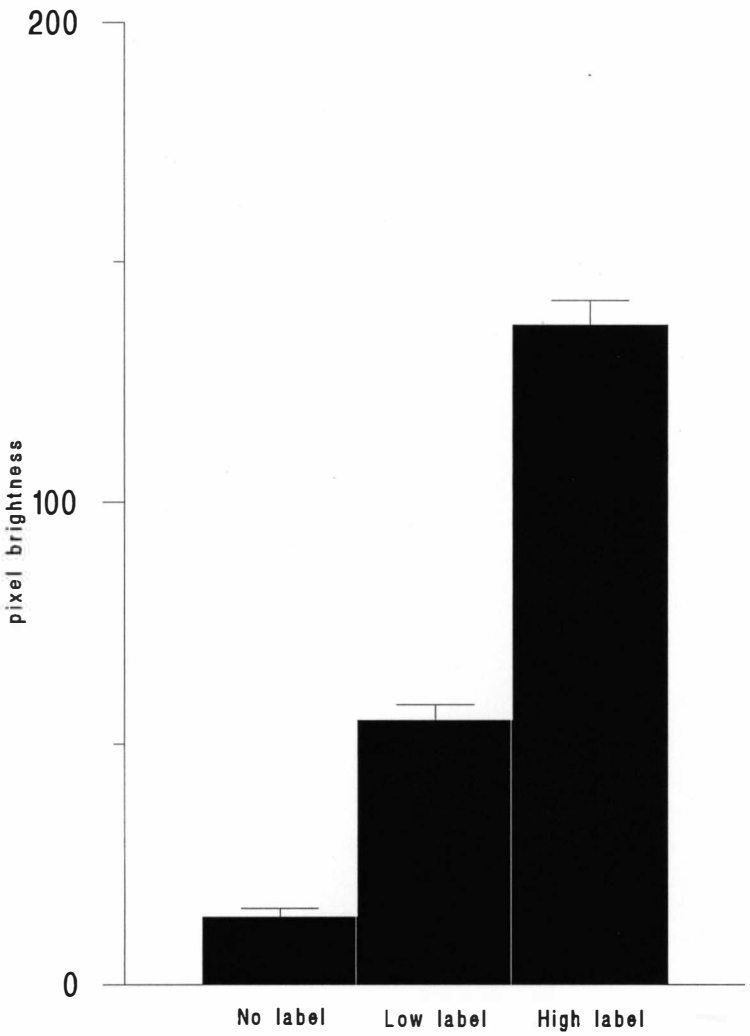


Figure 18. Image Analysis of Eosinophil Brightness.

Table 3

Brightness Levels as Determined by Image Analysis

	Mean	Standard Dev.	Range
Monocytes (neg)	14.9	9.41	2.4-39.8
Monocytes (pos)	59.8	42.17	19.8-164.2
Eosinophils (neg)	13.9	7.56	3.6-28.8
Eosinophils (low)	55.0	13.62	35.4-90.0
Eosinophils (high)	176.7	22.29	102.8-180.4
Mast Cells	9.0	5.08	2.0-20.0
Background	6.8	4.79	1.4-12.8

Because it was not always possible to distinguish between the various types of mononuclear cells with certainty, they were considered as a group. Small lymphocytes, which were grouped with the other types of mononuclear leukocytes, served as the positive control in this experiment, as some subsets of this population are known to express $\alpha_4\beta_1$ integrin (Pigott & Power, 1993). Additionally, previous trials in our laboratory had shown some of these cells to label, even under conditions in which no other cells in the preparation showed any detectable label.

While there were a limited number of mononuclear lymphocytes which showed labeling in the non-incubated cells from uninfected animals, the label was distinct and easily visualized, and was limited to cells which were smaller than those which were obviously macrophages. With incubation, the numbers of labeling mononuclear cells increased (Figure 19), but it was beyond the scope of this study to determine which specific cell types were most affected.

Figure 20 shows what appear to be two different types of mononuclear lymphocytes; the smaller cell, which shows a more intense label, was the type of cell used as a positive control, while the lager, less intense cell appears to be a macrophage. Figure 20 shows cells from an uninfected animal which had been incubated with no antigen. Mononuclear lymphocytes from infected animals showed significantly higher labeling initially, and the percentage of cells labeling increased significantly with incubation, although incubation with antigen was not clearly more effective in eliciting $\alpha_4\beta_1$ integrin expression on these cells than incubation alone (Figures 19, 21-22).

Image analysis of mononuclear cells revealed that differences in brightness were detectable between cells considered labeled and those considered non-labeled (see Figure 23). Within the labeled cells, there were examples of cells which were

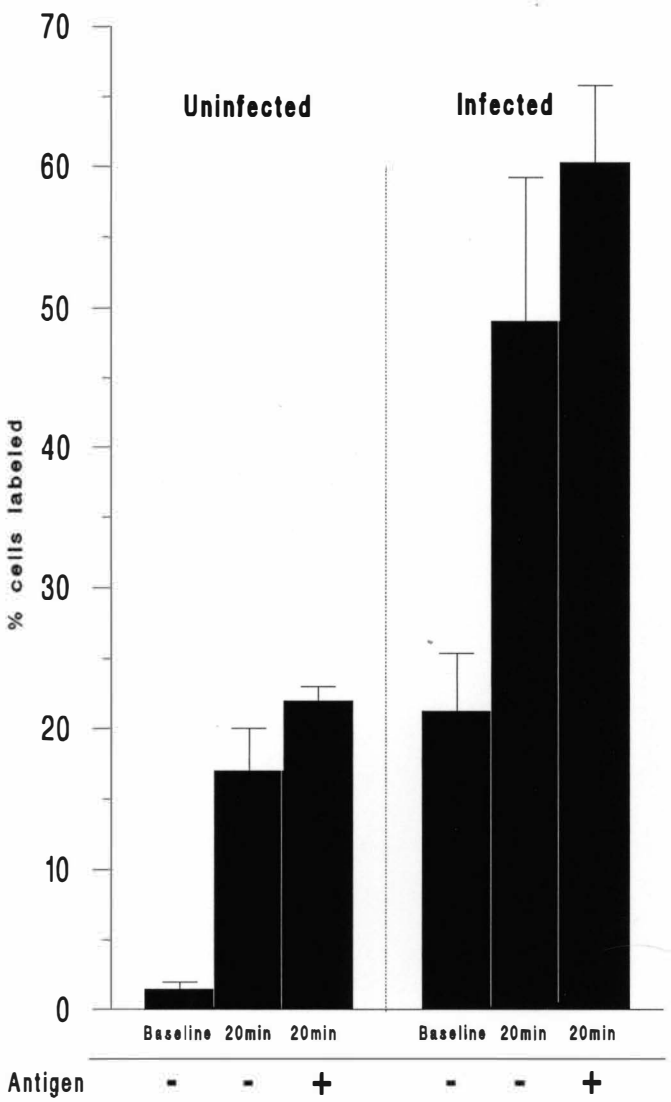


Figure 19. Percentages of Labeled Mononuclear Lymphocytes.

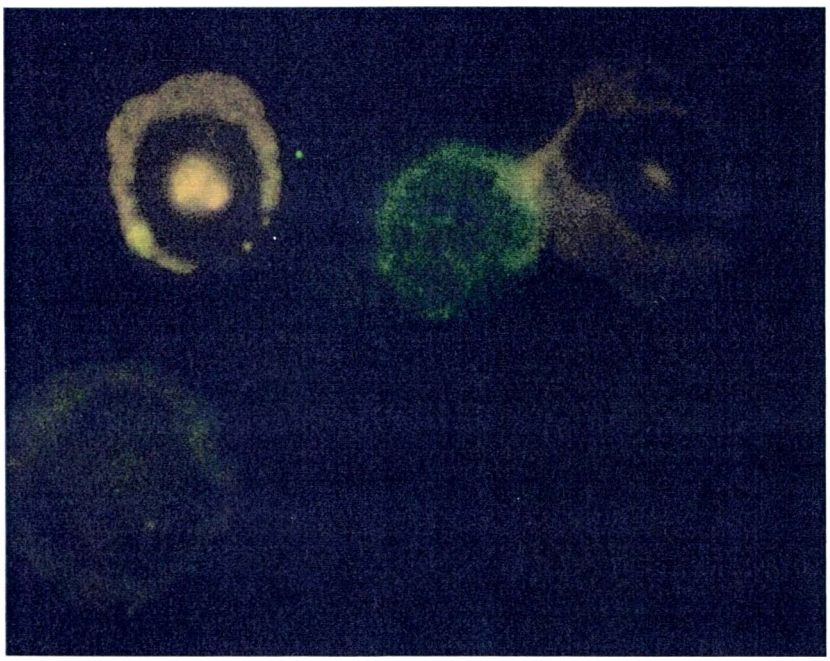


Figure 20. Epifluorescent Micrograph of Labeled Mononuclear Lymphocyte. This micrograph is from an uninfected animal with 20 minutes of incubation at 37°C, but no *Nb* antigen. This micrograph shows several cells, including two unlabeled eosinophils, a large mononuclear cell, perhaps a macrophage, and a distinctly-labeled small lymphocyte. These small lymphocytes were the cells which served as a positive control in this experiment.

over twice as bright as the average, but these cells were too few to consider separately, so they were incorporated into the group of all labeling mononuclear cells.

Mast cells did not label in this assay. Figure 24 shows a group of cells under phase-contrast microscopy, while Figure 25 shows the same group of cells under UV-excitation, with the mast cell becoming virtually invisible. Figure 26 shows the relationship between the brightness of mast-cells under UV-excitation and cell-free regions of the same micrographs (see also Table 2). Interestingly, the image analysis equipment was able to detect the minute differences in brightness between mast cell and background even when these differences were not visible to the eye.

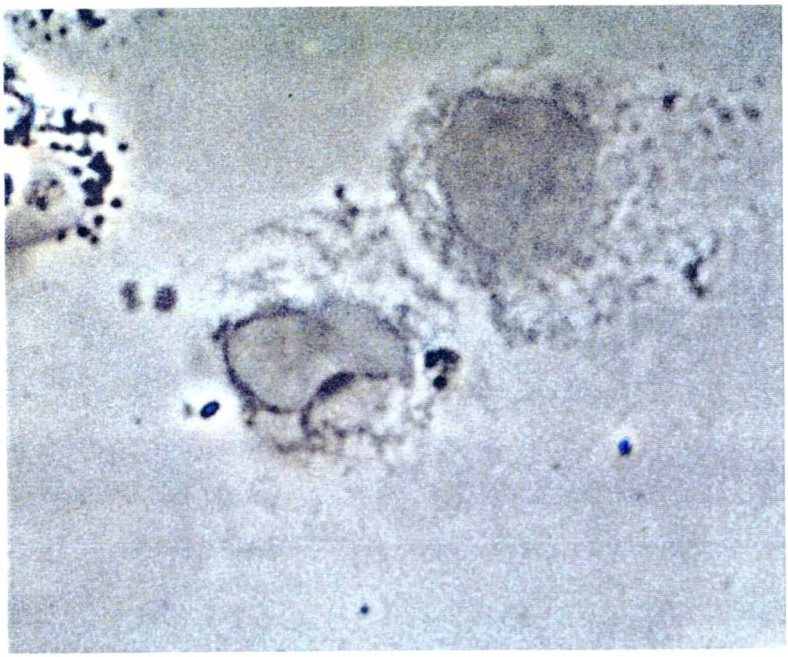


Figure 21. Epifluorescent Micrograph of Highly Labeled Mononuclear Cells. This micrograph shows cells from an infected animal which had been incubated with *Nb* antigen for 20 minutes at 37°C.

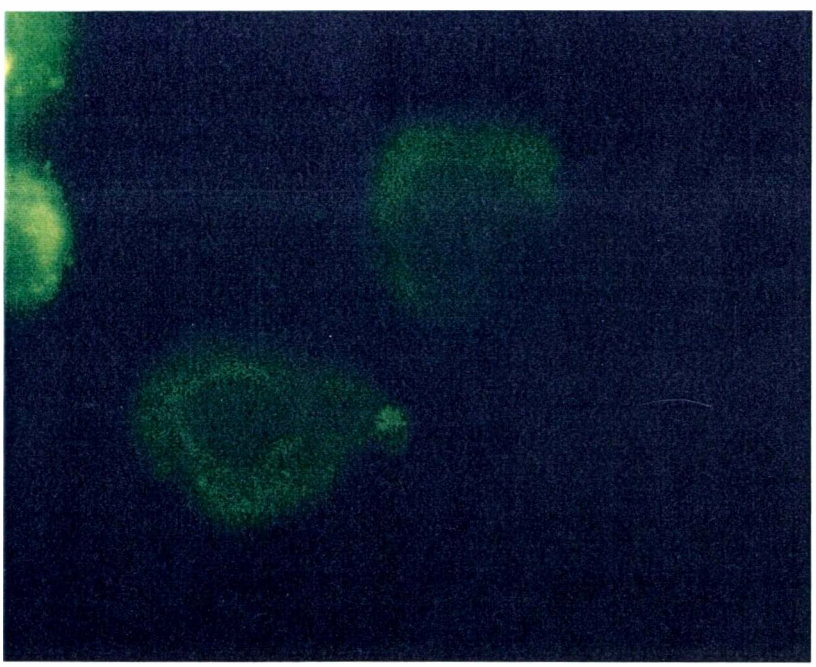


Figure 22. Phase Contrast Micrograph from the Same Field-of-view as Figure 21.

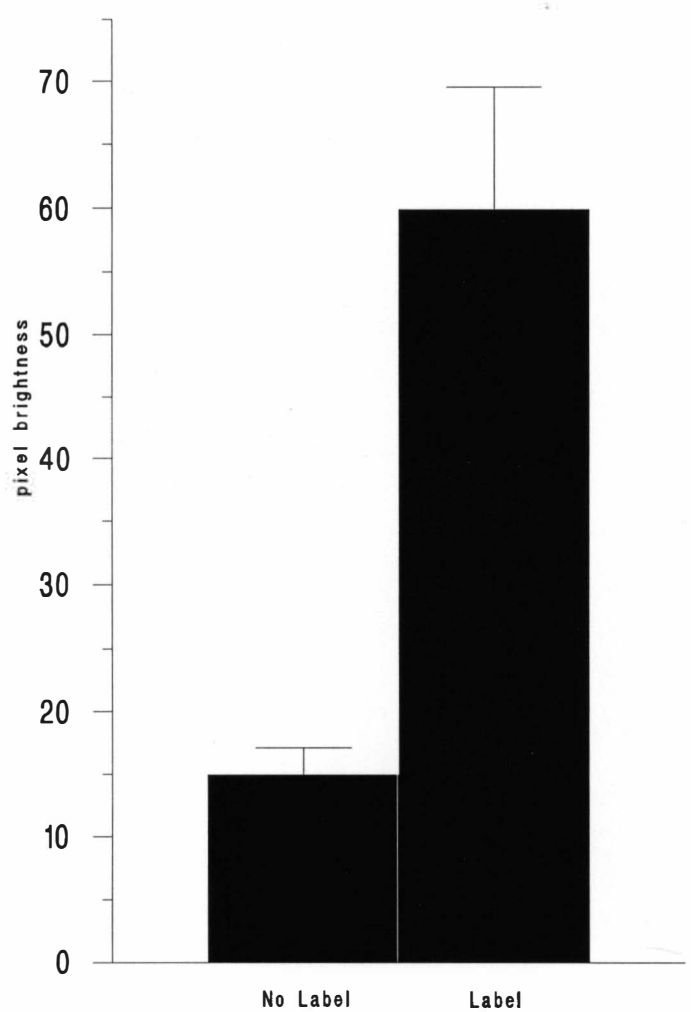


Figure 23. Image Analysis of Mononuclear Leukocyte Brightness.

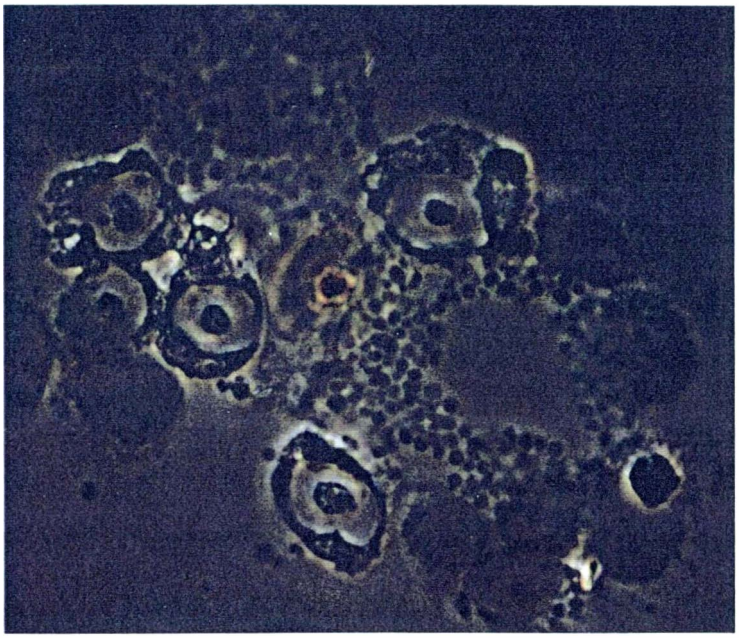


Figure 24. Phase Contrast Micrograph of Peritoneal Leukocytes, Including a Mast Cell.

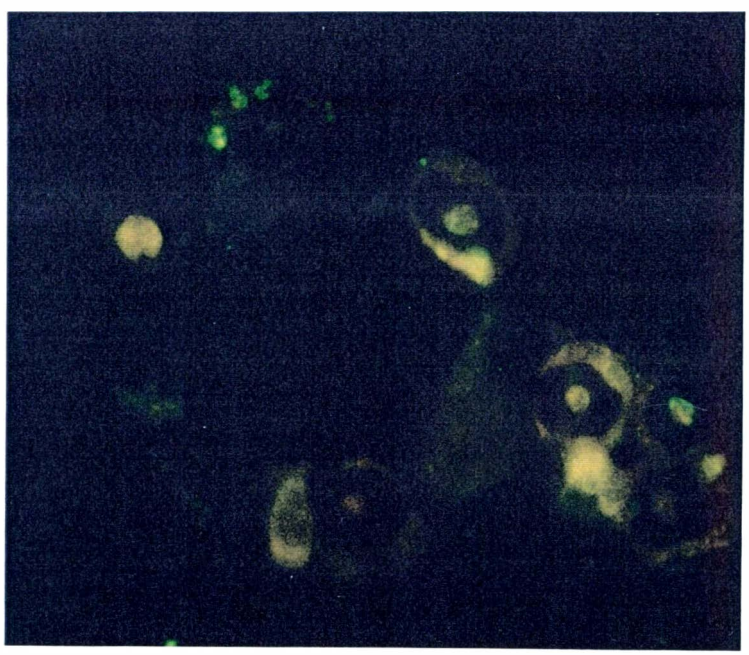


Figure 25. Epifluorescent Micrograph of the Same Field-of-view as Figure 24.

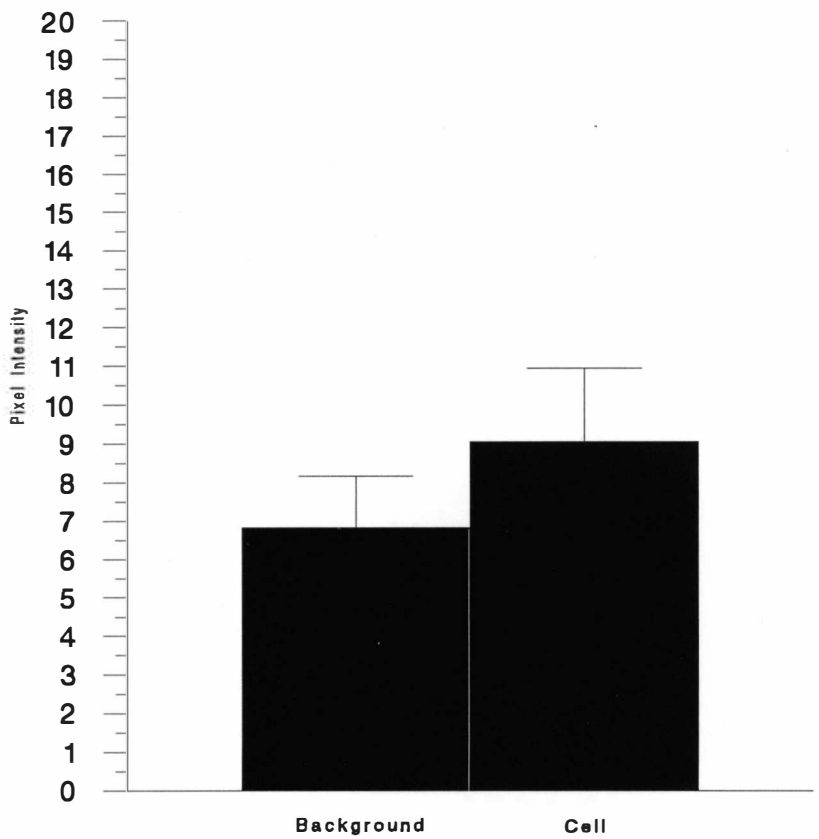


Figure 26. Image Analysis of Mast Cell Brightness Compared to Background.

DISCUSSION

In this study, we have shown that the mAb designated as TA-2 is useful for *in vitro* immunohistochemical protocols using AUG rat eosinophils. We have also developed a novel protocol for labeling experiments using AUG rat peritoneal leukocytes *in vitro*, with emphasis on eosinophils. This protocol can now be used, with the addition mAb against other cell-surface molecules, to continue research on this important cell population. Previous studies of eosinophil adhesion molecule expression, which focused solely on binding phenomena, do not address specific expression of the $\alpha_4\beta_1$ molecule itself. As described earlier, cells appear capable of regulating integrin expression at the cell surface and integrin/ligand binding independently. Our method of direct visualization offers a straightforward means of localizing adhesion molecule expression *in situ*, which will be important to further understanding of the detailed mechanisms of eosinophils' cell-cell and cell-ECM interactions.

Furthermore, this study confirms that rat eosinophils express $\alpha_4\beta_1$ integrin, and that this expression can be increased with appropriate stimuli. It is interesting that in our model, few eosinophils from uninfected animals showed significant amounts of $\alpha_4\beta_1$, nor did large numbers of these cells express this molecule after *in vitro* exposure to *Nb* antigen, while eosinophils from infected animals not only showed more of the molecule directly from the animal, but also expressed more $\alpha_4\beta_1$ after antigen challenge *in vitro*. This points to some form of *in vivo* functional priming connected with the infection which was required for $\alpha_4\beta_1$ expression by the cells. Previous studies in our lab (Phipps, unpublished observations) have used a number of treatments

including antigen, extracellular matrix molecules, and specific culture techniques, in attempts to stimulate eosinophils *in vitro* to produce $\alpha_4\beta_1$. None of the protocols we have tried has resulted in a level of expression even remotely comparable to the levels produced by the eosinophils from the infected animals. The exact mechanism of this *in vivo* priming remains unknown. It is worth noting that eosinophils in our preparation have shown that degranulation and $\alpha_4\beta_1$ integrin production can be controlled independently, since cells from non-infected animals showed morphological indications of degranulation, but did not express $\alpha_4\beta_1$.

If our contention that an unknown and possibly complex priming mechanism is necessary for $\alpha_4\beta_1$ integrin expression, how then does one account for studies describing *in vitro* binding of eosinophils? In one well-known study, human eosinophils were shown to bind to fibronectin-coated plates, with optimum binding taking place at one hour. The fact that this binding was significantly inhibited by the anti-$\alpha_4\beta_1$ mAb HP2/1 shows rather strongly that it was mediated by $\alpha_4\beta_1$. The source of the eosinophils, however, helps to explain this rapid binding: human eosinophils were obtained from the blood of donors with allergy or asthma-associated eosinophilia which suggests, on the basis of our results, that the cells had already undergone some form of functional alteration. While this study of blood-derived eosinophils is useful in illuminating how $\alpha_4\beta_1$ mediates a specific signal transduction mechanism, it is of doubtful usefulness in understanding eosinophil expression of the $\alpha_4\beta_1$ integrin. This process for eosinophil collection is found throughout the literature. In other studies, peripheral blood eosinophils from non-eosinophilic individuals are used, but separation methods such as density gradients preferentially select eosinophils with specific morphological profiles which may be linked to some form of activation. One advantage of our animal model is that it uses a heterogeneous cell population.

Our study cultured cells with immune serum from rats which had been infected with Nb 14-21 days. This serum presumably contained antibodies or other factors which may have had some effect on the eosinophils. Eosinophils are known to possess receptors for several classes of Ig's (reviewed in Giembycz & Barnes, 1993). It would be interesting to determine what if any change would occur in the results if various factors were removed from the serum. Similarly, our *Nb* antigen consisted of whole killed worms, which certainly contain numerous factors, both soluble and insoluble. The eosinophils in our preparation could have been reacting to a few very specific antigens, or in a more general way toward the combination of many antigens.

Although we demonstrated the presence of $\alpha_4\beta_1$ on mononuclear leukocytes the assay in its present state is not optimized for the study of these cells. Future investigations would be improved with the addition of some staining protocol or other method for discrimination of the different subsets of this cell population.

Although other investigators (Yasuda, Hasunuma, Adachi Sekine, Sakanishi, Hashimoto, Ra, Yagita & Okumura, 1995), found $\alpha_4\beta_1$ on phorbol ester-stimulated mast cells from the peritoneal cavities of rats using the anti-rat α_4 mAb MRalpha4-1, in our model of immune cell stimulation, mast cells did not at any time express $\alpha_4\beta_1$ integrin, even at incubation times up to 1 hour (Phipps, unpublished data). The reason for this remains unclear, but may be due to differences in species, stimulation or epitope recognition.

Even with the obstacles encountered in using computer-assisted image analysis to study this model, we were able to demonstrate very significant differences between what we considered labeled and non-labeled cells, thus lending an objective component to our assessment of cell labeling. Subjective observation had indicated that there were within the group of all labeling eosinophils some which showed dramatically

higher label, but without the aid of the image analysis system, this difference would have been difficult to describe in a meaningful way.

The results of this study lead to several interesting questions. What mechanisms are involved in the *in vivo* functional priming of the eosinophils in the infected animals? Were there factors in the immune serum which contributed to increased $\alpha_4\beta_1$ integrin expression? And, perhaps more importantly, are these mechanisms and factors part of the normal paradigm of eosinophil-mediated immunity?

In conclusion, we have developed an effective assay for localization of $\alpha_4\beta_1$ integrin on eosinophils from the peritoneal cavity of the AUG rat, which allows us to determine with confidence whether or not this molecule is being expressed on these cells. Our model provides an important means through which the expression of adhesion molecules, in particular $\alpha_4\beta_1$ on eosinophils, and the interactions between the extracellular matrix and this important class of adhesion molecules can be investigated.

Furthermore, using this new technique we have clearly shown a connection between *Nb* infection and the expression of $\alpha_4\beta_1$ integrin on tissue eosinophils and lymphocytes in this animal model. Finally, we have demonstrated that an *in vitro* exposure of peritoneal leukocytes to antigens from *Nb* resulted in an increased expression of $\alpha_4\beta_1$ integrin by cells from infected animals which dramatically exceeded the expression by identically exposed cells from uninfected animals.

Appendix A

Protocol Clearance From the Institutional Animal Use and Care Committee

IACUC Number 94-08-01
Date of Receipt 8/3/94
Date of Approval 8/29/94

WESTERN MICHIGAN UNIVERSITY
INSTITUTIONAL ANIMAL CARE AND USE COMMITTEE (IACUC)

Application to use Vertebrate Animals for Research or Teaching

The use of any vertebrate animals in research and/or teaching without prior approval of the Institutional Animal Care and Use Committee (IACUC) is a violation of Western Michigan University policies and procedures. This Committee is charged with the institutional responsibility for assuring the appropriate care and treatment of vertebrate animals.

Mail the signed original and five (5) copies of the typed application and any supplements to Research and Sponsored Programs, Room A-221 Ellsworth Hall, (616) 387-3670.

Any application that includes use of hazardous materials, chemicals, radioisotopoes or biohazards must be accompanied with SUPPLEMENT A.

Any application that includes survival surgery must be accompanied with SUPPLEMENT B.

R. Eversole	BIOS	387-5640
Principal Investigator/Instructor	Department	Campus Phone
Signature	Date 8/1/94	
L. Beuving	BIOS	387-5628
Responsible Faculty Member (if PI not faculty member)	Department	Campus Phone
Signature	Date 8/2/94	

Title of Project/Course Nippostrongylus brasiliensis infection in the August rat.

Check One: Teaching ____ Research X Other _____

I. ANIMAL USE CATEGORIES (check ONLY one category)

A. X Projects that involve little or no discomfort (including injections).

B. ____ Projects that may result in some discomfort or pain, but of short duration. Anesthetics, analgesics or tranquilizers will be used.

C. ____ Projects that may result in significant discomfort or pain. Anesthetics, analgesics, or tranquilizers will not be used.

Date Received 4/29/96
IACUC Number 96-08-01
First Renewal Request X
Second Renewal Request

WESTERN MICHIGAN UNIVERSITY
YEARLY RENEWAL FORM APPLICATION TO USE VERTEBRATE ANIMALS FOR RESEARCH OR TEACHING

GENERAL INFORMATION: Fill in all appropriate information

R. Eversole	BIOS	7-5640
Principal Investigator/Instructor	Department	Campus Phone
L Beuving	BIOS	7-5628
Responsible Faculty Member (if PI not faculty member)	Department	Campus Phone

Title of Project/Course Nippostrongylus brasiliensis infection in the August Rat

PRINCIPAL INVESTIGATOR/INSTRUCTOR DECLARATION

I assure that I have obtained IACUC approval prior to implementing this project and that there are no changes in the protocol submitted in the original application to use vertebrate animals for research or teaching. I understand that if at any time changes are made in the use of animals as described in the original application, a letter or amended protocol must be filed for review. I assure that the activities do not unnecessarily duplicate previous experiments.

Signatures:

Principal Investigator/Instructor — Date 4/29/96

Responsible Faculty Member (If PI not a faculty member) — Date 4-29-96

INSTITUTIONAL ANIMAL CARE AND USE COMMITTEE APPROVAL

IACUC Chairperson — Date 4-29-96

PLEASE MAIL COMPLETED APPLICATION TO:

Research and Sponsored Programs
Western Michigan University
301 Walwood Hall
Kalamazoo, MI 49008
(616) 387-8270

10/91

IACUC Number 94-08-01

WESTERN MICHIGAN UNIVERSITY
ANIMAL USE APPLICATION - SUPPLEMENT A
HAZARDOUS MATERIALS

NOTE: It is the principal investigator's responsibility to insure that all individuals who may come in contact with a project are aware of hazards involved in the project. Review and approval by Environmental Health and Safety office must be obtained before submission to IACUC.

R. Eversole	Electron Microscopist	387-5640
Principal Investigator/Instructor	Position	Campus Phone
BIOS	5330 McCracken	
Department	Address	

Title of Project: Nippostrongylus brasiliensis infection in the August rat.

Biohazards: (Please List)

Infectious Agent(s): Nippostrongylus brasiliensis
Route(s) of Administration: subcutaneously
Dosage(s): 3000 L3 larvae/animal: one dose
Route(s) or Excretion: Eggs by feces
Indicate species at risk and virulence of agent(s): Rats only: Low (mild diarrhea)
State specie(s) that will be exposed to the hazardous material: Rats
Method of disposal of contaminated animal tissue including carcass(es): Standard operating proc

Chemical Hazards: (Please List)

Agent(s) or Chemicals(s):
Route(s) of Administration:
Dosage(s):
Route(s) or Excretion:
Is(are) the Agent(s) or Chemical(s) known or suspected carcinogens?
Method of disposal of contaminated animal tissue including carcass(es):

Radioisotopes: (Please List)

Agent(s) or Chemicals(s):
Route(s) of Administration:
Dosage(s):
Route(s) or Excretion:
Time Period(s) for which the isotope(s) present(s) a Hazard:
Method of disposal of contaminated animal tissue including carcass(es):

Describe measures that will be taken to minimize the risks from all hazards indicated in this section. Specify the procedures that will be used to decontaminate equipment prior to routine washing.

All caging will be segregated from common animal facilities until study completion. Upon completion all equipment will be autoclaved to prevent egg contamination. This study will be conducted in isolation from other rodent species (1090 McCracken).

Reviewed and Approved by:

Manager, Environmental Health and Safety		Co-Director, Radiation Safety (If applicable)	
Pat M. Hatton	8/2/94		
Signature	Date	Signature	Date

II. ANIMAL USE FACILITIES

The animal(s) will be housed and maintained in accordance with the WMU Humane Care and Use of Animals Policies and Procedures.

Yes X No _____

If no, give explanation.

Please indicate the building and room(s) where the animal(s) will be housed and cared for as well as the location of the experiments and procedures if different from where housed.

1090 McCracken

III. ANIMAL USE SUMMARY

In language understandable to a layperson, summarize your primary aims and describe the proposed use of animals as concisely as possible. Bear in mind that the IACUC is primarily interested in the responsible, necessary, humane use of animals. Include a description of procedures designed to assure that discomfort and pain to animals will be minimized. It should include method of restraint; method of dosing with test compound; and methods of euthanasia or disposition of the animal after the experiment.

Six week old August rats will be injected subcutaneously with 3000 L3 stage larvae of the rat nematode Nippostrongylus brasiliensis in 5ml of saline. These animals will be killed by carbon dioxide inhalation on day 14 and day 21 post-infection. The cells of the peritoneal and vascular compartments will then be removed, counted and samples prepared for immunocytochemistry. The primary aims of this research is to ascertain the accumulation and activation status of the eosinophil leucocytes in the said compartments in this model of parasite immunity The dead animals will be disposed according to standard operating procedures. Deat will be assured by open thorax cardiac puncture prior to disposal.

Ref: Olgilvie, B.M., and Jones, V.E.(1971). Nippostrongylus brasiliensis: A review of immunity and the host/parasite relationship in the rat. Exp. Parasitol, 29, 138-177

IV. JUSTIFICATION FOR ALL ANIMAL EXPERIMENTS

Please provide a narrative with reference sources which addresses each of the following:

A. What assurance can be provided to indicate that the procedure is not duplicative?

Several searches done to date have provided no references in the National Library of Medicine of this model in the August rat.

B. Have non-live animal techniques (e.g. in vitro biological systems, computer simulation, audiovisual demonstration) been considered? Explain why they have not been utilized.

Yes. This model of parasite immunity requires in vivo study in rats.

C. Why has this species been selected for this procedure?

This rat possesses an inherent peritoneal eosinophilia and thus provides a unique opportunity to study eosinophil biology.

D. How many animals will be used in this project? How often will its procedures be done and over what duration?

Three groups of six animals for a total of 18 will be utilized for one infec to be run over the next few months.

E. In light of concern to minimize the number of animals used in experimentation, how will you determine the number of animals to be used?

An (N) of six for these types of in-vivo experiments has proven to be a statistical minimum. This study has 3 groups.

NOTE: Items F, G, H and I require the approval of the Consulting Veterinarian.

F. What is the anticipated pain or distress response of the animal; and what is the duration of discomfort? (Injections not included.)

Mild diarrhea is the only indicated pathology documented for this level of Nippostrongylus infection. Ten days.

G. How will the pain in the animal be monitored?

Daily behavioral observation

H. What sedative, analgesic, or anesthetics will be used, if any? Include dose, route and frequency of administration.

None

I. What is the justification if pain relieving drugs are not used?

Animals that display any adverse pain response will be killed. Death by carbon dioxide inhalation and open thorax cardiac puncture.

RD Sierio DVM

Signature: Consulting Veterinarian

8/29/94

Date

WESTERN MICHIGAN UNIVERSITY
INVESTIGATOR IACUC CERTIFICATE

Title of Project: Nippostrongylus brasiliensis infection in the August rat.

The information included in this IACUC application is accurate to the best of my knowledge. All personnel listed recognize their responsibility in complying with university policies governing the care and use of animals.

I declare that all experiments involving live animals will be performed under my supervision or that of another qualified scientist. Technicians or students involved have been trained in proper procedures in animal handling, administration of anesthetics, analgesics, and euthanasia to be used in this project.

If this project is funded by an extramural source, I certify that this application accurately reflects all procedures involving laboratory animal subjects described in the proposal to the funding agency noted above.

Any proposed revisions to or variations from the animal care and use data will be promptly forwarded to the IACUC for approval.

_____ Disapproved ✓ Approved _____ Approved with the provisions listed below

Provisions or Explanations:

IACUC Chairperson — Date: 8/29/94

Acceptance of Provisions

Signature: Principal Investigator/Instructor — Date

IACUC Chairperson Final Approval — Date

Approved IACUC Number 94-08-01

Rev. 3/92

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