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Estrogen Induced Eosinophil Migration into the August Rat Uterus is Associated with Intercellular Adhesion Molecule Expression

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ESTROGEN INDUCED EOSINOPHIL MIGRATION INTO THE AUGUST RAT UTERUS IS ASSOCIATED WITH INTERCELLULAR ADHESION MOLECULE EXPRESSION

by

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Estrogen causes an influx of eosinophils into the uterus in female rats. The diapedesis of eosinophils out of the vasculature has also been observed in allergic reactions, parasitic infections, and atopic dermatitis, and has been associated with cellular adhesion molecules (CAMs). Antibody blocking studies against ICAM-1/ LFA-1 and VCAM-1/VLA-4 have suggested that both ligand/receptor sets of CAMs are involved in the selective recruitment of eosinophils out of the vasculature. ICAM-1 is expressed on the vascular endothelium and decreases in expression when IL-4 is introduced. VLA-4 is present on eosinophils and is suspected to allow selective migration of eosinophils out of the blood system. Uterii of immature rats treated with either diethylstilbesterol (DES) or vehicle, were labeled with monoclonal antibodies against ICAM-1 and VLA-4. ICAM-1 expression decreased in DES treated animals and VLA-4 expression was up regulated on eosinophils within the uterus. Estrogens may signal eosinophils to increase VLA-4 expression while signaling uterine vasculature to decrease ICAM-1 expression, enabling selective eosinophil migration.

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STATEMENT OF THE PROBLEM

Estrogen is an ovarian hormone secreted in response to a CNS regulated pattern of follicle stimulating hormone (FSH) secretion. FSH is released from the adenohypophysis during the proestrous phase of the estrus cycle. During proestrus, plasma estrogen elevates and the uterus goes through many preparative steps for egg implantation. One of these steps may be the extravasation of eosinophils into uterine tissues. If estrogen is injected into an immature or ovariectomized rat, similar migration of eosinophils can be induced (Bjersing & Borglin, 1963). This study was designed to try to determine whether changes in two specific cellular adhesion molecules, ICAM-1 and VLA-4, were associated with migration of the eosinophil from the blood supply into the uterus during times of elevated blood estrogen. VLA-4 was chosen due to its putative role in selective migration of eosinophils out of the vasculature. ICAM-1 was selected because of its role in leukocyte migration during acute inflammation.

INTRODUCTION

The Eosinophil

The eosinophil is a white blood cell involved in many disease processes and has been generally associated with allergies and parasitic infections. These cells carry specific cytosolic granules that contain cationic proteins which have been shown to damage or destroy their target. Some of these targets include helminths, protozoa, and bacteria. Apart from challenging foreign organisms eosinophils play a role in cutaneous diseases. These include; syndromes associated with edema (i.e. Wells disease), dermatitides (i.e. atopic dermatitis), and syndromes associated with fibrosis (i.e. Spanish toxic oil syndrome) (Gleich et al., 1992a). It is well documented that the released granules of the eosinophil cause some of the major symptoms of asthma and other hypersensitivity diseases (Frigas & Gleich, 1986).

The granules in the cytoplasm of the eosinophil are electron dense and readily stain with acidic dyes such as eosin, from which the name eosinophil is derived. There are four major eosinophil granule proteins. The first is major basic protein (MBP), located in the core of the granule which has an isoelectric point of pH 10.9 (Gleich et al., 1992b). This protein is particularly damaging to parasites such as helminths and protozoa and can kill bacteria and mammalian cells. MBP also stimulates histamine release from mast cells and basophils and activates neutrophils and platelets (Gleich et

al., 1992b). Eosinophil Cationic Protein (ECP) is a very basic protein also, with an isoelectric point of pH 10.8. This protein is located in the eosinophil granule matrix and is a member of the ribonuclease gene superfamily. This gene family includes Eosinophil Derived Neurotoxin (EDN) and pancreatic ribonuclease. ECP is toxic to bacteria, helminths, protozoa, and mammalian cells. A less toxic granule protein is found in Eosinophil Derived Neurotoxin (EDN). EDN only weakly affects parasites and mammalian cells (Gleich et al., 1992b). This protein is extensively deposited along with MBP in atopic dermatitis (Leiferman, 1991). A fourth granule protein is eosinophil peroxidase (EPO). It is localized in the matrix of the eosinophil granules and has two mechanisms of toxicity. If there is a peroxide generating system available, e.g., NADPH oxidase, it oxidizes a halide producing a hypohalus acid. This peroxidase activity is very toxic to parasites, bacteria, viruses, microplasma and fungi. In the absence of a peroxide, EPO is still potent in killing parasites and mammalian cells via its toxic cationic effects; however, this is probably not its major mechanism (Gleich et al., 1992b). Another protein is a small constituent in the granules called the Charcot Leyden Crystal protein (CLC). This protein has a lysophospholipase, activity but the role that it plays in disease is still undetermined (Gleich et al., 1992a).

Eosinophils also synthesize many different inflammatory mediators. These mediators signal other immune cells as well as the vascular endothelium. Platelet activating factor (PAF) is one of these mediators. PAF causes an increase in cutaneous vascular permeability, and bronchoconstriction. PAF is also an eosinophil

chemoattractant (Gleich et al., 1992a). Leukotriene C_4 (LTC_4) is produced by eosinophils and causes bronchoconstriction and changes vascular permeability (Gleich et al., 1992a). Eosinophils also produce other leukotrienes (LTA_4, LTB_4, LTD_4, and LTE_4), but the dominant product is LTC_4 (Weller, 1991). Other inflammatory mediators that eosinophils can produce are; transforming growth factor-alpha (TGF-α) (Wong et al., 1990), transforming growth factor-beta (TGF-β) (Wong et al., 1991), interleukin-1 (IL-1) (Del Pozo et al., 1990), granulocyte macrophage colony stimulating factor (GM-CSF) (Moqbel et al., 1991), interleukin-5 (IL-5) (Desreumaux et al., 1992), and interleukin-3 (IL-3) (Kita et al., 1991). It is not now possible to describe the secretory response pattern of the eosinophil to specific stimuli. It seems better to comment that depending on the stimulus, as well as the surrounding cells, eosinophils may elicit events beyond their effector functions of degranulation, oxidative burst, and eicosanoid release.

Enhancement in the ability of eosinophils to produce proinflammatory mediators is caused by many cytokines and inflammatory mediators. Included in these activators are mononuclear derived factors; IL-5, GM-CSF, IL-3, and tumor necrosis factor-alpha (TNF-α) (Silberstein et al., 1989). Some other eosinophil activators are the complement component, C5a (Keman et al., 1991), and PAF (Kroegel et al., 1989). Activation also includes an increased ability to bind to endothelium. Adhesion of eosinophils to endothelium can be increased with PAF, IL-5, IL-3, as well as IL-4 stimulation (Thornhill & Haskard, 1991; Moser et al., 1992a).

There are many different types of membrane associated molecules on eosinophils that play functional roles. They can be divided into three groups; immunoglobulin and complement receptors, cytokine receptors, and adhesion molecules. There are receptors for immunoglobulin G (IgG), immunoglobulin E (IgE), immunoglobulin A (IgA), and immunoglobulin D (IgD). Complement receptors include; Clq, C3b/C4b (CR1), C3b (CR3) and C5a (Gleich et al., 1992a). The cytokine receptors on eosinophils include IL-5, IL-3, GM-CSF (Chihara et al., 1990), interferon-gamma (IFN-δ), IFN-β, TNF-α (Valerius et al., 1990), PAF (Kroegel et al., 1989), and leukotriene B_4 (LTB_4) (Maghini et al,, 1991). Adhesion molecule classes that eosinophils possess include those of the immunoglobulin gene superfamily (Springer, 1990), integrins (Hartnell et al., 1990), and selectins (Fakuda et al., 1984). Adhesion molecules and eosinophil stimulation are most likely important in eosinophil migration into the uterus.

Eosinophils possess and can synthesize CD 4 positive molecules as well as major histocompatability complex II. These transmembrane proteins may allow eosinophils to interact with CD 4 positive lymphocytes as antigen presenting cells (Lucey et al., 1989; Weller et al., 1991). The CD 4 positive protein and the major histocompatability proteins may enable eosinophils to fulfill effector functions analogous to those found in helper T cells and macrophages.

Inflammation and Adhesion

When there is inflammation, cytokines released at the site activate circulating leukocytes, especially neutrophils and monocytes, as well as the nearby endothelium. The activation of the endothelium causes a change in adhesion receptors on both the leukocyte and the endothelium. These changes in the expression of adhesion molecules can lead to increased adhesion and extravasation of specific white blood cells (Resnick & Weller, 1993). These changes may not be limited to up regulation or down regulation, but may also include conformational or affinity changes (Detmers et al., 1988), resulting from phosphorylation (Chatila et al., 1989).

When a leukocyte or endothelial cell becomes activated they are more adhesive, leading to blood cell margination to the post capillary veinule walls (Springer, 1990). This margination results in loose associations, or the rolling of the cells along the endothelium. They then adhere firmly to the endothelial wall and migrate between the junctions of the cells. Adhesion probably plays a very important role in the activation of these cells as well as their migration into the underlying tissues. Different cells possess various adhesion molecules and express them in unique ways, which can allow specific cell immigration.

There are three major families of adhesion molecules involved in leukocyte migration out of the vasculature. The immunoglobulin gene superfamily is one of these groups. This family includes intercellular adhesion molecule-1 (ICAM-1) and

intercellular adhesion molecule-2 (ICAM-2). They are both constitutively expressed on the endothelium, and ICAM-1 can be up regulated by TNFα, IL-1, lymphotoxin, IFN-δ, and lipopolysaccharides (LPS) (Dustin et al., 1986). ICAM-1 and ICAM-2 can bind to lymphocyte function-associated antigen-1 (LFA-1, CD11a/CD18) (Dobrina et al., 1991). ICAM-1 is also present on the eosinophil's surface and can be up regulated by IFN-δ, TNF-α, and IL-3 (Czech, 1993). Vascular cell adhesion molecule-1 (VCAM-1) is also in this supergene family and is only minimally expressed on unstimulated endothelial cells (Schleimer et al., 1992). IL-5, IL-4, TNF-α, and LPS cause the synthesis and expression of VCAM-1 (Dobrina et al., 1991). IL-4 seems to uniquely up regulate VCAM-1 on the endothelium (Thornhill & Haskard, 1991).

The second family of CAMs is the integrin family made up of heterodimers with one alpha and one beta subunit. There are at least 11 different alpha subunits and 7 different beta subunits (Walsh et al., 1993). β_2 integrins are limited to white blood cells and include LFA-1, CR3, and p150,95. LFA-1 is widely expressed on all leukocytes (Krensky et al., 1983). CR3 is a receptor for the third component of complement cascade, and is up regulated by exposure to IL-5, IL-3, GM-CSF, and PAF (Lopez et al., 1988; Walsh et al., 1990). P150,95 is a necessary ligand for the adhesion of monocytes and neutrophils to endothelial cells (Lo et al., 1989).

The β_1 integrins are mainly receptors for extracellular matrix proteins. VLA-4 is a unique β_1 integrin because it is influential in cell-cell interactions (Helmer, 1990). VLA-4 is constitutively expressed on eosinophils and is not expressed on neutrophils

(Walsh et al., 1993). VLA-4 selectively binds to VCAM-1 (Anderson & Springer, 1987). Increase in expression of VLA-4 in blood lymphocytes has been seen in patients with systemic lupus erythematosus (SLE) (Takeuchi et al., 1993).

The third major family of adhesion molecules are the selectins. These molecules are prominent in lymphocyte and eosinophil interactions with vascular endothelium. Endothelial-leukocyte adhesion molecule-1 (ELAM-1, E-Selectin) is not constitutively expressed on the endothelium but can be induced by IL-1, LPS, and TNF-α (Bevilacqua et al., 1987). ELAM-1 is a lectin recognizing various forms of the Lewis X glycans (Kyan-Aung et al., 1991). L-selectins contribute to neutrophil emigration at inflammatory sites (Jutila et al., 1989). They are involved in the initial adherence, binding to sialoglycoproteins (Smith et al., 1992). P-selectin is transiently and rapidly translocated to the cell surface in response to inflammatory stimuli such as histamine or thrombin (Hottori et al., 1989). ELAM-1, L-selectin, and P-selectin function in the early steps of neutrophil and eosinophil binding to the endothelium before migration (Lawrence & Springer, 1991; Vadas et al., 1993).

There are a number of steps involved in recruitment of white blood cells into the tissue depending on the inflammatory response. The general steps remain similar between cell types, however the specific adhesion molecules involved differ in expression and timing. The following paragraphs describe the migration of neutrophils out of the vasculature generally observed in acute inflammation such as the wheal and flare response of the skin when exposed to an antigen; and the migration of eosinophils

generally observed in chronic inflammation such as atopic dermatitis (Malech & Gallin, 1987; Weller, 1991).

In neutrophil migration, the vascular endothelium is first activated by a cytokine such as, TNF-alpha, IFN-gamma, or one of a number of interleukins (Pober et al., 1989). This activation causes up regulation of the selectins and the immunoglobulin gene superfamily adhesion molecules (Bevilacqua et al., 1987; Wegner et al., 1990). The neutrophil binds to the selectins using its Sialyated Lewis X sugars. This is a loose association, causing the neutrophil to roll along the vascular wall. Activation of the neutrophil by endothelial derived cytokines enables it to bind to up regulated ICAM-1 via LFA-1. This firm binding is followed by the migration of the neutrophil between the endothelial cells into the tissue (Ebisawa, 1992).

Eosinophil migration across the vascular endothelium is similar to neutrophil migration in that the endothelium is first activated by cytokines, causing up regulation of the selectins and the immunoglobulin gene superfamily adhesion molecules (Bevilacqua et al, 1987). The eosinophils are first bound to the selectins via their Sailylated Lewis X sugars (Vadas et al., 1993). They then bind more firmly using the VLA-4/VCAM-1 binding set (Walsh et al., 1991). Once bound firmly, the eosinophil migrates between endothelial cells into the tissue (Lamas et al., 1988).

Just as the role of cytokines and inflammatory mediators are being described in the adhesion of eosinophils to endothelium, other substances such as hormones are being studied to observe their effects on adhesion molecule expression. For example,

estrogens may have the ability to alter adhesion molecule expression. This altered expression may be important in determining how eosinophils enter the uterus in many female mammals during the estrus stage of the estrous cycle. Alterations in endothelial adhesion molecules via estradiol have been observed in certain endothelium-dependent responses (Gisclard et al., 1988). Estradiol has been shown to up regulate expression of ICAM-1, VCAM-1, and E-selectin in bovine aortic endothelial cells pretreated with TNFα (Cid et al. 1994). This up regulation increased the adhesion of poly-morphonuclear cells to the endothelium (Cid et al., 1994). However, different endothelial types react differently under the influence of estrogen and therefore extrapolation of this relationship to uterine endothelium should be guarded.

Hormone Fluctuations During the Female Reproductive Cycle

Two critical events in the sexual cycle of mammals are the development of the ovarian follicle to a mature egg and the preparation of the uterus for implantation of the fertilized egg. During the follicular phase of the cycle, the hypothalamus releases gonadotropin releasing hormone (GnRH). This in turn causes the anterior pituitary to release follicle stimulating hormone (FSH) and leutinizing hormone (LH). These hormones promote follicular development in the ovary, which in turn secretes low levels of estradiol. This low level of estradiol stimulates the uterus and other sexual organs. As the follicle develops its estrogen secretion increases. Peak blood estrogen levels have a stimulatory effect on the hypothalamus and the anterior pituitary causing

an LH/FSH surge, leading to ovulation. During the luteal phase the remaining follicular cells transform to luteal cells which synthesize and secrete moderate levels of estrogen and large amounts of progesterone. These two hormones coordinate preparation of the uterus for implantation (Hadley, 1992).

The LH and FSH released by the anterior pituitary indirectly cause cyclical changes in the uterus from the resulting estrogen and progesterone levels. During the proestrus phase, when FSH and LH levels rise, estrogen is also increased leading to the LH surge. During this phase, the endometrial glands begin to increase in number and length, the endometrial arterioles lengthen, and glycogen accumulates in the gland basal epithelia. In the estrous stage of the cycle, endometrial glands increase further in number, glycogen accumulates throughout the gland cells, the gland cells begin to hypertrophy and there is stromal edema. Increase in vascularization within the uterus enables the blood supply to extend further into the endometrium. During proestrus in the rat there is an influx of eosinophils into and throughout the uterine body. This influx continues throughout estrus, and then diminishes in diestrus (Rytomaa, 1960). In diestrus the degeneration of the corpus luteum leads to the contraction of spiral arteries decreasing the blood flow to the endometrium. This ischemia causes the degeneration of the luminal endometruim, and the decrease in size of both the endometrium and myometrium (Hadley, 1992)

Specific Uterine Responses to Estrogens

The rat uterus responds to estrogen in various ways. These responses can be divided into 3 different categories: genomic, non-genomic, and "other" (Jensen & Desombre, 1972; Grunert & Tchernitchin, 1982). The genomic responses are a direct result of estrogens effects on gene transcription, while the non-genomic responses are due mainly to the actions of infiltrating eosinophils. Genomic responses include increases in uterine RNA and protein synthesis, increases in uterine enzyme concentrations, and functional differentiation of target cells (Jensen & Desombre, 1972). The non-genomic responses are uterine eosinophilia, edema, increased vascular permeablility, and histamine release (Tchernitchin, 1972). The "other" effects include the increase in glycogen content via adenyl cyclase activation (Singhal et al., 1972). It is possible to separate nongenomic and genomic responses to estrogen. Compounds such as actinomycin D and puromycin inhibit RNA and protein synthesis, blocking only the responses defined as genomic (Tchernitchin & Galand, 1987). Cochicine, which blocks eosinophil migration by disrupting microtubule formation has been shown to block the nongenomic responses to estrogen in the rat uterus (Soto & Tchernitchin, 1979).

The possibility of the uterine vasculature having high affinity receptors for estrogens has also been studied. Tchernitchin & Tchernitchin (1976), using autoradiography showed evidence that high affinity estradiol receptors were present on the inner

surface of small uterine blood vessels of immature rats. There is also evidence to support the possibility that bovine aortic endothelial cells have receptors for estrogen (Colburn & Buonassisi, 1978).

Eosinophils and Estrogen Receptors

Generally eosinophil migration is observed in chronically inflamed tissues (Weller, 1991), but not in acute inflammation. The migration of eosinophils into the uterus is very different. Eosinophils in this situation migrate into the uterus quickly. This infiltration of eosinophils occur concomitantly with characteristic indicators of acute inflammation such as edema, increased vascular permeability, and histamine release (Tchernitchin et al., 1976).

Rytomaa (1960) and Bjersing & Borglin (1963) measured the fluctuation of eosinophils into rat the uterus at the different stages of the estrous cycle. The number of eosinophils per 0.1 sq. mm. reached its highest level at estrus (105) and its lowest level during diestrus (2). This magnitude of eosinophil migration is unique to rats.

There have been several studies exploring the migration of eosinophils into the rat uterus under the influence of estrogen. Tchernitchin et al. (1974) used immature Wistar rats that had not yet begun to produce high levels of estrogen and injected them interveinously with estradiol. The number of eosinophils in the whole uterus increased from 90 to 150,000 in twenty-four hours. The population of eosinophils was always

more within the endometrium as compared with the myometrium (Bjersing & Borglin, 1963).

The specific action that eosinophils have once they enter the uterus is not known, however eosinophils are thought to be involved in restructuring of the uterus as well as the protection of the blastocyst (Tchernitchin et al., 1989). Eosinophil enzymes; β-glucuronidase, arylsulfatase, and collagenase have been reported to depolymerase uterine extracellular matrix glycosaminoglycans and collagen fibers (Tchernitchin et al., 1985). Protection of the blastocyst may be accomplished by eosinophil phospholipase D which inactivates PAF, and EPO which decreases the bioactivity of LTC_4 and LTD_4 (Tchernitchin et al., 1985). EPO is also able to inactivate heparin released by mast cells within the uterus (Tchernitchin et al., 1985). Other eosinophil granule proteins and enzymes may further prepare the uterus and protect the blastocyst (Tchernitchin et al., 1989).

Estrogen induced cell migration into the rat uterus is selective for eosinophils. The mechanism for this unique migration has been postulated as follows; eosinophils and/or the uterine vascular endothelium are activated by estrogen via their estrogen receptors. The activation of these two cell types cause a change in them leading to migration of the eosinophil, allowing it to elicit its effects on the uterus (Tchernitchin & Tchernitchin, 1976).

Since the discovery that eosinophils migrate into the uterus under the influence of estrogen there have been many studies to determine if eosinophils have a receptor

for estrogen. There have been several procedures applied to demonstrate the localization of this receptor on eosinophils; wet radioautography (Tchernitchin, 1967), dry radioautography *in vivo* (Tchernitchin et al., 1978) and, dry radioautography *in vitro* (Tchernitchin et al., 1974). Lyttle et al. (1984) used radioactive estrogens and Scatchard analysis to identify a specific estrogen receptor present in the uterus only when eosinophils were in the uterus. Lyttle identified two different estrogen receptors, type I and type II. Isolated peritoneal eosinophils had an estrogen receptor with a lower affinity (type II), while a higher affinity receptor (type I) was located in the uterus. When eosinophils had infiltrated the uterus after estrogen injection Scatchard analysis revealed two different estrogen receptors. Tchernitchin & Tchernitchin (1976) did similar Scatchard analysis resulting in a Kd of 5.6×10^{-10} M for the eosinophil estrogen receptor.

Selective migration of eosinophils is also observed in allergic reactions and asthma. In asthma there is a unique increase of VCAM-1 expression on the lungs' vascular endothelium, with no increase in ICAM-1 expression (Nakajima et al., 1994). IL-4, which is typically released by T-cells in allergic responses interacts with the surrounding endothelium causing a unique up regulation of VCAM-1 and down regulation of ICAM-1 (Thornhill & Haskard, 1990). The binding of VLA-4 on the eosinophil with its ligand VCAM-1 on the vasculature, may be an integral association needed for eosinophil migration into the uterus under the control of estrogen. In this

study we looked at the possible association between ICAM-1 and VLA-4 expression, and estrogen induced migration of eosinophils into the uterus.

MATERIALS AND METHODS

Preliminary Study: Eosinophil Cycling in the August Rat Uterus

Due to its inherent peritoneal eosinophilia, the August rat was chosen for this study. A preliminary study was undertaken to determine if these rats responded similarly to other rat strains (i.e. Sprague Dawley and Wistar rats) in terms of eosinophil cycling into the uterus throughout the stages of estrus.

Animals and Treatment

Mature female inbred August rats (Harlan Olac, UK) were used in all the preliminary studies. Vaginal smears were obtained daily from each animal to ascertain the status of their estrous cycle. The stage of estrus was determined by the criteria stated by Ross & Klebanoff (1966). At a predetermined stage of estrus each animal was euthanized by carbon dioxide asphyxiation.

Removal, Fixation, and Preparation of Uterine Tissue

The uterii were dissected out of six rats (2 from the proestrous stage, 2 from the estrous stage, and 2 from the diestrous stage) and placed in 10% normal buffered formalin. All uterine tissue in the preliminary, as well as the subsequent studies, under went the following procedures in preparation for light microscopy. After fixation the

tissue was dehydrated in a graded ethanol series (70%-100%) followed by the transition solvent, xylene, and embedded in paraffin (Tissue-Tek, Fisher Scientific). The samples were then sectioned at 5 microns on a Reichert-Jung Biocut 2030 microtome and stained with Luna's, or Gills hematoxylin and eosin. The sections were viewed using a Nikon Microphot FXA microscope and photographed with Kodak 160T film.

Eosinophil Quantification

In both preliminary and subsequent studies uterine eosinophils were counted in 60 non-overlapping fields of view, covering the length of the uterine horn. These fields of view included 20 observations in the two layers of myometrium, 20 in the lower portion of the endometrium and 20 in the upper portion of the endometrium The field of view was set at 30 μm^2 using Image-1®, image analysis software. The endometrium was separated into upper and lower portions due to its non-uniform eosinophil densities.

Estrogen Induced Eosinophil Migration into the August Rat Uterus

Immature female August rats injected with DES were used in this study to determine the association between ICAM-1 and VLA-4 expression, and eosinophil migration into the rat uterus.

Animals and Treatment

Inbred female August rats (Harlan-Olac, UK), were used in this study. These rats were 27 days old, and weighed 40-50 grams. Each rat was treated and then placed back with its mother until euthanasia.

On the day of the experiment, the rats were divided into two groups of four animals; control and experimental animals. The control animals were injected with the vehicle, 0.1 ml of 10% ethanol in saline. The experimental animals were injected with 0.1 ml of a 200 μg /ml DES solution. DES (Sigma, St. Louis, MO) was dissolved in 10% ethanol and brought to 200 μg/ml with saline. DES was chosen due to its water solubility and increased estrogen efficacy. The dose of DES was selected because it increases blood "estrogen" levels to at least as high as they are during proestrus in the rat. The diameter of the uterine horns, the height of the uterine epithelial lining of the lumen, and the infiltration of eosinophils were measured to assess the estrogenic response. All dosing was accomplished with a single subcutaneous injection in the subscapular region. All animals were euthanized via carbon dioxide asphyxiation 24 hours after the injections. The twenty-four hour time point was selected due to peak eosinophilia and ICAM-1 expression in the uterus at this time.

Removal and Fixation of Uterine Tissue

After asphyxiation the abdominal wall was cut exposing the uterii. Both uterine horns were excised at the cervix. One uterine horn from each animal was fixed in an aqueous 10% neutral buffered formalin solution in preparation for light microscopic analysis. The other horn was snap frozen in isobutanol at -160°C (cooled by liquid nitrogen) and stored at -70°C in preparation for immunohistochemical analysis. Immunocytochemistry determining ICAM-1 and VLA-4 expression was measured using monoclonal antibodies.

Immunohistochemical Analysis

For both ICAM-1 and VLA-4 studies, 8-12 frozen sagittal uterine sections were cut from each of the 8 rats. Each uterus was cryosectioned at 6 μm in thickness with a Tissue-Tek 4553 cryostat-microtome and placed on Poly-Lysine coated slides. These slides were then fixed with cold acetone (4°C) for 10 minutes and air dried. Indirect staining was conducted with a mouse IgG alkaline-phosphatase-ABC kit (Vector Labs Inc., Burlingame, CA) following the manufacturers instructions. Briefly, tissues were incubated with normal horse serum (blocking serum) to decrease non-specific staining. Primary, and secondary antibodies were diluted with 10 mM Tris buffer, pH 7.5, containing 5% horse IgG (500 μg/ml)(Sigma ImmunoChemicals, St. Louis, MO) to additionally reduce non-specific background. Sections were then

incubated with primary antibody for 30 minutes at room temperature using either monoclonal anti-rat ICAM-1 (provided by T. Manning, The UpJohn Co., Kalamazoo, MI) at 1.56 μg/ml in 2% BSA-Trisma buffer, or monoclonal anti-rat VLA-4 (provided by T. Manning, The UpJohn Co., Kalamazoo, MI) at 275 μg/ml in 2% BSA-Trisma buffer. After 10 dip and blot rinses with 10% ethanol-Tris buffer (pH 7.5) on an aqueous blotting pad, sections were incubated with a secondary antibody, biotinylated horse anti-mouse IgG (1.25 ug/ml)(Vector Laboratories Inc., Burlingame, CA) for 30 minutes and rinsed again. The enzyme, alkaline phosphatase, was incubated on the sections for 60 minutes followed by another Tris buffer rinse. Finally, sections were incubated with the substrate, aminoethyl carbazole containing levamisole, for 30 minutes and rinsed with distilled water. The sections were counterstained with Mayer's hematoxylin for 3 minutes and rinsed with tap water. After staining, the slides were mounted and coverslipped using an aqueous mounting medium.

Specific antibody binding was determined by comparing the antibody labeled sections with a negative control section. In the negative controls, the primary antibodies were replaced with non-immune mouse IgG's at the same protein concentration. To assure uniform staining, all tissue sections for each antibody studied were labeled at one time.

ICAM-1 staining intensity was classified according to the criteria as described by Briscoe et al. (1992) and Nakajima et al. (1994). The evaluation of ICAM-1

expression on the endothelium was scored as 0 (absent staining or faint staining of an occasional vessel only), 1+ (faint staining of several vessels), 2+ (moderate intensity staining of most vessels), or 3+ (intense staining of most vessels). The sections were randomly coded, examined, and the average section score for each animal was used for subsequent calculation of ICAM-1 expression. The DES-treated animals exposed to the VLA-4 antibody labeling system were compared to normal August rat blood eosinophils, because there are very few eosinophils within the immature control uterii. Fifty eosinophils from the blood were identified and scored, uterine and blood eosinophils were evaluated using the same intensity classification used in the ICAM-1 evaluation.

Eosinophils from the cryosections were identified by their high nuclear to cytoplasm ratio, location in the uterus, as well as density within the uterus. Density and location criteria was determined by the eosinophils present in hematoxylin and eosin stained DES treated uterine sections. Eosinophils in the blood slides were identified by their nuclear to cytoplasm ratio, their size, and their donut shaped nucleus.

Data Analysis

Data are presented as mean + / - SEM. All statistical analyses were performed by a Student-T-test using Quattro Pro ®. A p value of 0.05 was used to determine significance.

RESULTS

General Observations

The preliminary study showed the August rat uterine changes during estrus; this included uterine weight and size changes, as well as the influx of eosinophils. All three criteria increased from proestrus through estrus and thereafter decreased in metestrus.

Twenty-four hours after a subcutaneous injection, DES induced morphological changes in the immature rat uterus included edema, increased vascularization, increased epithelial height, and the influx of eosinophils.

Morphological Evaluation

Control Rats

Each uterine horn of the control immature August rats had a diameter of approximately 1 mm. Upon examining sagittal sections of the uterii it was noted that the tissue was not edematous and had little vascularization. The three major layers of the uterus; the endometrium and the two myometrial layers, were easily distinguishable as seen in Figure 1. The glands were small with a simple cuboidal epithelium. The

epithelium lining the uterine lumen was composed of simple and cuboidal cells, as see in Figure 2.

DES Treated Rats

The uterine horns of DES treated rats were markedly different from those of control animals. Upon gross examination the vasculature was more easily seen and the diameter of the uterine horns had increased to over twice that observed in controls, as seen in Figure 3. Longitudinal sections of the treated rat uterus revealed a much more vascularized uterus which was highly edematous, as seen in Figure 4. The uterine glands were more twisted with columnar epithelial cells and the height of the epithelial lining of the uterus was approximately three times greater, as seen in Figures 5 and 6. The uterus also was infiltrated with eosinophils as seen in Figures 7 and 8. A high density of eosinophils was evident near the endometrial/myometrial border.

Immunohistological Staining

General Observations

Immunostaining of the immature control uterine sections for ICAM-1 expression resulted in intense staining of the uterine vasculature. However, there was substantially less labeling on immature DES treated rat uterine sections as compared to the control rat uterine sections. VLA-4 expression on the eosinophils within the

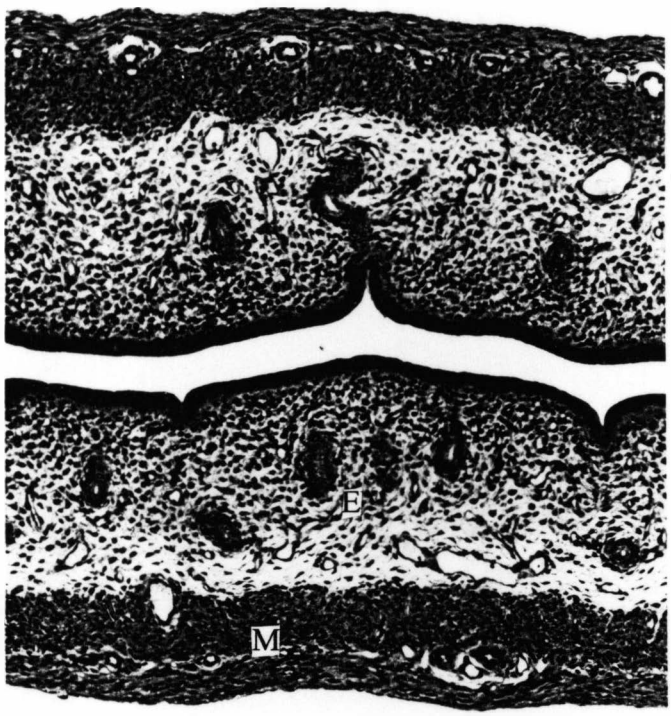

Figure 1. Photomicrograph of a Cross Section of Uterine Horn From an Untreated Rat. (x 100)

In this micrograph, observe the myometrial layers (M) and the endometrial layer (E) with its epithelium. Also observe the compactness of the uterine tissue with no fluid accumulation.

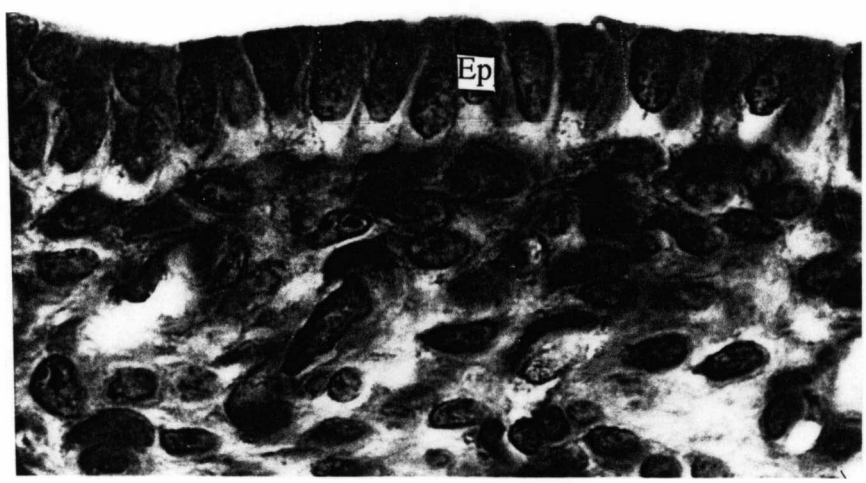

Figure 2. Photomicrograph of the Epithelial Lining From the Uterine Horn of an Untreated Rat. (x 1000)

In this micrograph, observe the cuboidal epithelial cells (Ep) lining the luminal side of the horn.

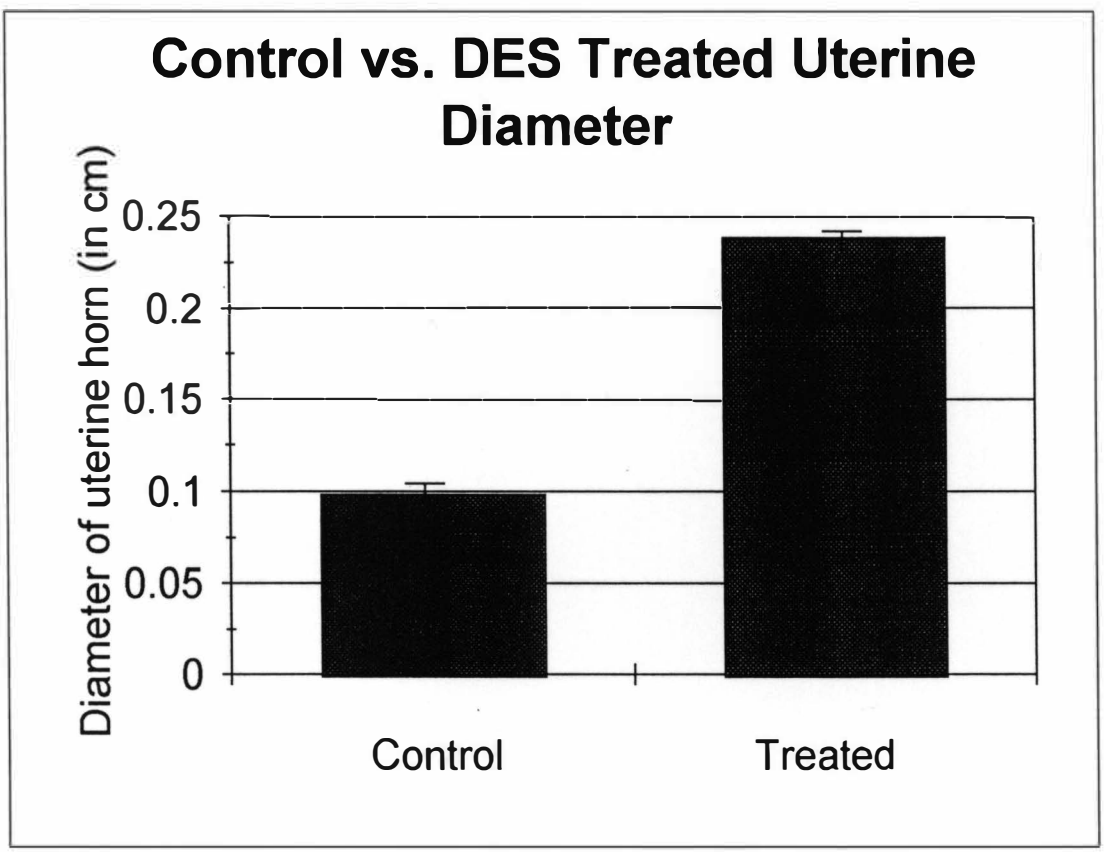

Figure 3. Comparison of Uterine Diameter Between Control and DES Treated Rats.

There were 3 animals in each group. Each uterus was cross sectioned and 4 random positions along the uterine horn were measured using Image-1® software. Significant at $p \leq 0.05$ by Student-T-test.

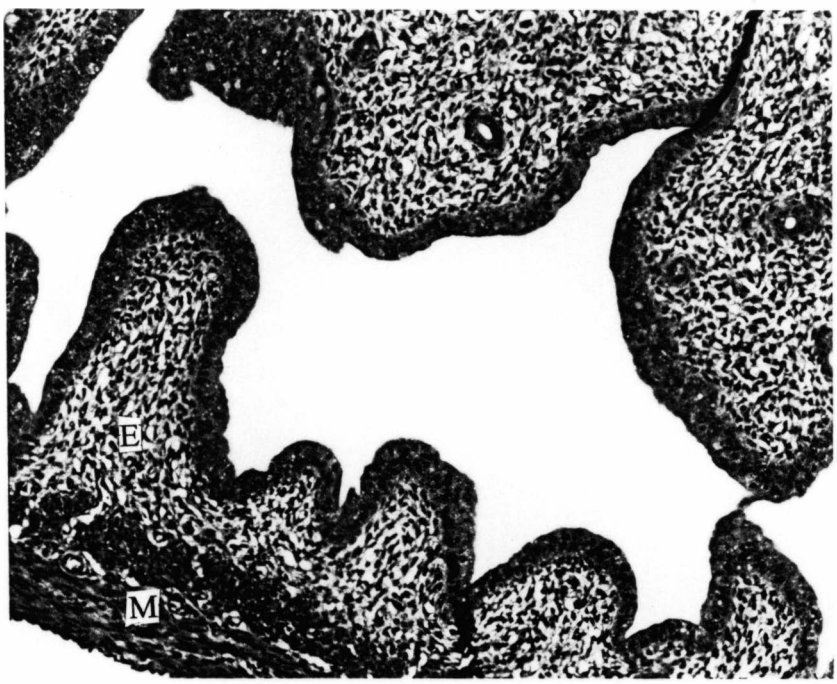

Figure 4. Photomicrograph of a Sagittal Section of the Uterine Horn From a DES Treated Rat. (x 100)

In this micrograph, observe the myometrial layers (M) and the endometrial layer (E) with its epithelial luminal covering. Also observe the edema in the submucosa.

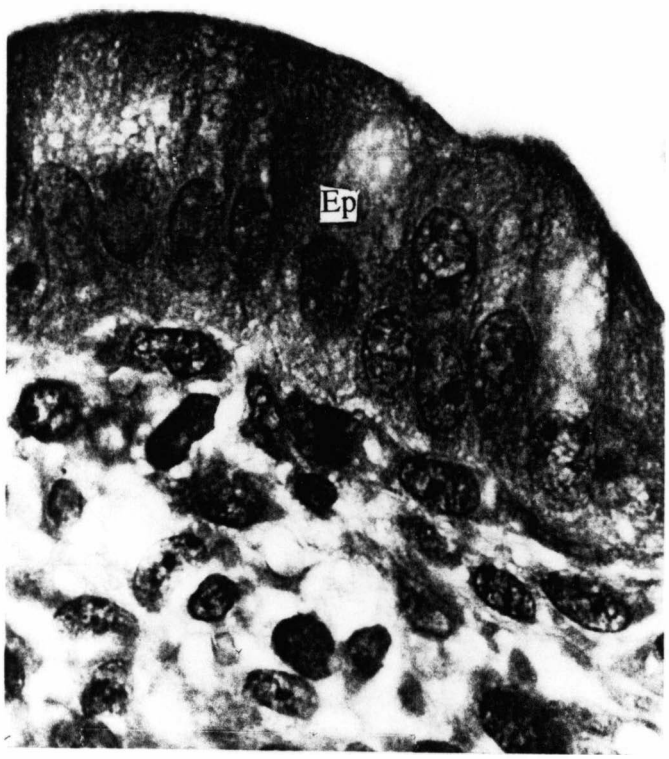

Figure 5. Photomicrograph of the Epithelial Lining in a DES Treated Uterine Horn. (x 1000)

In this micrograph, observe the tall columnar epithelial cells (Ep) lining the luminal side of the uterine horn.

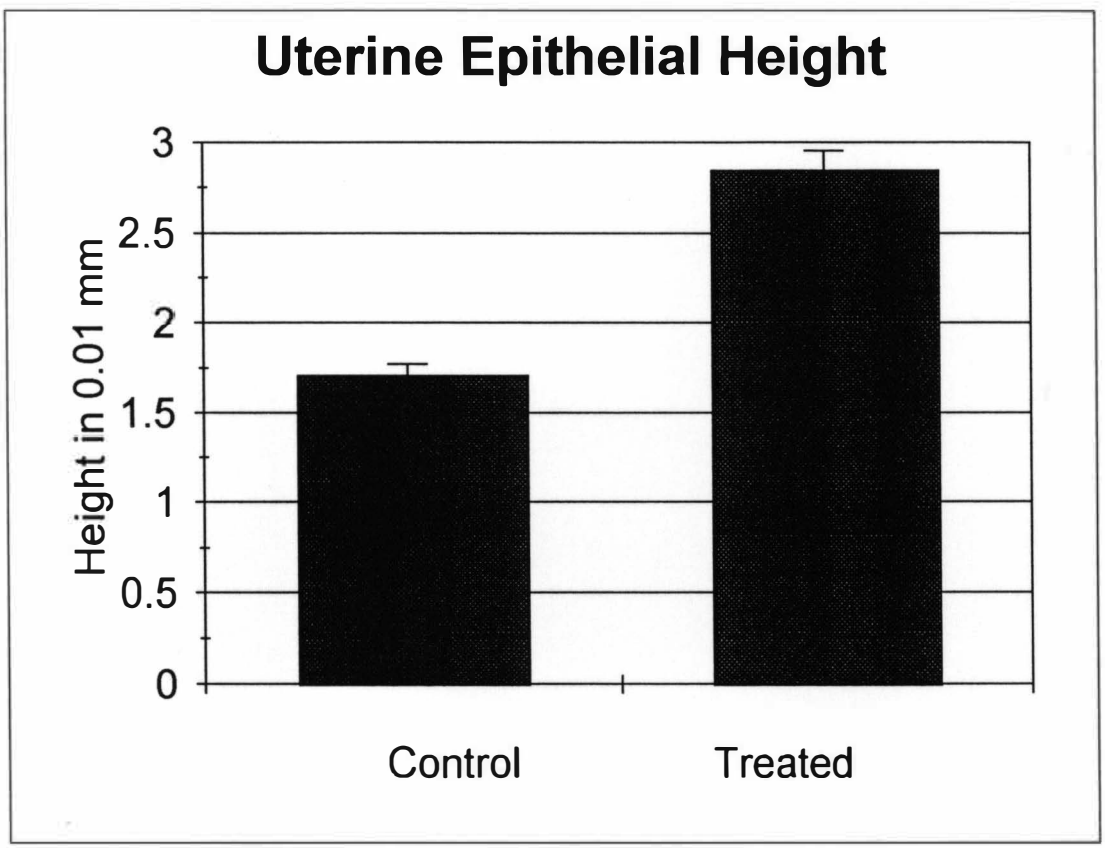

Figure 6. Comparison of the Epithelial Cell Height Between the Control and DES Treated Groups.

10 height measurements were made for each animal. There were 4 animals in each group. The animals in the treated group were injected subcutaneously with 20 μg of DES. Significant at $p \leq 0.05$ by Student-T-test.

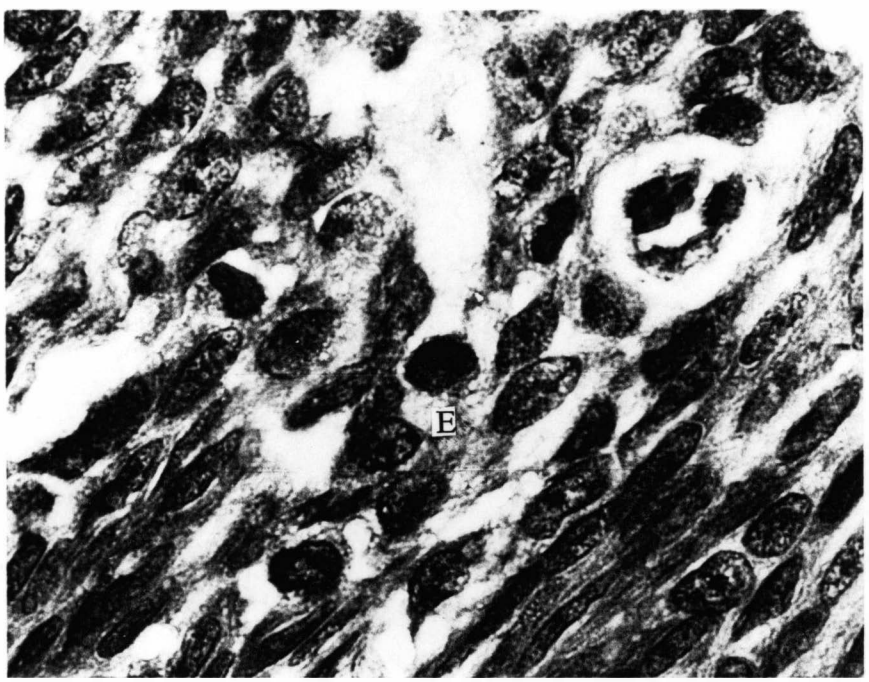

Figure 7. Photomicrograph of Eosinophils Within the Uterus of the DES Treated Rat. (x 1000)

In this micrograph, observe an eosinophil (E) with the intense coloring of its "donut" shaped nucleus. This eosinophil is located in the lower portion endometrium where a high concentration of these cells are found.

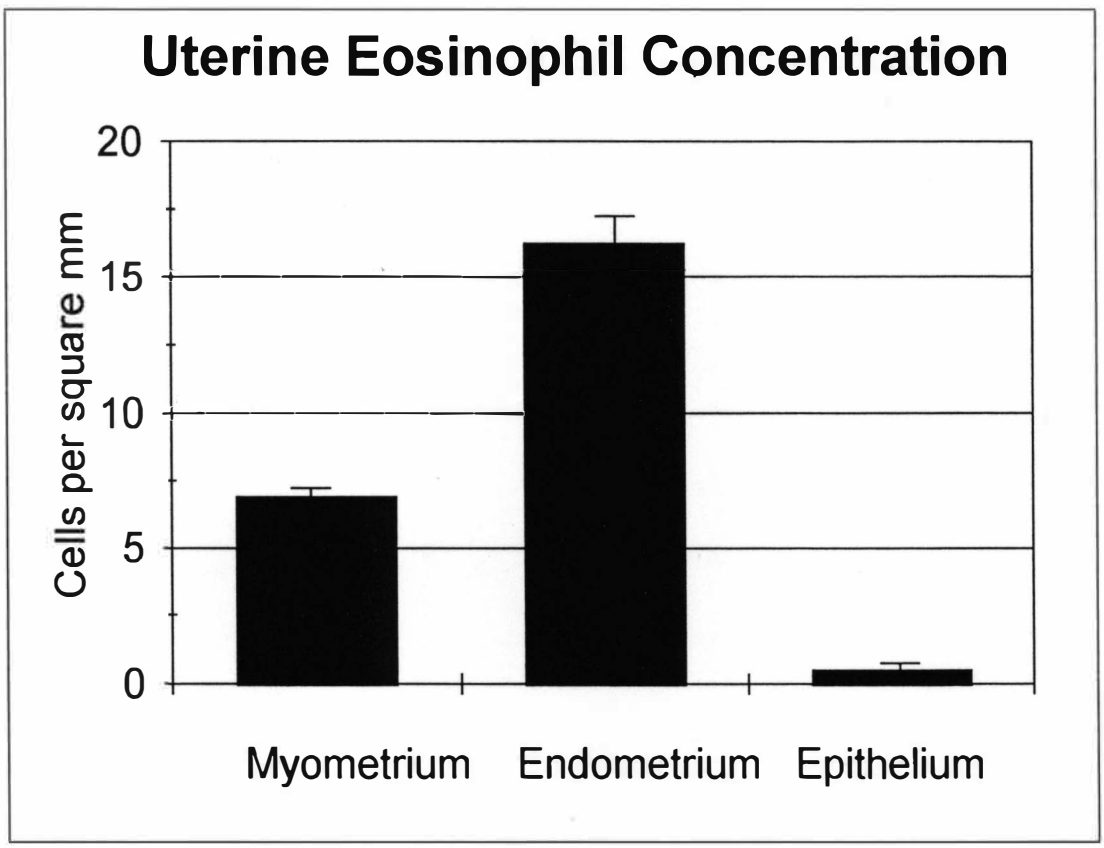

Figure 8. Comparison of the Concentration of Uterine Eosinophils Within the Myometrium, Basement Endometrium, and the Upper Endometrium. of the DES Treated Rat.

20 fields were measured in each tissue layer for each animal. There were 4 animals in this group which were injected subcutaneously with 20 μg of DES 24 hours before uterine harvest.

vasculature and interstitial tissues of the uterus had a higher intensity than normal blood eosinophils.

ICAM-1 Uterine Staining.

There was an observable difference between the control tissue and the treated tissues response to the monoclonal antibody against ICAM-1. In Figure 9, a section of an untreated uterine horn is stained by the product of the enzyme alkaline phosphatase which was coupled to IgG directed against ICAM-1. Figure 9 shows the labeling of the endothelial cells in the control uterine vasculature. Figure 10 shows the labeling of the endothelial cells of the uterine vasculature in the DES treated uterus. The staining is much lighter in the treated uterus, indicating a decrease in labeling for ICAM-1. When the tissue sections were scored using the criteria defined by Briscoe (1992) there was a significant difference between the DES treated uterii and the control uterii, as seen in Figure 11.

VLA-4 Uterine Eosinophil Staining

When the control uterine tissues were examined for VLA-4 there was only background staining of the untreated tissue and no eosinophils were observed, however there was a substantial amount of intensely stained eosinophils in the DES treated uterii. Examples of this staining can be seen in Figure 12 and 13. The criteria used to identify the eosinophils is described in the Material and Methods section. The

uterine eosinophils were intensely stained indicating the presence of VLA-4 on these eosinophils. Using the criteria described by Briscoe et al. (1992), the eosinophils in the uterus of the DES treated rats were compared to blood eosinophils in controls. The scores for uterine eosinophils were consistently higher than those of the blood in untreated rats. The increase in labeling intensity on the eosinophils in the uterus is seen in Figure 12, as compared with the blood eosinophils shown in Figure 14. This suggests an increase of VLA-4 on eosinophils within the uterus and uterine vasculature.

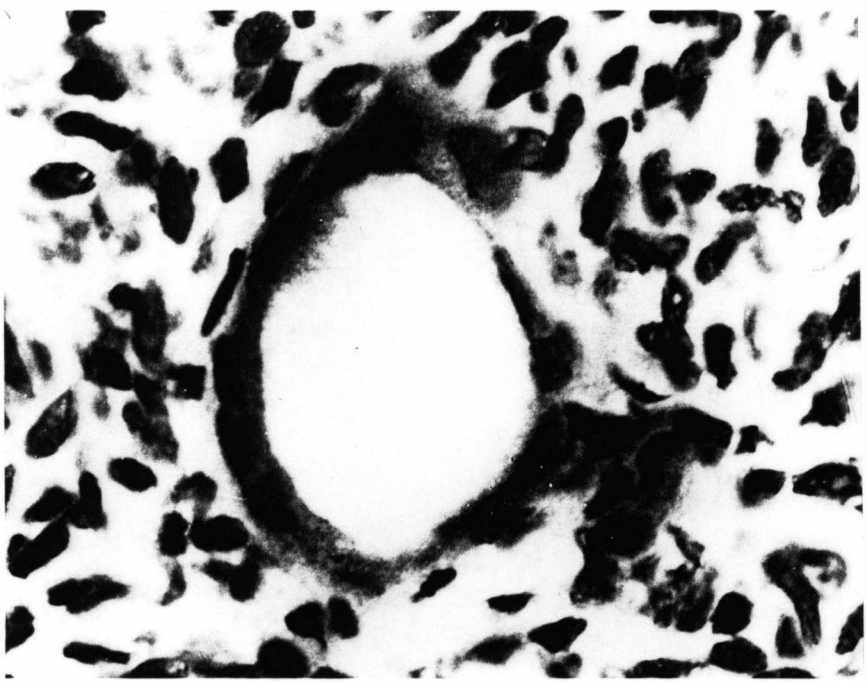

Figure 9. Photomicrograph of the Staining of the Untreated Uterus With a Monoclonal Antibody Against ICAM-1. (x 1000)

In this micrograph, observe the endothelial cells which are staining for an antibody against ICAM-1. Also observe the rest of the section which is not stained, indicating no ICAM-1 expression.

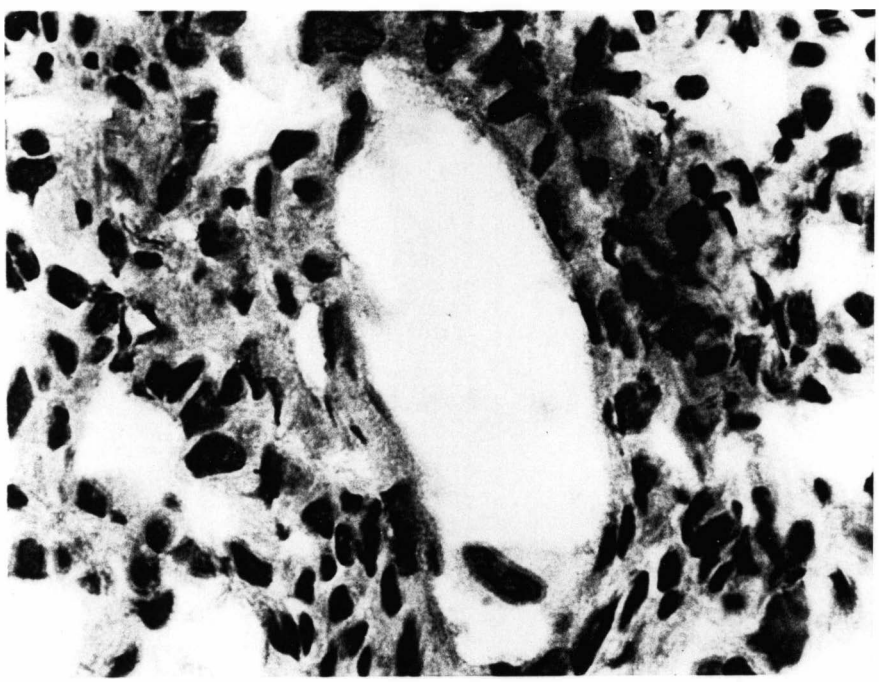

Figure 10. Photomicrograph of a DES Treated Uterus Stained With a Monoclonal Antibody Against ICAM-1. (x 1000)

In this micrograph, observe the endothelial cells which are lightly staining for ICAM-1. Also observe the rest of the section which is not stained, indicating no ICAM-1 expression.

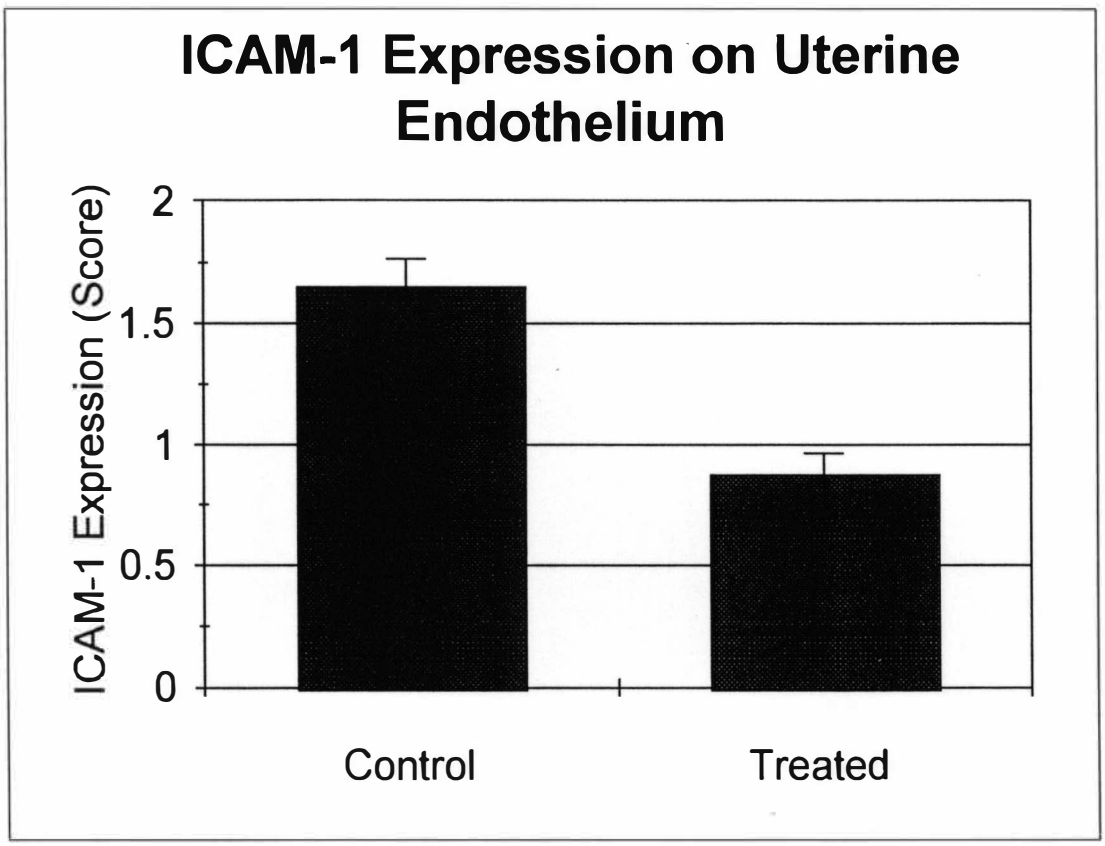

Figure 11. Estrogen Induced ICAM-1 Expression on the Endothelium of Rat Uterii 24 Hours After a Subcutaneous Injection of DES.

The intensity of staining was scored according to the following criteria; 0 = absent staining or faint staining of an occasional vessel only, 1 = faint staining of several vessels, 2 = moderate intensity staining of most vessels, and 3 = intense staining of most vessels. Significant at $p \leq 0.05$ by Student-T-test.

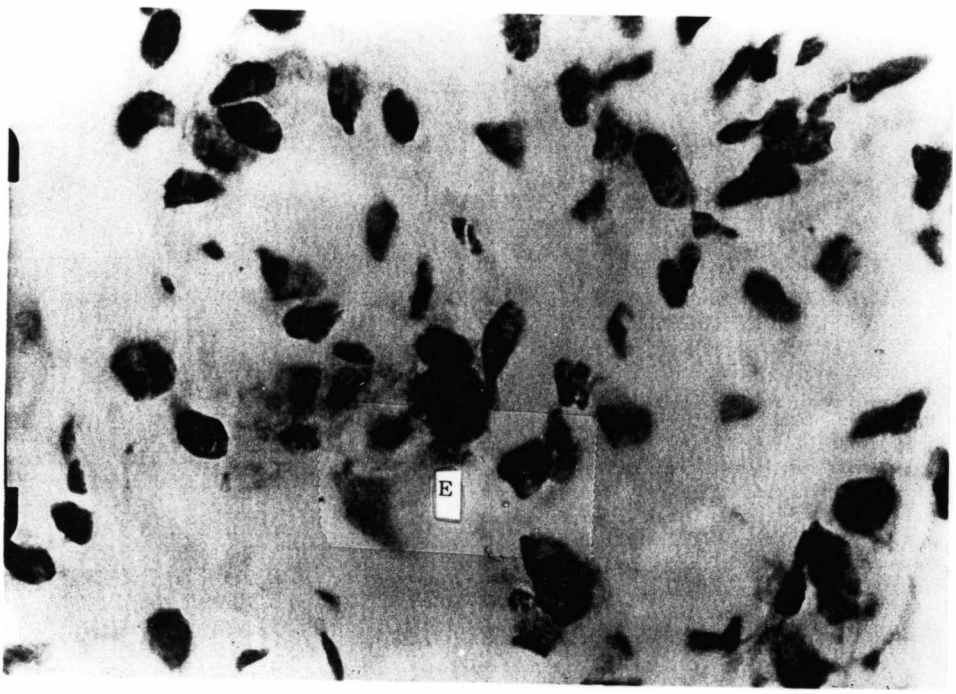

Figure 12. Photomicrograph of the DES Treated Uterus Stained With a Monoclonal Antibody Against VLA-4. (x 1000)

In this micrograph, observe the eosinophil which is staining for an antibody against VLA-4. Also observe the remainder of the section which is not stained indicating no VLA-4 expression.

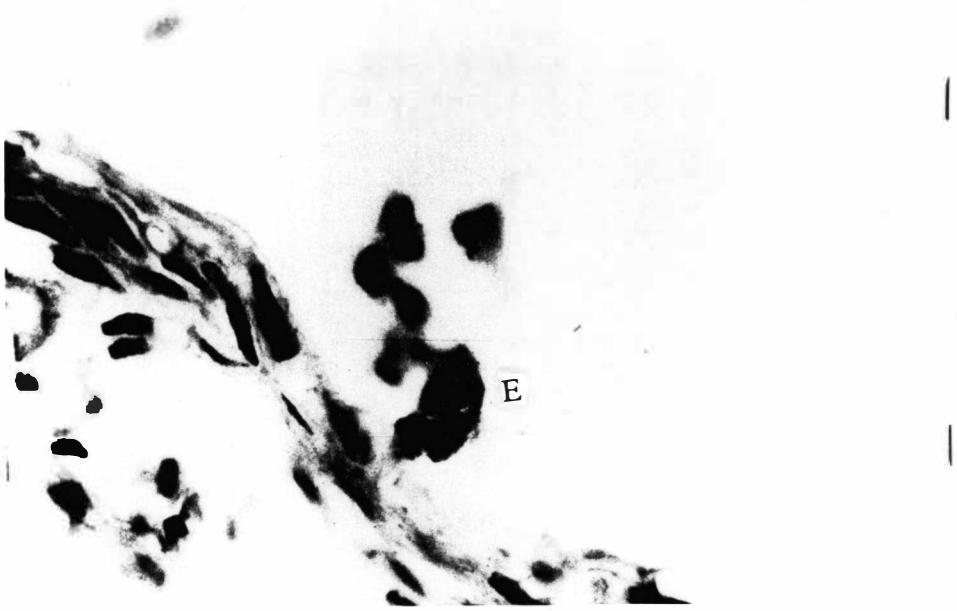

Figure 13. Photomicrograph of the DES Treated Uterus Stained With a Monoclonal Antibody Against VLA-4. (x 1000)

In this micrograph, observe the eosinophil (E) which is in the vasculature stained with an antibody against VLA-4. Also observe the adjacent cell of the section which is also stained. Because of its size and expression of VLA-4, it is probably a T-lymphocyte.

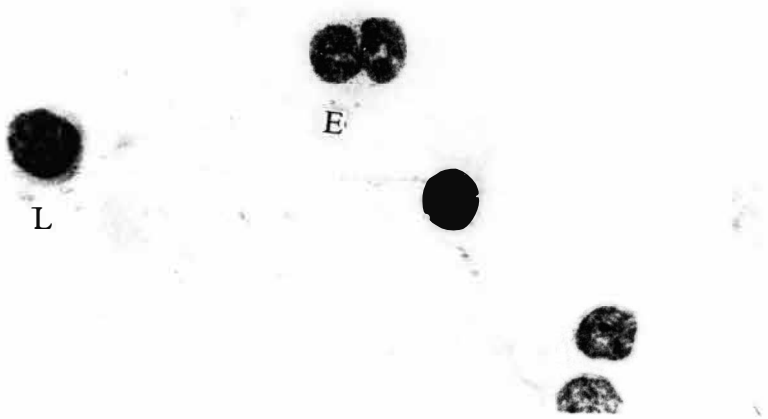

Figure 14. Photomicrograph of the Staining of a Control Blood Sample Including Eosinophils With a Monoclonal Antibody Against VLA-4. (x 1000)

In this micrograph, observe the eosinophil (E) which is staining for an antibody against VLA-4. Also observe the rest of the section which contains a lymphocytes (L) staining for VLA-4.

DISCUSSION

The purpose of this study was to determine the association between intercellular adhesion molecule expression and eosinophil recruitment into the rat uterus in response to DES. We hypothesized that the contributory adhesion molecule expression pattern would be similar to that seen in an eosinophilic selective response, such as asthma. The typical response in asthma is an increase in the expression of VCAM-1 (the ligand for VLA-4) with no increase in ICAM-1 or its ligand LFA-1 (Nakajima et al., 1994). The up regulation of VCAM-1 may cause a selective increase in extravasation of eosinophils into the lungs because other leukocytes, such as neutrophils, do not possess the ligand for VCAM-1 and therefore may not as readily migrate. Increasing evidence from *in vitro* models indicates that induced eosinophilia is associated with an increase in expression of VCAM-1 while there is no substantial increase in ICAM-1 expression (Thornhill & Haskard, 1991). In other studies monoclonal antibodies to VCAM-1 or VLA-4 cause a 75% decrease in eosinophil adherence to endothelium (Schleimer et al., 1992), while the remaining adherence was demonstrated to be caused by ICAM-1 (near total blocking of adherence was demonstrated when monoclonal antibodies to both ICAM-1 and VCAM-1 were administered). The importance of ICAM-1 in this instance may be over estimated since subjects with LADS (leukocyte adhesion deficiency syndrome), which destroys

LFA-1's ability to bind to ICAM-1, still have eosinophils in extravascular sites of inflammation (Anderson et al., 1987). This ICAM-1 adhesion may be a type of nonspecific binding for all leukocytes which does not necessarily lead to immigration into the tissues (Weller, 1991).

One of the underlying signals for specific eosinophil infiltration with allergic reactions seem to be IL-4 (Thornhill & Haskard, 1991). IL-4 is an inflammatory mediator which specifically enhances eosinophil adherence and migration through endothelial cell layers (Moser et al., 1992b). The enhanced adherence and extravasation of the eosinophil across the endothelial layer is postulated to be due to the ability of IL-4 to specifically up regulate VCAM-1 (Thornhill & Haskard, 1990). IL-4 has also been shown to partially suppress constitutive expression of ICAM-1 on the endothelium. (Thornhill & Haskard, 1990). In our study we observed an up regulation of the ligand for VCAM-1, VLA-4.

In this study DES was administered 24 hours before the uterine horns were removed for examination. The typical effects of estrogen were observed in the uterus including; increased vascularization, edema, epithelial proliferation, and eosinophil migration. Immunohistochemical analysis demonstrated a decrease in the constitutive expression of ICAM-1 on the uterine vasculature as well as an increased expression of VLA-4 on uterine eosinophils as compared to control blood eosinophils. Since there is no available rat monoclonal antibody to VCAM-1 it was not possible to study the presence of this molecule in this study. However ICAM-1 and VLA-4 findings

suggest a similarity between eosinophil migration in a true, eosinophil selective, inflammatory response and in an inflammatory "like" response. The decrease in ICAM-1 expression on uterine vasculature suggest that ICAM-1 and its ligand LFA-1 are not important in this eosinophil migration. The up regulation, or at least expression, of VLA-4 on uterine eosinophils allows us to speculate that VLA-4/ VCAM-1 binding may be involved in selective eosinophil migration into the uterus. Adhesion molecule expression was measured in this study, not function, therefore we can only speculate how this expression may relate to function.

The majority of VLA-4 research has focused on blocking assays and assays using human *in vitro* models. Our research is unique because we used a rat model in which we labeled VLA-4 *in situ*. This is important because it is a labeling experiment, not a blocking experiment, that is specific for localizing VLA-4. It also demonstrates that VLA-4 is present on the August rat eosinophils. This confirms that eosinophils in this rat are similar to human eosinophils in their VLA-4 expression. This similarity could allow for the important switch from research emphasis on *in vitro*, to the more realistic setting of *in vivo* models without necessitating the use of primates.

At this time it is difficult to determine if eosinophils are activated without using electron microscopy. It would be interesting to observe VLA-4 expression on eosinophils in different inflammatory events. It is possible that up regulation of VLA-4 could be an indicator of eosinophil activation.

The exact mechanism for the up regulation of VCAM-1 and down regulation of ICAM-1 during eosinophil migration have not been elucidated. Tchernitchin (1976) suggested that vascular endothelial cells in the uterus have receptors for estrogen, and Colburn & Buonassisi (1978) presented evidence to support this supposition in bovine aortic endothelial cells. Tchernitchin and coworkers also reported in a series of papers between 1967 and 1978 that eosinophils have specific estrogen receptors. These receptors unlike uterine estrogen receptors were always available, not fluctuating in number under hormone control (Tchernitchin, 1976). It is possible that estrogen binds to eosinophils, up regulating or activating VLA-4. Estrogen may also interact with uterine vascular endothelium, causing up regulation of selectins and VCAM-1, enabling selective eosinophil binding and migration into the uterus.

Looking further beyond the scope of this study, the rapidity of eosinophil migration into the uterus is difficult to explain. Tchernitchin et al. (1972) observed a rapid influx of eosinophils into the uterus after an IV injection of estrogen. Because of the time frame required for up regulation, adhesion molecules which require protein synthesis cannot account for this migration.

This suggests several possibilities: First, P-selectin (GMP-140) may be released from Weibel-Palade bodies, rapidly translocating to the surface of the uterine endothelium due to estrogen stimulation. This is seen in endothelial cell cultures with histamine activation (Hattori et al., 1988). The process of increasing cell surface P-selectin seems to be by means of translocation of Weibel-Palade Body vesicles fusing

with the endothelial plasma membrane. Eosinophils bind to P-selectin on the endothelium (Vadas et al., 1993). P-selectin is specifically mentioned because of its unique ability to translocate rapidly, peaking 10 minutes after stimulation with histamine (Hottori et al., 1988). Secondly, down regulation of ICAM-1 may alter the eosinophils' ability to bind to endothelial cells, somehow allowing it more direct access to VCAM-1. This possibility is unlikely since the kinetics of ICAM-1 down regulation is in terms of hours, not minutes (Thornhill & Haskard, 1991). Thirdly, the presence of estrogen may alter the conformation of VLA-4 which in itself allows VLA-4 and VCAM-1 to bind to one another more efficiently. This "activation" of cell adhesion molecules has been seen with Mac-1, LFA-1 and VLA-4 under the control of different cytokines (Chatila et al., 1989; Springer, 1990). It is suspected that this activation may result from the phosphorylation of these molecules. Chatila et al. (1989) used a protein kinase C activator, which in turn phosphorylated the CD18 residue of the CD11/18 heterodimer. This phosphorylation caused a conformational change in the complex, increasing its binding ability. Obviously these options do not have to act exclusively, and possibly a combination of one with another could explain this rapid migration.

Because of the rapidity of eosinophil migration into the rat uterus and the unique expeditious expression of P-selectin on endothelial cells after histamine stimulation, it would be interesting to block P-selectin and observe what affects it may have on eosinophil migration into the uterus. It would also be interesting to examine

the VLA-4 molecule on both resting and estrogen "activated" eosinophils *in vitro* to determine if there are different degrees of phosphorylation.

CONCLUSION

The results of this study support the findings in other models involving selective eosinophil recruitment out of the vasculature, such as asthma. The presence of adhesion molecules specific to eosinophils (not neutrophils) such as VLA-4 have been observed in other studies. August rat eosinophils were shown to express VLA-4 in this study. We noted a decrease in ICAM-1 expression from its constitutive expression on the vasculature. This is similar to studies where IL-4 was shown to also down regulate ICAM-1 in a model of eosinophil recruitment.

Appendix A

IACUC Approval Form

WESTERN MICHIGAN UNIVERSITY
INVESTIGATOR IACUC CERTIFICATE

Title of Project: Estrogen regulation of cellular adhesion molecules in eosinophil emigration in the rat uterus.

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:

_____

_____

_____

_____

_____

_____

D Dierso, DVM — IACUC Chairperson

2/28/94 — Date

Acceptance of Provisions

D L R C — Signature: Principal Investigator/Instructor

3/1/94 — Date

_____ — IACUC Chairperson Final Approval

_____ — Date

Approved IACUC Number 94-02-03

Rev. 3/92

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