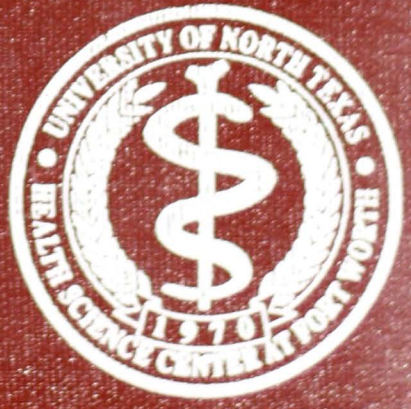
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Lee, Carol Hamberlin, A Study of Some Aspects of the Role of Mast Cells in Experimental Autoimmune Uveitis. Doctor of Philosophy (Biomedical Sciences), June 1994, 141 pp., 6 tables, 29 illustrations, bibliography, 115 titles.

Choroidal mast cells have been implicated in experimental autoimmune uveitis (EAU), an ocular inflammatory disease induced by S-antigen (Sag). Activation of ocular mast cells in Lewis rats was evaluated by determining changes in numbers of mast cells, levels of histamine, and wet weights of ocular tissues. A decrease in choroidal mast cells was confirmed statistically, and limbal mast cells were found to be activated earlier than choroidal mast cells.

The ocular histamine distribution was altered during EAU, decreasing in the anterior eye, and increasing in the posterior eye. Retinal histamine levels increased when EAU symptoms occurred, but decreased while the disease was still intense. Levels of histamine methyltransferase, which degrades histamine, increased significantly in retinal tissue when histamine levels fell. Significant weight increases indicated edema, which can result from mast cell mediator action.

Leflunomide, an immunomodulating drug that is known to affect mast cells *in vitro*, prevented induction of EAU. Leflunomide also suppressed changes in the mast cell-related parameters, histamine levels and wet weights.

Mechanisms for activation of ocular mast cells in EAU were investigated. Results suggest that mast cell activation does not occur through mast cell surface IgE-antigen crosslinking. The adjuvant used, complete Freund's adjuvant, is not conducive to IgE production. Histamine releasing factors, HRFs, are produced by various immune system cellular components. Preliminary efforts did not demonstrate HRF activity.

Mast cell numbers, histamine levels, and wet weights were also evaluated in a milder form of EAU induced by M-peptide (Mpep), a peptide fragment of Sag. Mpep/EAU produces few disease symptoms in the anterior eye, but destroys the same retinal area as Sag/EAU--photoreceptor cells and their outer segments. Inflammation is less intense, restricted primarily to the target area. Mast cell numbers did not change, but histamine levels and wet weights changed significantly, suggesting that mast cells are also involved in Mpep/EAU.

Overall, the results of this study add to evidence that mast cells are involved in the pathogenesis of EAU. The results also point to topics of further investigation into the role of mast cells in EAU and in normal function in ocular tissues.

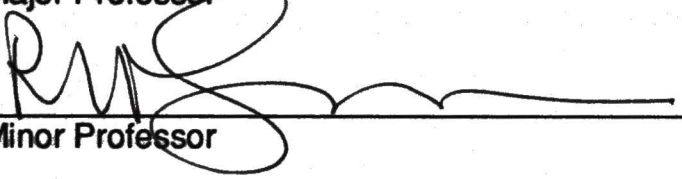
A STUDY OF SOME ASPECTS OF THE ROLE OF MAST CELLS

IN EXPERIMENTAL AUTOIMMUNE UVEITIS

Carol Hamberlin Lee, B.A., M.S.

APPROVED:

Major Professor



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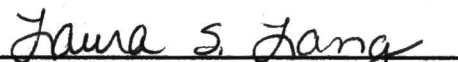

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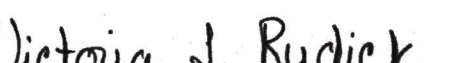

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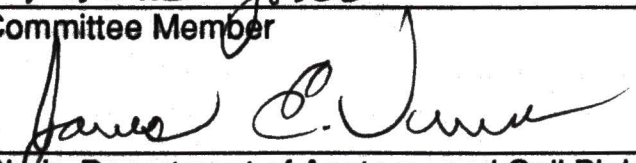

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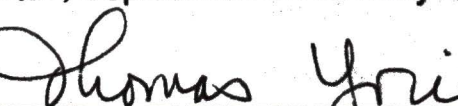

Dean, Graduate School of Biomedical Sciences

A STUDY OF SOME ASPECTS OF THE ROLE OF MAST CELLS IN EXPERIMENTAL AUTOIMMUNE UVEITIS

DISSERTATION

Presented to the Graduate Council of the
Graduate School of Biomedical Sciences
The University of North Texas Health Science Center at Fort Worth
in Partial Fulfillment of the Requirements

For the Degree of

DOCTOR OF PHILOSOPHY

By

Carol Hamberlin Lee, B.A., M.S.
Fort Worth, Texas

June 1994

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I would like to thank Dr. E.L. Orr for his patience, support, and direction during my work at the University of North Texas Health Science Center at Fort Worth; and Dr. L.S. Lang, of Alcon Laboratories, Inc., for her continued support, interest, and encouragement during this time.

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LIST OF ABBREVIATIONS

BSA	Bovine serum albumin
CFA	Complete Freund's adjuvant
CNS	Central nervous system
CsA	Cyclosporin A
EAAU	Experimental autoimmune anterior uveitis
EAE	Experimental autoimmune encephalomyelitis
EAN	Experimental autoimmune neuritis
EAU	Experimental autoimmune uveitis
HLA	Human leukocyte antigen
HMT	Histamine methyltransferase
HRF	Histamine releasing factor
^{3}H-SAM	^{3}H-S-adenosyl methionine
IgE	Immunoglobulin E
IL-1, 2, 6, 8	Interleukin-1, 2, 6, 8
INF-γ	Interferon γ
LEF	Leflunomide
MNL	Mononuclear leukocyte
Mpep	M-peptide
NBF	Neutral buffered formalin
PAF	Platelet activating factor
PB	Phosphate buffer
PKB	Phosphate buffer with azide
PMC	Peritoneal mast cell

PMN	Polymononuclear leukocyte
PT	Pertussin toxin
SA	Specific activity
Sag	S-Antigen
SEM	Standard error of the mean
TB	Tyrode's buffer
TNFα	Tumor necrosis factor α

CHAPTER 1

INTRODUCTION

Uveitis, an inflammation of the uveal tract, is estimated to be the cause of about 10 percent of cases of severe visual handicap in the United States (76). Uveitis is usually chronic, often relapsing, and may occur in association with systemic diseases such as Behçet's disease, a disease of the mucous membranes. Uveitis is usually regional in nature, involving the pars planitis (intermediate uveitis), the anterior eye (anterior uveitis), or the posterior eye, including the retina, choroid, and vitreous (posterior uveitis) (76).

Some agents that cause uveitis, such as toxoplasmosis and cytomegalovirus, are known. However, in many cases the etiology of uveitis has not been determined (101). Autoimmunity to uveal antigens was suggested by Elschnig in 1910 as the cause for sympathetic ophthalmia, which is a form of uveitis that sometimes results in the loss of the contralateral eye after a puncture injury to the eye (107). Autoimmunity has also been suggested as a contributory or causative factor in several other forms of uveitis, usually based on cellular and humoral responses to ocular antigens. These diseases include birdshot retinochoroidopathy (named for characteristic lesions on the fundus), Vogt-Koyanagi-Harada's disease (a bilateral granulomatous uveitis) and Behçet's disease (35).

Corticosteroids are commonly used for the treatment of uveitis (76). The immunosuppressants, cyclosporin A (78) (despite its nephrotoxic properties) and FK506 (69), have also been used effectively to treat uveitis. In addition, some routes of immunization (including oral, intravenous, and intracameral routes) can specifically suppress or tolerize the immune system to the immunizing antigen. Oral administration of retinal antigen to patients who demonstrate prior or current ocular inflammation by their positive *in vitro* response to this antigen, is currently being tested in clinical trials to investigate immune tolerance (77, 111).

Experimental Autoimmune Uveitis

Animal models of uveitis have been used to gain insight into the pathogenesis of human uveitis. Animal studies have demonstrated the changes that occur in T-cell subsets during experimental autoimmune uveitis (EAU), established the genetics-related (HLA associated) susceptibility to EAU, and tested the effectiveness of various drugs as potential treatments for uveitis (36, 76, 77).

The work of Wacker et al. (107) established that retinal proteins are uveitogenic in animals. An animal model, EAU, was subsequently developed that can be induced in laboratory animals, such as the Lewis rat, by inoculation with one of several retinal antigens emulsified in an appropriate adjuvant (108). EAU is a T-lymphocyte-mediated autoimmune disease, and can also be induced through adoptive transfer using T-helper cells sensitized to retinal antigen (36, 108). The uveitogenic proteins include arrestin, also referred to as S-antigen (Sag), and interphotoreceptor retinoid binding protein (IRBP). Large

doses of rhodopsin can also induce EAU (107). Most EAU research has used Sag as the sensitizing antigen (107).

The severity and pathogenesis of EAU can vary widely, depending on the animal, the retinal antigen, the adjuvant(s), and the dosage of antigen used (35). For example, although EAU in rats is an acute disease that involves the entire retina and the choroid, in guinea pigs EAU affects only the choroid and the photoreceptor cell layer. This appears to occur because the guinea pig retina has only a single, choroidal blood supply without the additional retinal vasculature found in humans, rats, monkeys, and other animals (35). EAU in the monkey requires a much longer induction time (up to a month, versus 9-12 days for the rat) than EAU in the rat (77), and more closely resembles a human uveitis, birdshot retinochoroidopathy, than EAU induced in other laboratory animals (35). The use of *Bordetella pertussis* as an adjuvant shortens the time required to induce EAU and intensifies the disease (35).

Bovine-derived retinal Sag in complete Freund's adjuvant (CFA) is commonly used to induce EAU in rats. Sag-induced EAU (Sag/EAU) in the Lewis rat is characterized clinically and histologically by intense bilateral involvement of both anterior and posterior portions of the eye (17, 35). The onset of Sag/EAU is fulminant and occurs 9-12 days postinoculation (pi) (33). At the peak of the disease, the limbal vessels are dilated and engorged; corneal cloudiness and, at times, perforation occur; fibrin deposits are visible in the anterior chamber; and the iris is unresponsive to mydriatics. Histological examination of the retina shows that the inflammation is characterized by edema and a massive influx of leukocytes, culminating in total disruption of the retina and destruction of the target protein, Sag, which is located in the outer

segments of photoreceptor cells. Because Sag is also diffusely localized in the pineal gland, pinealitis is induced at the same time as EAU (27).

S-Antigen was first identified as a uveitogenic component of retina and purified from the soluble fraction ("S"-fraction) of a retina preparation by Wacker and his associates (109, 110). Sag has been purified from retinas of several species and localized immunohistochemically to the rod outer segments of the photoreceptor cells (108). Also known as the 48K protein or arrestin, Sag functions in the visual process in downregulating light-activated and phosphorylated rhodopsin (59, 86). Sag has been sequenced (95) and uveitogenic sites identified at the molecular level (27, 28).

Cyanogen bromide digestion of Sag produces several peptide fragments, two of which, peptides M and N, are uveitogenic (28). These digestion products, as well as synthetic forms of M-peptide (26, 40), induce EAU that is identical in symptoms to Sag/EAU, when *B. pertussis* and CFA are used together as adjuvants. An M-peptide-induced EAU (Mpep/EAU) reported by Smith et al. (97), induced using CFA (supplemented with additional lyophilized *Mycobacterium tuberculosis*) as the adjuvant, is similar to Sag/EAU in that the outer segments (where Sag is localized in the eye) and associated cell bodies of photoreceptor cells are destroyed. However, the degree of disruption of the retinal architecture is significantly less than that observed in Sag/EAU, or in Mpep/EAU induced with *B. pertussis*/CFA, and little or no involvement of the anterior portion of the eye occurs (97).

Mast Cells

Mast cells, first described by Ehrlich in 1879, are of hematopoietic, myeloid origin, and are located in tissues throughout the body. Mast cells are cells filled with granules containing preformed stores of biogenic amines and other physiologically active substances that are bound to a matrix composed of either heparin or chondroitin sulfate (92). Mast cells can be isolated from tissues, but have not been cultured successfully in large numbers. Cell lines with many features of mast cells have been developed from bone marrow precursors, using growth factors. Some spontaneous growth factor-independent cell lines exist, and viral-infected mast cell lines also are used for evaluation of mast cell function and chemistry (34). Some characteristics of mast cells, such as granule density, granule matrix, and specific enzymes, vary within and between different species.

Two major types of mast cells are recognized in rats, and are classified on the basis of the chemistry of the granule matrix, as well as on distinctive developmental characteristics and pharmacological responses (37). Ocular mast cells are of the connective tissue (serosal) type (2), and have a heparin granule matrix. Serosal mast cells are distributed throughout connective tissue and are specifically found in tissue interfacing with surfaces, such as in conjunctiva, skin and nasal mucosa. In the rat, serosal mast cells are abundant in the peritoneal cavity. The other major type of mast cell, the mucosal mast cell, has a chondroitin sulfate matrix, and is located primarily in intestinal mucosa and bronchial epithelium (10). Mast cell precursors isolated from bone marrow differentiate into either mucosal or serosal mast cells *in vitro*, depending on the growth factors used (34).

Rat serosal mast cells each contain 10 to 30 pg of histamine and about 1 pg of serotonin, bound to heparin in the mast cell granules (92). The granules also contain neutral proteases and acid hydrolases. Heparin is stained metachromatically by acidified toluidine blue. This characteristic stain provides a method for identification of the rat serosal mast cell (7). When mast cells are activated, the granules are released, and heparin, with the other granule contents, is dispersed into the surrounding tissue and is no longer stainable with toluidine blue. Thus, the activated mast cells are no longer stained metachromatically with toluidine blue, and the "disappearance" of stained mast cells then serves as a method for assessing and quantitating mast cell activation.

Activation and release of granule contents produces the effects commonly recognized in inflammation. Histamine and serotonin both enhance vasopermeability and result in edema. Upon release of mast cell granules, the acid hydrolases are released into the surrounding tissue, but the neutral proteases remain bound to the heparin matrix (92).

Synthesis and release of other potent mast cell mediators follows activation and release of granules (10, 38). Substances synthesized upon activation include some products of arachidonic acid metabolism that contribute to inflammation (e.g., some of the prostaglandins, leukotrienes, thromboxanes, and hydroxyeicosatetranoic and monohydroxyeicosatetranoic acids) (10), platelet activating factor (PAF), and a number of cytokines, including tumor necrosis factor α (TNFα) (38).

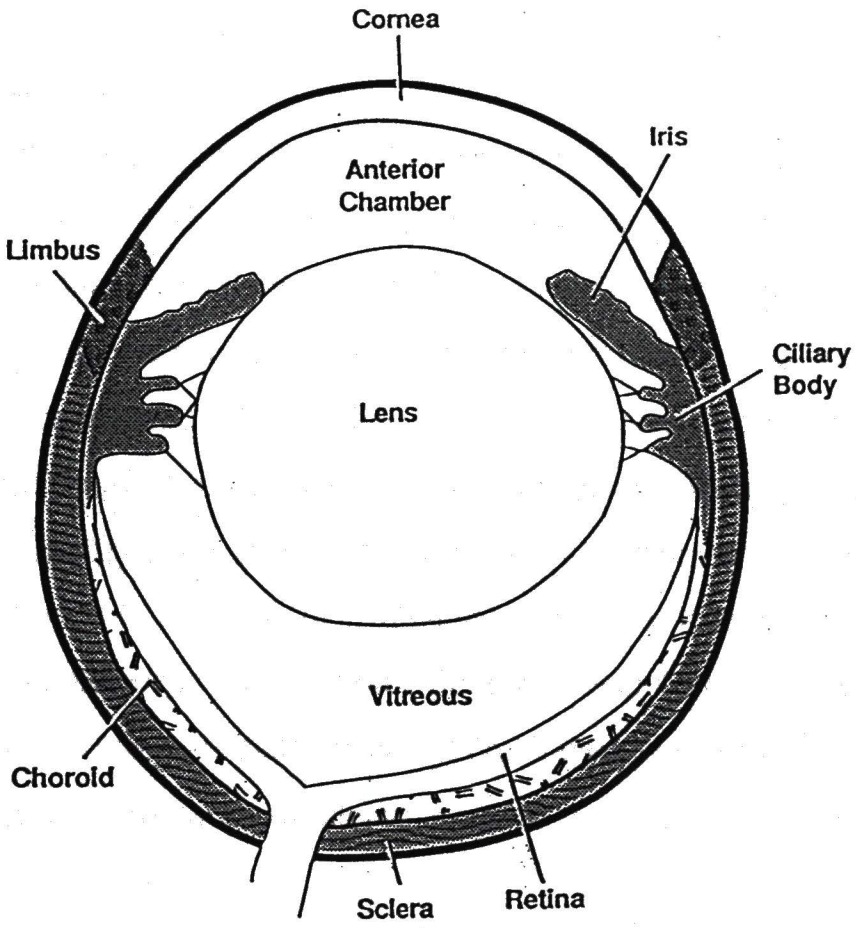
Classically, activation of mast cells occurs when the cells are stimulated by the crosslinkage of antigen-specific IgE bound to Fc^{ε} receptors on the cell

surface (10). Mast cells are also activated by "histamine releasing factors" (HRFs). HRFs do not involve antigen-antibody crosslinkage, and are produced by a number of different immune system cells, including macrophages, polymorphonuclear cells, and T lymphocytes (10). The close association of ocular mast cells with blood vessels suggests that mast cells could be encountered and possibly activated by HRFs elaborated by T lymphocytes or other immune cells exiting the vasculature (88). In addition to immune system-related activation, mast cells are activated by numerous chemical agents and by physical stimuli, such as mechanical and thermal disturbances (81).

Historically, connective tissue-type mast cells have been categorized as significant cellular mediators of immediate hypersensitivity and allergic reactions (10). More recent findings suggest that mast cells have additional functions, such as a regulatory role in inflammation (25), control of microvasculature dynamics (89), and a possible role as antigen presenting cells (87). At present, the mechanisms for mast cell involvement in these processes have not been fully elucidated.

Mast cell distribution in the rat eye has been described by Smelser and Silver (96) and Allansmith et al. (2). The distribution of ocular mast cells is illustrated by the diagram in Figure 1, modified from Allansmith et al. (2). Excluding adnexal tissues of the eye, such as lids and conjunctiva (which contain very large numbers of mast cells), mast cells are found in highest density in the limbus and the choroid (2, 96), generally arrayed along blood vessels. Specifically, choroidal mast cells are associated with arterioles (37). Numerous mast cells are also associated with the blood vessels of the human choroid and limbus (47). Rat choroidal mast cells are most numerous in the

Figure 1. Diagrammatic representation of mast cell distribution within the rat eye with shaded areas indicating the location of mast cells. Areas with mast cells include the limbus, choroid, sclera, and iris/ciliary body. The highest density of mast cells occurs in the limbus, and the mast cell density decreases in the tissues in the order listed (2). No mast cells are found in the retina.


Cornea
Iris
Anterior
Chamber
Limbus
Ciliary
Body
Lens
Vitreous
Choroid
Sclera
Retina

posterior choroid, with sparse distribution toward the anterior uvea (96). The density of mast cells in the limbus is greater than the density in the choroid (2). Mast cells are also located in the ciliary body (2, 65) and the outer surface of the sclera, but are less abundant in these areas (2, 37).

Mast Cells in Uveitis

de Kozak et al. (20) first reported activation of choroidal mast cells in Lewis rats with Sag/EAU, based on a temporal decrease in toluidine blue stained choroidal mast cell numbers. However, this observation was based on a sample number of only two eyes, apparently from only one or two rats for each time point considered. de Kozak et al. (20) suggested that vasoactive amines released from activated choroidal mast cells may contribute to the increased permeability of the blood-retinal barrier that occurs in Sag/EAU, and may open this barrier to the influx of inflammatory cells. de Kozak et al. (21) later reported that EAU can be attenuated through manipulation of mast cells with drugs that stabilize mast cells (disodium cromoglycate or ketotifen), or that deplete mast cells of their granular stores of active products (Compound 48/80).

Mochizuki et al. (70) found that rat strains with large numbers of choroidal mast cells (such as Lewis rats) are more susceptible to Sag/EAU than rat strains with relatively few choroidal mast cells (such as Brown Norway rats). The F1 hybrids of these rat strains fell between the parental strains both in numbers of choroidal mast cells and in susceptibility to EAU. Thus, mast cell numbers apparently correlated with susceptibility to Sag/EAU.

To account for the mechanism of activation of choroidal mast cells by an antigen located in the retina, de Kozak et al. (20) suggested that Sag leaked from the retina through the retinal pigmented epithelium and induced mast cell degranulation by crosslinking with Sag-specific (IgE) antibody bound to choroidal mast cells. Supporting this hypothesis, de Kozak et al. reported that Sag-specific IgE antibodies were present in the serum of EAU rats, and that peritoneal mast cells obtained from rats with EAU degranulated when exposed to Sag (20). The adjuvants that de Kozak et al. used in inducing EAU included either alum or *B. pertussis* (20), both of which stimulate IgE production (52). EAU is also induced when CFA alone is employed as adjuvant, but CFA alone is not conducive to IgE production (52).

Several cytokines have been implicated in uveitis, including TNFα, interleukin-1 (IL-1), IL-2, IL-6, IL-8, and interferon-γ (INF-γ), (22). All of these, and/or their precursors, are synthesized by mouse peritoneal mast cells and/or by mast cell lines (38).

In addition to the mast cell stabilizing drugs, disodium cromoglycate and ketotifen, that were employed by de Kozak et al. (21) to implicate mast cell involvement in EAU, the immunosuppressants, cyclosporin A (CsA), FK506, and rapamycin, have been shown to prevent or attenuate EAU (42, 43, 98). CsA, FK506, and rapamycin also inhibit mast cell cytokine production in murine mast cell lines (42, 43) and CsA has been shown to inhibit exocytosis of granules in a rat mast cell line (106). Smith Lang et al. (98) have reported that an immunomodulating drug, leflunomide (LEF, HWA 486), is effective in suppressing the inflammation of EAU. LEF has also been shown to inhibit the

release of histamine from rat peritoneal mast cells (8). Together, these results further implicate mast cells as active cellular participants in Sag/EAU.

Mast cell involvement has been described in other T-cell-mediated autoimmune disease models in the Lewis rat, such as experimental autoimmune encephalomyelitis (EAE) (23, 83) and experimental autoimmune neuritis (EAN) (16). Significant decreases were found in mast cell numbers during EAN (16). Both inhibitors of mast cell degranulation (23, 103) and treatment with antagonists to the receptor for the mast cell mediator, serotonin, (23) have been shown to block development of EAE.

Research Aims

The overall purpose of this research was to evaluate the role of ocular mast cells in EAU. In particular, the first aim was to confirm and extend research that suggested that choroidal mast cells may be important cellular mediators of EAU (20, 21). Inflammation of the anterior portion of the eye is a prominent feature in Sag/EAU (20, 21), but possible involvement of limbal mast cells had not been investigated in EAU. Therefore, the second aim of the study was to assess the possibility that limbal mast cells may also mediate EAU, especially the inflammation of the anterior eye. Control of Sag/EAU through the use of drugs that prevent mast cell activation has been reported (20, 21), and the third aim of this study was to evaluate additional drugs that block mast cell activation. Because several alternate mechanisms of mast cell activation exist (20, 21), the fourth aim was to study possible mechanism(s) involved in mast cell activation in Sag/EAU. Finally, the fifth aim of the study was to compare the role of ocular mast cells in the milder form of EAU induced by Mpep to the role

of ocular mast cells in Sag/EAU. These aims were implemented as indicated below.

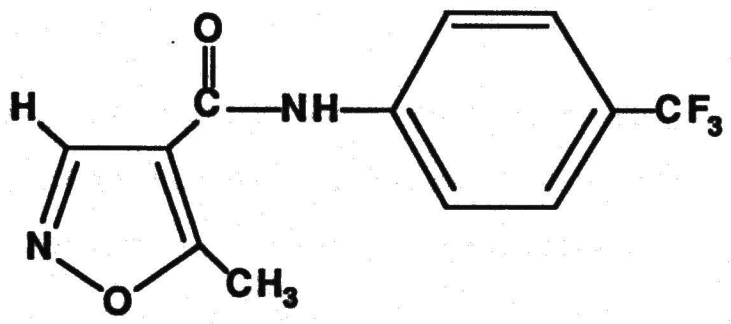
Two techniques, assessment of numbers of mast cells and determination of changes in histamine levels, were used to evaluate mast cell activation in EAU. Changes in choroidal and limbal mast cell numbers before and during Sag/EAU (61, 62, 64) and Mpep/EAU (63) were determined. Changes in histamine levels, potentially a more sensitive method of evaluating activation of mast cells, were monitored in ocular tissues during Sag/EAU (61, 62, 64) and Mpep/EAU (63). These tissues included the choroid, retina, sclera, and aqueous humor, and the anterior portion of the eye (cornea with limbus, lens, iris, and ciliary body, treated as one tissue).

Mast cell activation was evaluated during suppression of Sag/EAU by an immunomodulating drug, leflunomide, made available by Alcon Laboratories, Inc. (Fort Worth, Texas). The structure of this drug and the active metabolite, (A77 1726) are shown in Figure 2. Because leflunomide inhibits the release of histamine from mast cells (8), histamine levels were determined to assess inhibition of mast cell activation.

Both IgE-dependent and IgE-independent mast cell activation mechanisms were evaluated during EAU. *In vitro* release of histamine by both peritoneal and ocular mast cells was undertaken to investigate mechanisms involved in the activation of ocular mast cells in EAU.

Figure 2. The structure of a) leflunomide and b) the active metabolite of leflunomide, A77 1726.

a



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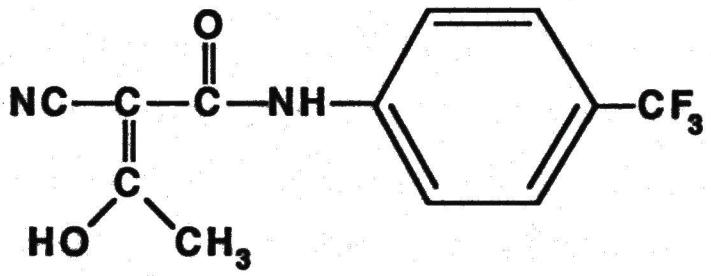


CHAPTER 2

MATERIALS AND METHODS

Alcon Laboratories, Inc., (Fort Worth, Texas) provided animals, housing for animals, and supplies, including antigens, for this study. In addition, EAU was induced and disease scoring was performed by Alcon personnel. Histological preparations were also supplied by Alcon and were prepared as described in (99). In some instances, serum and aqueous humor were collected by Alcon personnel.

Animals

Male Lewis rats (Charles River Laboratories, Wilmington, MA) were used for all studies. Weights for groups of rats were consistent within each experiment, with variations of only a few grams. The range of average weights for the groups of rats was from 185 g to 275 g. Ocular disease was inducible independent of the variations in weight. Rats were housed using constant temperature and controlled lighting (12 h light/dark cycle), with unrestricted access to food and water. All animals underwent ophthalmoscopic examination prior to the initiation of the study to certify that all were free of ocular inflammation.

Preparation of S-Antigen

Bovine S-antigen (Sag) was purified to homogeneity by Alcon Laboratories, Inc. (Fort Worth, TX) using a modification of the method of Dorey et al. (30). Pooled bovine retinas were suspended in 50 mM sodium potassium phosphate buffer (pH 7.6) containing 0.02 percent azide (PKB), and were stirred for 1 hr at 4°C. The extract was removed and the extraction repeated. The pooled extracts were centrifuged (40,000 g for 40 min). Saturated ammonium sulfate was then added to the supernatant to a concentration of 50 percent. The mixture was allowed to stand overnight at 4°C, and then it was centrifuged (7500 g for 15 min). The precipitate was then dissolved in a minimal volume of PKB and clarified by centrifugation at 140,000 g for 1 hr. The supernatant was dialyzed overnight at 4°C against PKB and then concentrated with sucrose to 3-5 ml.

Gel filtration was carried out using a 2.5 x 90 cm Sephacryl S200 (Pharmacia, Sweden) column, with the entire sample applied to the column. The column was eluted with PKB, and fractions containing Sag were identified on SDS-PAGE using purified Sag and molecular weight markers. Fractions containing Sag were pooled and vacuum concentrated/dialyzed against 0.5 M NaCl in 10 mM sodium potassium phosphate buffer, pH 7.6.

Sag was further purified using adsorption chromatography on a 2.5 x 50 cm Phenyl Sepharose CL-4B (Pharmacia, Sweden) column. Sag was eluted from the column with 0.5 M NaCl in 10 mM sodium potassium phosphate buffer, pH 7.6, and was identified on SDS-PAGE as described above. The purified Sag was vacuum concentrated/dialyzed against PKB to 1 mg protein/ml. Dialyzed Sag was stored at -20°C.

Preparation of M-Peptide

Mpep, an octadecapeptide corresponding to amino acid positions 303 to 320 in bovine Sag, was synthesized by Peptide Technologies, Inc. (Washington, D.C.) using the method of Knight et al. (58).

Induction of EAU

Purified bovine S-antigen or synthetic M-peptide dissolved in phosphate buffered saline at a concentration of 1 mg/ml was emulsified 1:1 (v/v) with complete Freund's adjuvant (Difco Laboratories, Detroit, MI) supplemented with 5 mg/ml *Mycobacterium tuberculosis* H37Ra (Difco) (sCFA). One or the other of the emulsified antigens was injected in a volume of 0.1 ml (containing 50 µg of antigen) into the right hind footpad of each experimental rat. Control rats were injected with an equal volume of the same adjuvant emulsified 1:1 (v/v) with saline.

Administration of Leflunomide

Leflunomide (HWA 486, Hoechst AG, Wiesbaden, Germany) was administered orally to rats daily beginning on the day of inoculation with Sag/sCFA or saline/sCFA. Dosage was 4 mg/kg/day, which had previously been established as a minimum effective dose (98). A third group of rats received Sag/sCFA only.

Ocular Disease Scoring

Each eye was evaluated for clinical evidence of EAU on the day of inoculation and on days 10 through 13 postinoculation (pi) for Sag/EAU or on

days 9 through 19 pi for Mpep/EAU. These days included the day(s) of onset (day 10 pi for Sag/EAU) and the disease peaks for both forms of EAU. When saline/sCFA-inoculated control animals were used, evaluation was performed on the same days as for EAU eyes. Eyes were observed for disease following dilation of the pupil with a 0.5 percent solution of a mydriatic agent, MYDRIACYL® (Alcon Laboratories, Inc., Fort Worth, TX). A modification of the ocular disease scoring method of Hackett and McDonald (41) was used (see Appendix A). This scoring method employs direct ophthalmoscope observation to evaluate disease severity for five regions of the eye, and facilitated rapid handling of large numbers of animals. The regions scored included: 1) limbal vessels; 2) cornea; (2a, intensity of disease; 2b, percent area of cloudiness); 3) anterior chamber; 4) iris (response to mydriatic); and 5) vitreous. The range for scoring was from 0 (no disease) to 4 (most severe disease) for all the regions scored except for the limbal vessel evaluation, which was given a maximum score of 3. The vitreous was arbitrarily assigned the maximum score when lack of response to mydriatic prevented observation of the posterior eye. The specific parameters evaluated are described in Appendix A.

Collection of Ocular Tissue

Following light anesthesia with halothane, the animals were sacrificed by cervical dislocation or decapitation and the eyes were enucleated. Eyes from treated and control rats were collected on days 5, 7, 9, 11 and 13 pi for Sag/EAU and on days 9, 11, 13, 15, 17 and 19 pi for Mpep/EAU. For mast cell counts, whole eyes were placed in 10 percent neutral buffered formalin (NBF). Eyes to be assessed for histamine were placed on ice and, after an aqueous

humor aliquot was obtained using a drawn-out capillary tube, each eye was dissected on ice into four parts: retina, choroid, sclera, and anterior segment. The anterior segment consisted of cornea with limbus, lens, iris, and ciliary body. Tissues were weighed and stored at -70°C until analyzed.

Blood samples were obtained by heart puncture after rats were anesthetized with halothane. The animals were sacrificed before regaining consciousness. Blood was refrigerated until a clot formed and then centrifuged for 10 min at 12,500 g. Serum samples were stored at -70°C until analyzed.

Collection and Purification of Peritoneal Mast Cells

The method used was a modification of that of Atkinson et al. (5). Following light anesthesia with halothane, rats were decapitated or cervically dislocated, and 10 ml of ice cold Tyrode's buffer (TB) was injected into the peritoneal cavity. Tyrode's buffer (5, 85) contained 137 mM NaCl, 2.7 mM KCl, 1.0 mM CaCl, 5.6 mM glucose, 10 mM Hepes, and 1.0 mg/ml BSA (Sigma) at pH 7.2. TB was stored at 4°C without the glucose and BSA, which were added on the day of use. The peritoneum was then massaged for 30 sec. and the fluid removed. The cells were washed once with TB using centrifugation at 450 g, and were resuspended in 1 ml TB. Cells from each rat were collected separately. The cell preparations were used as is or were partially purified on bovine serum albumin (BSA) gradients.

For purification of PMC on BSA gradients, each 1 ml cell suspension in TB was layered onto a 2 ml cushion of 38% BSA in a 15 ml polypropylene conical centrifuge tube. The cells were allowed to settle by gravity for 25 min. at room temperature before centrifugation at 450 g for 20 min. at room

temperature. The interface of TB/BSA was removed using a Pasteur pipette, and the top of the gradient was then carefully washed (maintaining the gradient) twice by slowly adding and aspirating 3 ml TB with a Pasteur pipette. The TB washes were transferred to a 50 ml centrifuge tube, additional TB was added to a final volume of 40 ml, and the cells were washed twice by centrifugation at 450 g at 4°C. The cell pellet was resuspended in 0.5 ml after the last wash and an aliquot was counted.

Peritoneal Mast Cell Counts

Peritoneal mast cells were stained with a 0.25% solution of toluidine blue in 70% ethanol acidified with HCl (pH 2.4) as described by de Kozak et al. (20). Cells were counted in a hemacytometer after appropriate dilution, and a differential count was performed to determine the percentage of purification obtained.

Limbal and Choroidal Mast Cell Counts

After fixation in NBF for at least one week, the choroid and the anterior portion of the eye (which included the limbus and cornea), were dissected from each eye and stained with acidified toluidine blue. Tissues were dipped into 0.5 percent acidified toluidine blue, blotted, and rinsed, first in tap water, then in acidified tap water. Both limbal and choroidal tissues were spread flat on glass slides and mounted with glycerol (80).

Mast cells in choroids and limbi were counted at a magnification of 75X, using a dissecting microscope equipped with a 1 mm^2 ocular grid. The grid squares were projected onto areas of highest mast cell density in both tissues

(Fig. 3a and b). The area counted for choroids was 4.4 mm^2. For limbi, 1.6 mm^2 was counted for Sag/EAU eyes and 3.2 mm^2 for Mpep/EAU eyes. The eyes were coded before counting, and all counting was done by the same observer. In order to assure that similar areas of choroid were counted in each eye, the long posterior ciliary artery was used as a marker to consistently orient placement of the microscope grids. In order to include the areas of greatest mast cell density, the limbal areas evaluated for mast cells spanned the entire width of the limbal vascular ring, and the grid used for counting was moved around the circumference of the limbus.

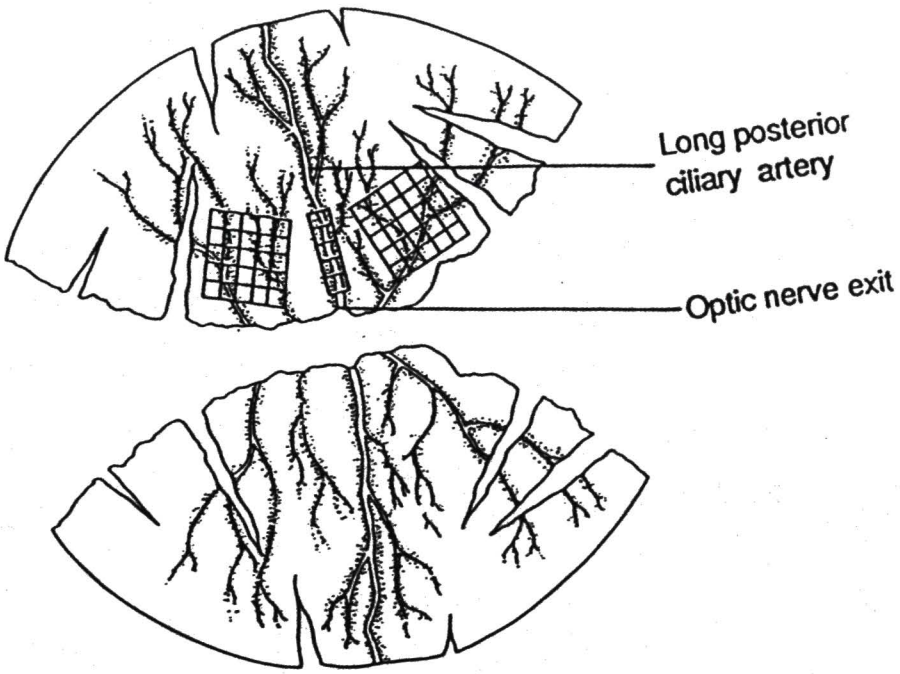
Preparation of Tissue for Histamine Evaluation

Retinas, choroids, anterior segments, and sclera of all eyes to be evaluated for histamine were homogenized or sonicated for 30 sec. in ice cold deionized, distilled water, using either 1) a Polytron homogenizer (Brinkman Instruments, Rexdale, Ontario, Canada) for sample volumes greater than 500 µl (choroid and anterior segment) or 2) a Kontes micro-ultra sonicator (Kontes, Vineland, N.J.) for smaller samples (retina). The amount of water added for tissue homogenization was based on the expected histamine content of the tissue.

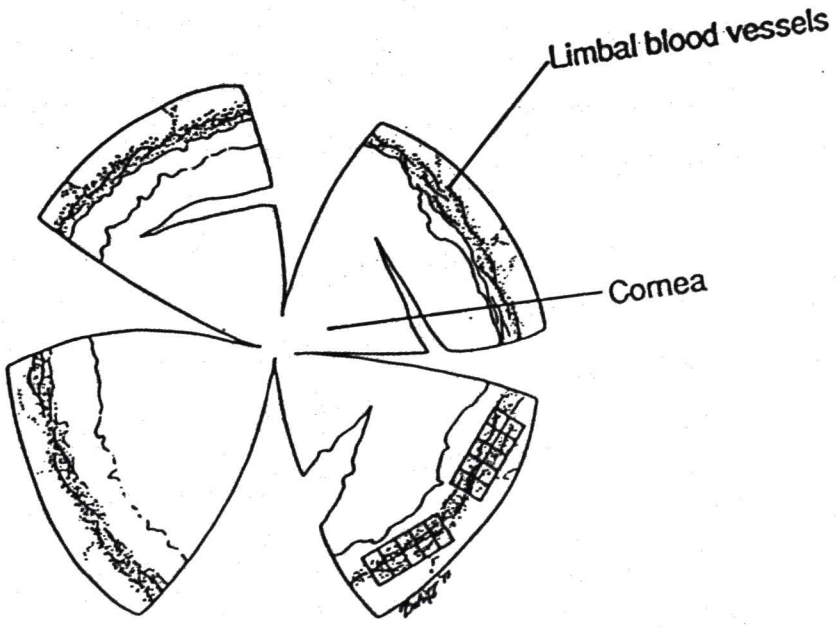
In order to destroy any endogenous histamine-N-methyltransferase and S-adenosyl-L-methionine, aliquots of tissue homogenates in microcentrifuge tubes were immediately placed in either a boiling water bath or a block heater (preheated to 100°C) for 10 min. Each tube was timed separately, then cooled on ice and subsequently stored at -70°C until assay. Because of the small

Figure 3. Areas counted in choroid and limbus. A 1 mm^2 grid was placed over areas of highest mast cell density and mast cells were counted as described. a) Diagram showing placement of grid on spread preparation of the choroid. b) Diagram showing placement of grid on spread preparation of the limbus.

a


Long posterior
ciliary artery
Optic nerve exit

b


Limbal blood vessels
Cornea

volumes, aqueous humor obtained from individual eyes was not sonicated and was subjected to only two minutes of heating to minimize evaporation.

Histamine Determination

Immediately before analysis, samples were thawed and centrifuged for 10 min. at 4°C in microcentrifuge tubes at 12,500 g or at 24,300 g. (The force of centrifugation varied depending on centrifuge availability. Either force was adequate for pelleting the sonicated material.)

Aliquots of each supernatant were assayed radioenzymatically for histamine using a partially purified preparation of rat kidney histamine-N-methyltransferase, prepared according to Shaff and Beaven (94). This enzyme transfers a radioactively labeled methyl group from S-adenosyl-L-[methyl-^{3}H] methionine to histamine, and forms radioactively labeled 1-methylhistamine. Aliquots (10 µl) of supernatant were added to duplicate 250 µl microcentrifuge tubes on ice (for blanks, ice-cold distilled water was substituted for supernatant). Then 10 µl 0.05 M phosphate buffer, pH 7.9 (PB7.9), alone or containing 50, 100, or 200 pg histamine (internal standards) was added to duplicate tubes. The tubes were then centrifuged briefly (3 sec) at 12,500 g. Finally, 10 µl of a reaction mixture containing 2 µl histamine-N-methyltransferase, 0.5 µl (0.5 µCi) S-adenosyl-L-[methyl-^{3}H] methionine (Amersham, Arlington Heights, IL, specific activity (SA) 15 Ci/mmol) and 7.5 µl PB7.9 was added to each tube. After a brief centrifugation (3 sec) at 12,500 g, the tubes were incubated at room temperature for 60 min. The reaction was stopped by the addition of 10 µl 0.5 mg/ml 1-methylhistamine dihydrochloride (Sigma Chemicals, St. Louis, MO) in 0.4 M perchloric acid ("Stop Mix") and the

tubes were placed on ice after another brief centrifugation (3 sec) at 12,500 g. After 20 min., the tubes were centrifuged at 12,500 g for 5 min., and an aliquot (20 µl) of each supernatant was spotted on one of the channels of an LK5D TLC plate (Whatman Chemical Separation, Inc., Clifton, NJ). The center channel of each plate was also spotted with a 5 µl aliquot of the Stop Mix, containing 1-methylhistamine. Prior to spotting, the TLC plates were activated at 120°C for 20 min. The spots were air dried in a hood for at least 1 hour, and then developed to a solvent height of 12 cm in chloroform/methanol/ammonium hydroxide (12:7:1, by volume). The TLC plates were then air-dried in a hood, subsequently sprayed with ninhydrin (Sigma, 200 mg/100 ml of 100% ethanol), and, finally, heated briefly in an oven to visualize the methylhistamine spots. After cooling, each methylhistamine spot was scraped into a scintillation vial, to which was added 1.0 ml 100% ethanol and 5 ml Ecolite (+) ® (ICN Biochemicals, Cleveland, OH). The vials were counted in a liquid scintillation spectrometer for 5 min. After subtraction of blanks, tissue histamine levels were determined from plots of dpm (or cpm) versus pg internal histamine standard. This assay is sensitive to about 25 pg histamine. Histamine content was calculated on the basis of each entire area of the eye evaluated (choroid, retina, sclera or anterior segment), because calculation on a per unit wet weight basis would not have taken into account the dilution of histamine resulting from the edema that occurs during inflammation, nor for the large proportion of the weight of the anterior segment that is contributed by the lens (approximately 70 percent, see Appendix B, Table 5). Aqueous humor histamine content was recorded on a per µl basis.

Histamine Methyltransferase Determination

The method used for histamine methyltransferase (HMT) determination was a modification of that described by Orr et al. (84). Frozen retinal tissue from rats with Sag/EAU (sacrificed on days 9, 11, and 13 pi) was thawed and homogenized in ice cold glass distilled water (1:1, w/v) as described above for histamine determination. The homogenates were centrifuged as described above, and aliquots of the supernatant removed for analysis. The remaining homogenate/supernatant was refrozen. Generally, reanalysis of the frozen tissue homogenate was not necessary; however, any reanalysis was performed on samples frozen less than 7 days.

Duplicate 10 µl aliquots of the supernatants were placed in 250 µl microcentrifuge tubes. Duplicate 10 µl water blanks were also included in the assay as a control. A working stock of S-adenosyl-L-[methyl-^{3}H] methionine (^{3}H-SAM), Amersham, Arlington Heights, IL) was made by diluting the stock ^{3}H-SAM (SA 500 mCi/mol) 1:10 v:v in 0.05 M phosphate buffer, pH 7.4 (PB7.4). Ten µl of the diluted ^{3}H-SAM solution was added to each assay tube. The reaction was initiated by addition of 10 µl 75 µM histamine in PB7.4. The total reaction volume was 30 µl. The tubes were incubated for 30 min. at room temperature, and the reaction terminated by the addition of the same Stop Mix used for histamine determination. Subsequent steps for the assay were the same as those described in the histamine determination, i.e., thin layer chromatography separation of 1-methylhistamine. Dpm for 10 µl of ^{3}H-SAM stock solution was determined and used to calculate results as nmol 1-methyl histamine/g tissue/30 min. incubation.

Release of Histamine from Peritoneal Mast Cells Exposed to S-Antigen

Peritoneal mast cells (PMC) from Sag-sensitized rats (sacrificed on days 5, 7, 9, 11, and 13 pi) were incubated *in vitro* with aliquots of Sag that had been previously dried in 1.5 ml microcentrifuge tubes at room temperature, as described by Benveniste (11) and modified by Sainte-Laudy et al. (90) and de Kozak et al. (21). Five dilutions of Sag in water, ranging downwards from 100 µg/ml to 1 ng/ml, and one control tube that had contained only water, were prepared in triplicate. For calibration of histamine release, some cells were incubated with varying concentrations of Compound 48/80 (Sigma). Ten µl of cell suspension (containing 1000 to 2000 BSA-gradient purified PMC) were placed into each tube. After a 15 min. incubation at 37°C, 190 µl of ice cold Tyrode's buffer (TB) was added to stop any antigen-antibody crosslinkage, resulting in a final volume of 200 µl. After the cell suspension was centrifuged for 10 min at 12,500 g, 150 µl of the supernatant was removed (containing 75 percent of the released histamine), increased in volume to 200 µl by the addition of 50 µl of water, and sonicated. The pellet was suspended in the remaining 50 µl of TB (containing the unreleased histamine and 25 percent of released histamine), 150 µl water was added, and the suspension was sonicated. All samples were heat treated as described for the histamine determination (to destroy endogenous HMT and S-adenosyl L-methionine) and were evaluated for histamine to calculate percentage histamine release. Samples not analyzed immediately were frozen at -70°C. The supernatants were analyzed undiluted and the supernatants from the cellular homogenates

were diluted as necessary before analysis to fall within the sensitivity range of the assay.

Release of Histamine from Ocular Tissue Exposed to S-Antigen

Choroids and limbal regions from a rat with Sag/EAU (sacrificed day 34 pi) were divided into fourths and each fourth was incubated in a volume of 100 µl TB with 0.1 µg/ml or 1.0 µg/ml Sag that had been previously evaporated into microcentrifuge tubes as described above. Exposure to Sag was terminated by the addition of 400 µl TB, after which the material was centrifuged at 12,500 g. After 250 µl of supernatant was removed (representing 50 percent of the released histamine), this aliquot, and the tissue with remaining liquid were sonicated, heat-treated, and assayed for histamine as described above.

Release of Histamine from Peritoneal Mast Cells Exposed to Aqueous Humor from Sag/EAU Rats

PMC from rats that had not been inoculated with antigen (naive rats) were incubated with aqueous humor from rats with Sag/EAU that were sacrificed on either day 11 or 13 pi (potential source of HRF) or with aqueous humor from naive rats (control). PMC (1000 cells in TB, prepared as described above) were pelleted into microcentrifuge tubes, resuspended in 10 µl of aqueous humor, and incubated at 37°C for 30 min. HRF action was stopped by the addition of 190 µl TB. In a second procedure, 1000 PMC from naive rats were incubated with 10 µl aqueous humor from day 11 pi Sag/EAU eyes or from the eyes of naive rats, undiluted or diluted in TB as follows: 1/2, 1/20, 1/100, 1/1000, and 1/10000. HRF action was stopped by the addition of 190 µl

additional TB. For either method of exposure to aqueous humor, the samples were processed and analyzed for histamine as described for EAU-sensitized PMC exposed to Sag.

Passive Sensitization of Peritoneal Mast Cells and Ocular Tissue to S-Antigen

PMC (prepared as described above), choroids, or limbal regions from naive rats were incubated in serum from rats with Sag/EAU that were sacrificed on day 7, 9, or 11 pi (potential source of IgE antibodies to Sag) or in TB (control). One half of the choroidal or limbal tissues were incubated with 200 µl serum; the remaining half was incubated in 200 µl TB (controls). 200 or 2000 PMC were centrifuged and resuspended either in 400 µl serum from Sag/EAU rats or in 400 µl TB. The PMC and tissues were incubated overnight at 4°C with gentle shaking. PMC and tissues were then washed twice in TB by centrifugation.

Release of Histamine from Peritoneal Mast Cells and Choroidal and Limbal Tissue Passively Sensitized to S-Antigen

After exposure to serum, PMC samples were exposed to Sag *in vitro*, processed, and assayed for histamine as described earlier. Choroids and limbal regions that had been exposed to serum from Sag/EAU rats as described above were divided in half (this represented one-fourth of each original tissue section). Choroid sections were exposed to 0.01 µg/ml Sag in 100 µl TB; sections of limbal regions to 0.1 µg/ml in 100 µl TB; for controls, the second portion of each tissue was exposed to 100 µl TB only. The exposure to

Sag was terminated by addition of 400 µl TB. After centrifugation, 250 µl supernatant was removed. Supernatants and tissue samples were subsequently processed and analyzed for histamine as described above for Sag-sensitized ocular tissue exposed to Sag.

Statistical Evaluation

A two-tailed Student's t-test was used to compare changes in mast cell numbers, histamine content of tissues, or HMT to control values. For the leflunomide study, differences between controls and rats treated with the drug were assessed using a one-factor ANOVA. The Fisher PLSD test was used posthoc to compare groups. Probabilities of $p \leq 0.05$ were considered significant.

CHAPTER 3

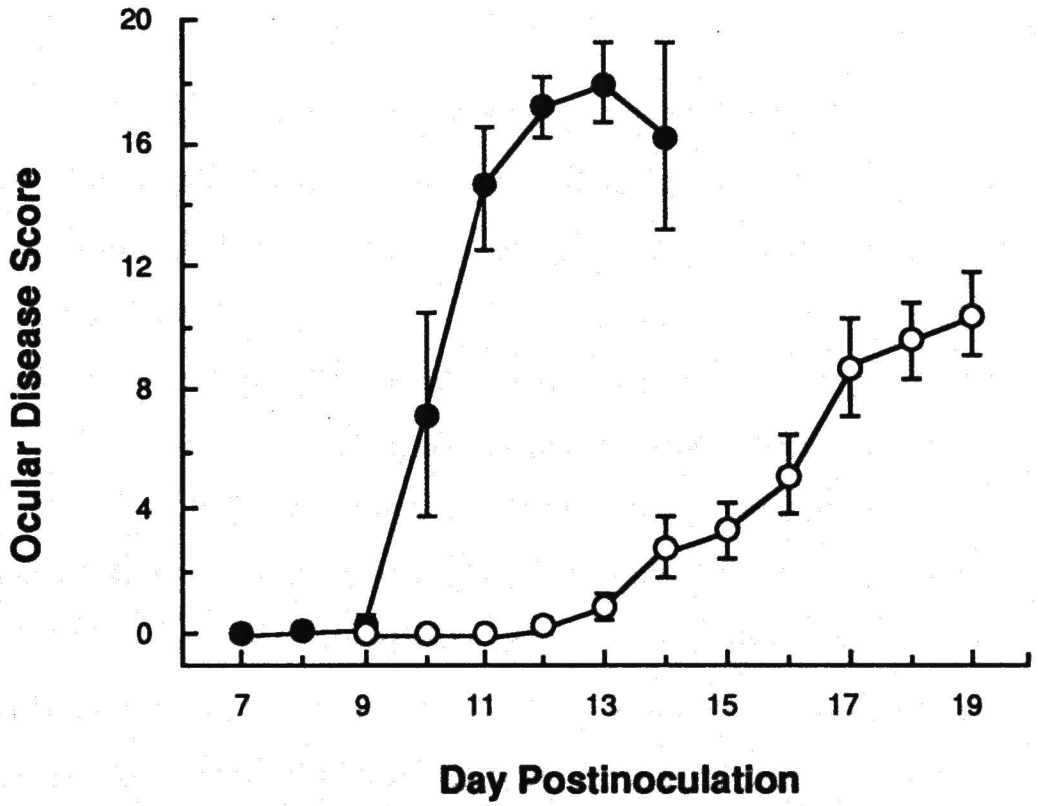
RESULTS

Sag-Induced EAU

Activation of ocular mast cells during Sag/EAU was assessed at various times after inoculation with Sag/sCFA, and then compared to data for saline/sCFA controls using two methods: 1) counting numbers of stainable (i.e., intact or partially degranulated) limbal and choroidal mast cells remaining in the limbus and the choroid on days 5, 7, 9, 11, and 13 pi; and 2) determining the histamine content of the anterior segment of the eye and of the choroid on the same days pi. In addition, retinal, scleral, and aqueous fluid histamine levels were determined. Wet weights of the tissues were also recorded to use in evaluating edema.

Induction of Sag/EAU. Historical data provided by Alcon Laboratories, Inc., illustrates a typical clinical disease course for Sag/EAU and for Mpep/EAU (Fig. 4). The onset of symptoms of Sag-induced EAU was sudden and bilateral, and occurred between days 10 and 11 pi in 100 percent of animals, with clinical scores at or near the maximum score on the day of onset. Correlation of histological evidence of disease with disease scores is highly significant ($p < 0.01$) (60). As described in Materials and Methods, eyes of rats in the current study were only scored from the point of disease onset through the disease

Figure 4. Typical ocular disease scores for Sag/EAU (●) and Mpep/EAU (O). Values are means ± SEM. For Sag/EAU eyes, $n = 16$; for Mpep/EAU eyes, $n = 12$.


Ocular Disease Score
20
16
12
8
4
0
7
9
11
13
15
17
19
Day Postinoculation

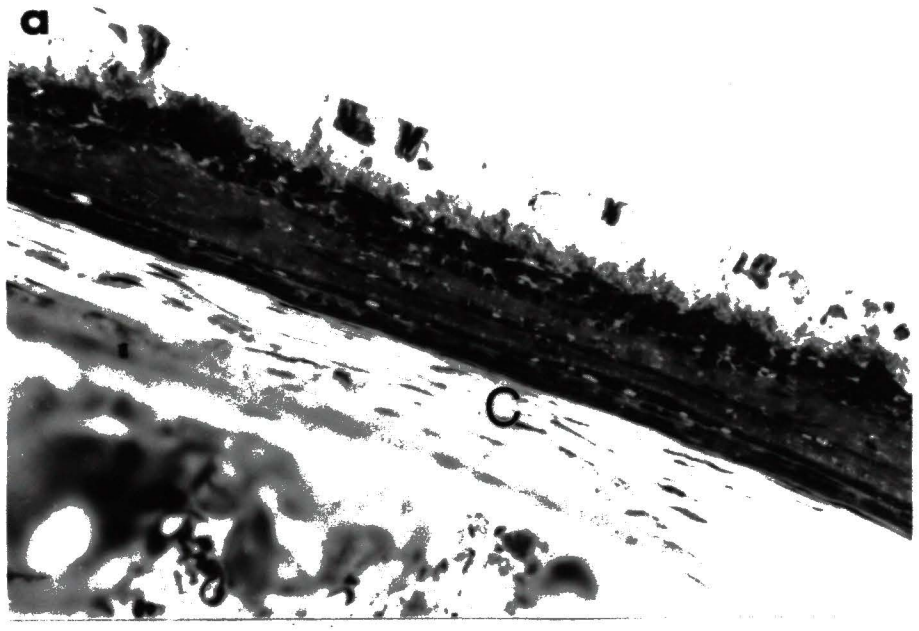
peak. Figure 4 data for disease scores of rats with Mpep/EAU will be discussed later in this chapter.

Figure 5a shows the extensive disruption of the retina that occurs in Sag/EAU. The retina is detached and edematous, and the photoreceptor cell layer is disrupted. Figure 5b, a micrograph that includes the angle of the eye, illustrates the severe inflammation of the anterior eye that occurs during Sag/EAU.

The toluidine blue-stained spread preparations in Figure 6 show the distribution of mast cells in the normal choroid (Fig. 6a) and limbus (Fig. 6b). The enlarged insert (Fig. 6a) shows the paucity of mast cells associated with venules in the choroid.

Ocular mast cell numbers in Sag/EAU. With disease progression, eyes of rats with Sag/EAU exhibited statistically significant decreases in numbers of both choroidal and limbal mast cells when compared to sCFA/saline controls. On days 5 and 7 pi, prior to onset of clinical signs of disease, Sag/EAU eyes and sCFA/saline controls exhibited equivalent numbers of choroidal and limbal mast cells (Figs. 7 and 8). A decrease in choroidal mast cell numbers in Sag/EAU eyes, compared to saline/sCFA controls, was not apparent until day 11 pi and reached significance by day 13 pi ($p = 0.0001$) (Fig. 7). A decrease in limbal mast cell numbers, compared to saline/sCFA controls, began by day 9 pi, and this decrease was significantly lower than control values for days 11 and 13 pi ($p < 0.005$ and $p = 0.0001$, respectively) (Fig. 8). Mast cell numbers did not change significantly over time in sCFA/saline control eyes.

Figure 5. Micrographs showing a) retina and b) part of anterior segment from eye with Sag/EAU obtained on day 11 pi (ocular disease score, 16). In micrograph a), the photoreceptor cell layer is labeled (**P**). In micrograph b), a limbal vessel (**V**) is located at the top of the micrograph with retinal tissue (**R**) to the left and the ciliary body (**C**) to the right. Stained with H & E. Original magnification X250.


a
C

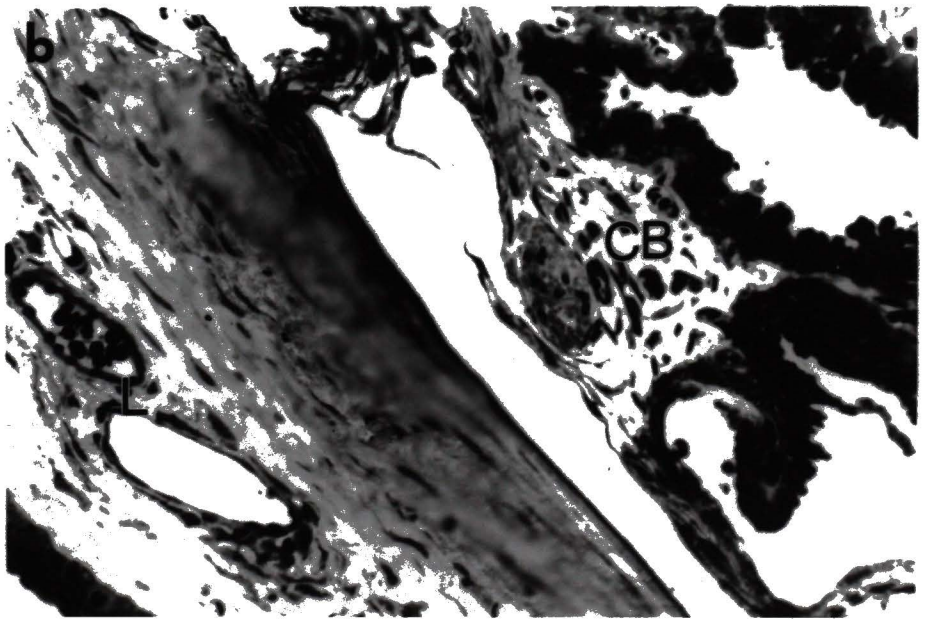
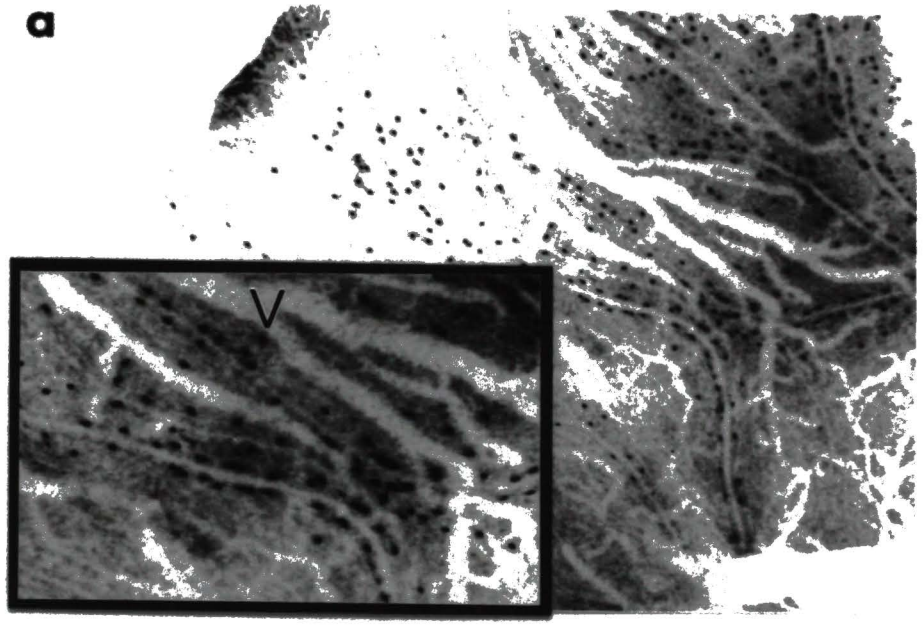

b
CB

Figure 6. Micrographs of toluidine blue-stained spread preparations showing normal a) choroidal and b) limbal mast cells arrayed along blood vessels. In a), the enlarged inset illustrates the paucity of mast cells associated with veins (**V**) (original magnification X50). In b), the center of the cornea (**C**) is toward the bottom of the micrograph; mast cells are seen along scleral blood vessels at the top of the micrograph (original magnification X60).


a
V

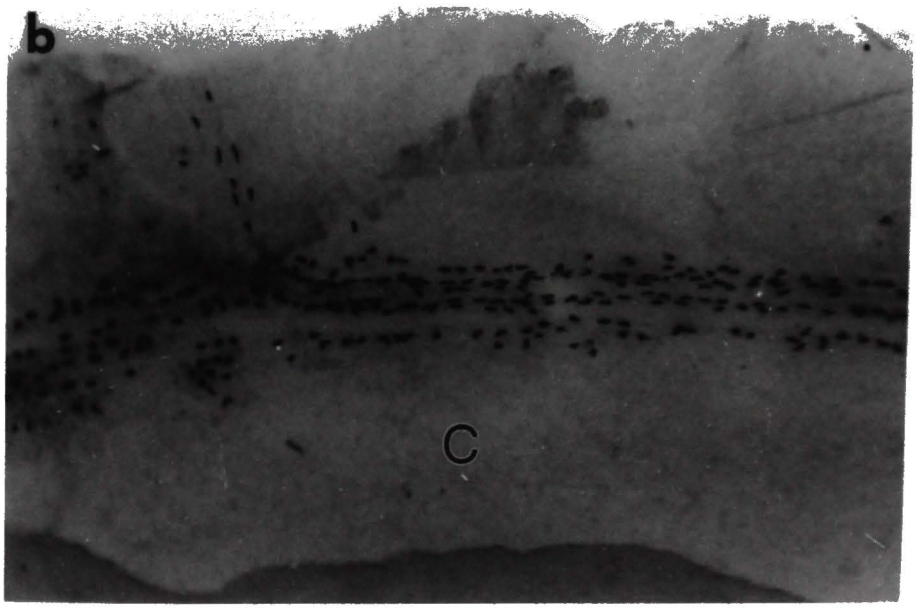

b
C

Figure 7. Choroidal mast cell numbers for eyes of animals with Sag/EAU (●) and for eyes of saline/sCFA control animals (O). Values for each sacrifice day are means ± SEM of 8 eyes. The arrowhead indicates time of onset of clinical symptoms. * indicates that $p < 0.05$ as compared to the corresponding control group.

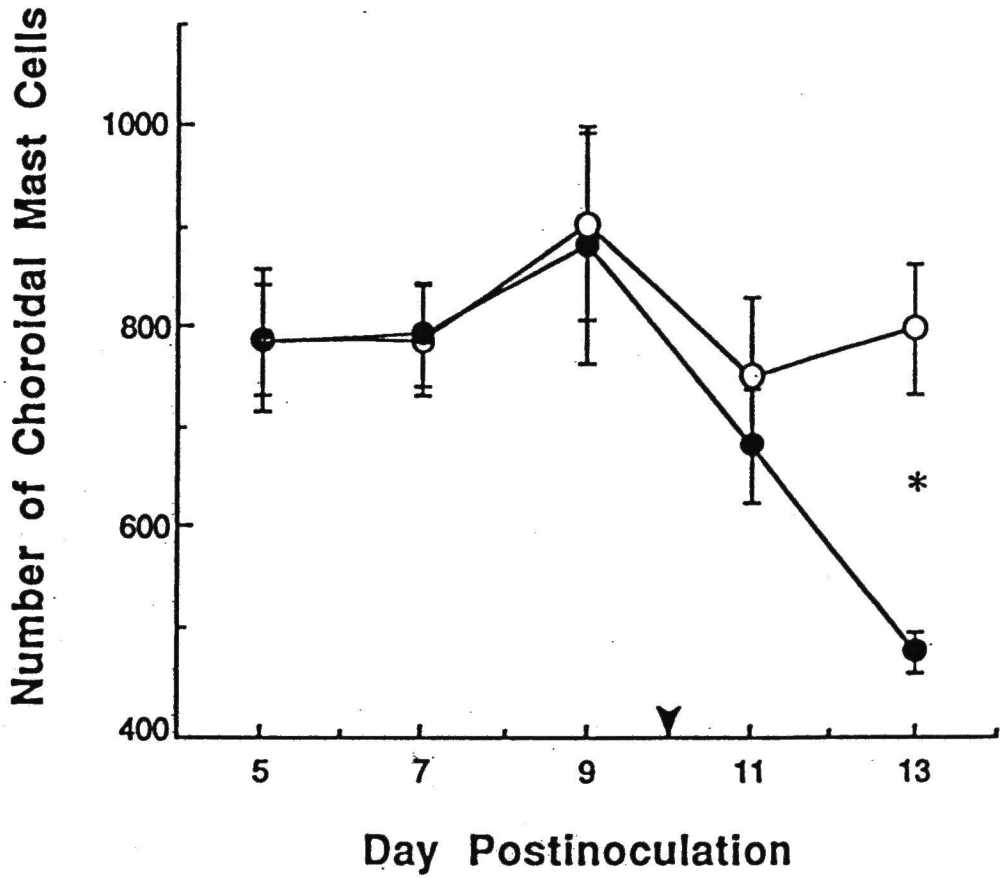
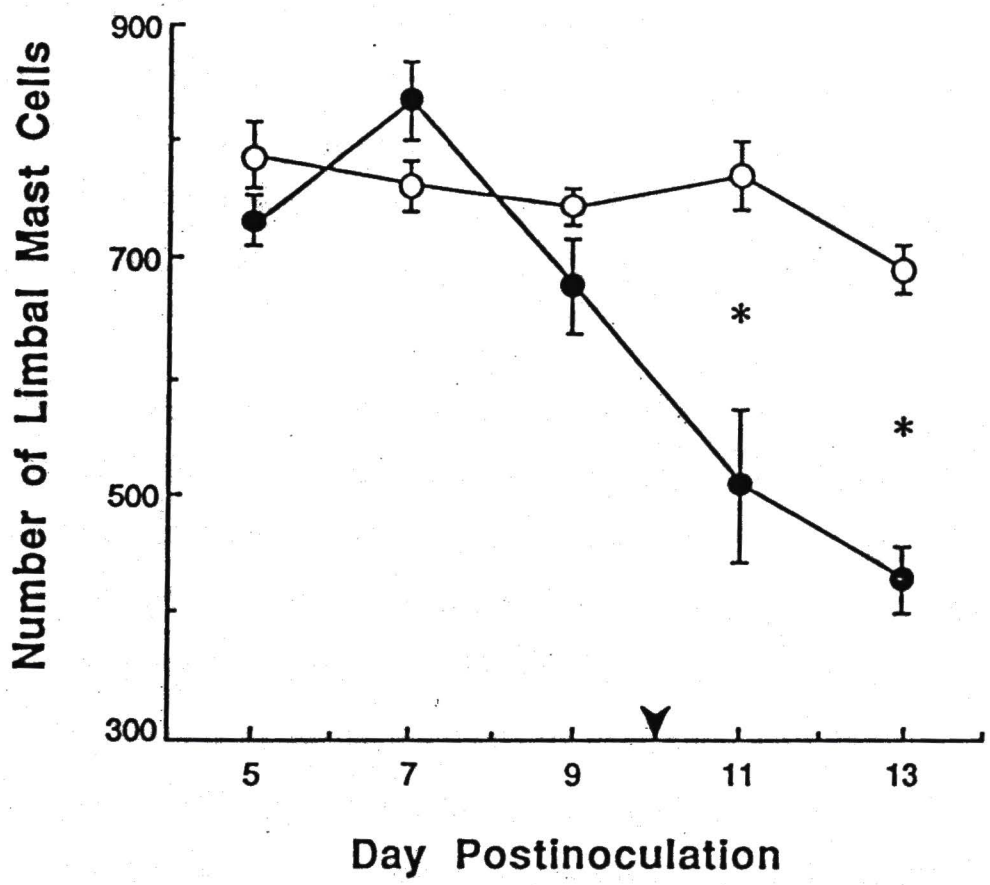

Number of Choroidal Mast Cells
1000
800
600
400
5
7
9
11
13
*
Day Postinoculation

Figure 8. Limbal mast cell numbers for eyes of animals with Sag/EAU (●) and for eyes of saline/sCFA control animals (O). Mast cells in 1.6 mm^2 of limbal tissue were counted as described in Chapter 2. Values for each sacrifice day are means ± SEM of 8 eyes. The arrowhead indicates time of onset of clinical symptoms. * indicates that $p < 0.05$ as compared to the corresponding control group.


Number of Limbal Mast Cells
900
700
500
300
5
7
9
11
13
Day Postinoculation
*
*

Relative to the onset of Sag/EAU symptoms, the decrease in limbal mast cell numbers in Sag/EAU rats, seen as early as day 9 pi, began one day prior to onset of observable symptoms (Fig. 8). In contrast, the drop in numbers of choroidal mast cells did not begin until day 11 pi, after the onset of symptoms of Sag/EAU (Fig. 7).

Histamine levels in ocular tissues in Sag/EAU. The total histamine content of the choroid (Fig. 9) and of the anterior segment (Fig. 10) of the eye reflected the presence of the large numbers of mast cells found in these tissues (Figs. 7 and 8). Consistent with the absence of mast cells in the retina, the histamine content of the retina in saline/sCFA control eyes (Fig. 11) was very low.

Significant changes in histamine levels were found in some of the ocular tissues during Sag/EAU. In the choroid (Fig. 9), a significant increase in histamine was observed on days 9, 11, and 13 pi ($p < 0.05$, $p < 0.01$, and $p = 0.001$, respectively). However, the decrease in numbers of choroidal mast cells was not evident until day 11 pi (Fig. 7). In the anterior segment of the eye (Fig. 10), the histamine content, as well as the numbers of limbal mast cells, decreased (Fig. 8). This decrease in histamine was significant for days 9, 11, and 13 pi ($p < 0.05$, $p < 0.05$, and $p = 0.0001$, respectively). Control levels of histamine did not vary significantly.

In retinas from rats with Sag/EAU, a significant, transient increase in histamine was seen on day 11 pi ($p < 0.05$) (Fig. 11). On day 13 pi, histamine levels had decreased and were not significantly higher than controls. However, inflammation in the posterior uvea was still severe (results not shown). To investigate the possibility that histamine degradation enzymes are

Figure 9. Changes in histamine content of choroids from Sag/EAU eyes (●) during the course of EAU. Values for saline/sCFA control eyes (O) are shown for corresponding days. Error bars represent SEM; $n = 8$, except as indicated by numbers in parentheses. The arrowhead indicates time of onset of clinical symptoms. * indicates that $p < 0.05$ as compared to the corresponding control group.

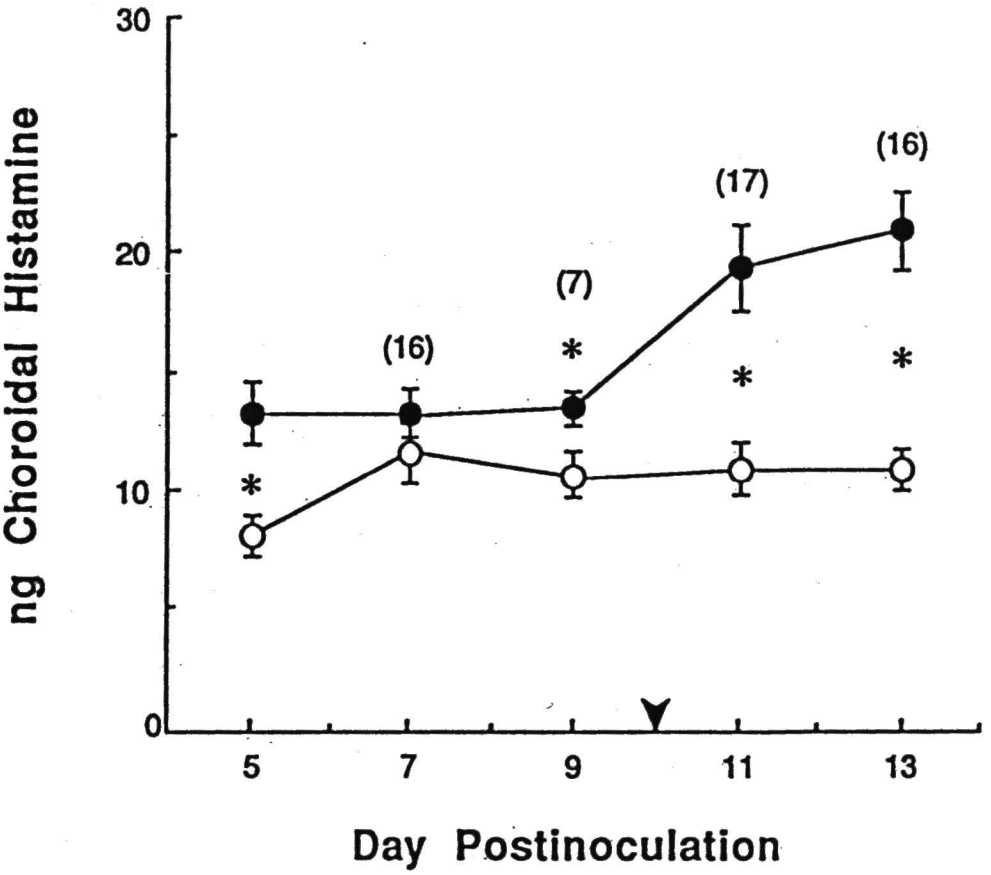

30
20
10
0
ng Choroidal Histamine
(16)
(7)
(17)
(16)
*
*
*
*
5
7
9
11
13
Day Postinoculation

Figure 10. Changes in histamine content of anterior segments from Sag/EAU eyes (●) during the course of EAU. Values for saline/sCFA control eyes (O) are shown for corresponding days. Error bars represent SEM; $n = 8$, except as indicated by numbers in parentheses. The arrowhead indicates time of onset of clinical symptoms. * indicates that $p < 0.05$ as compared to the corresponding control group.

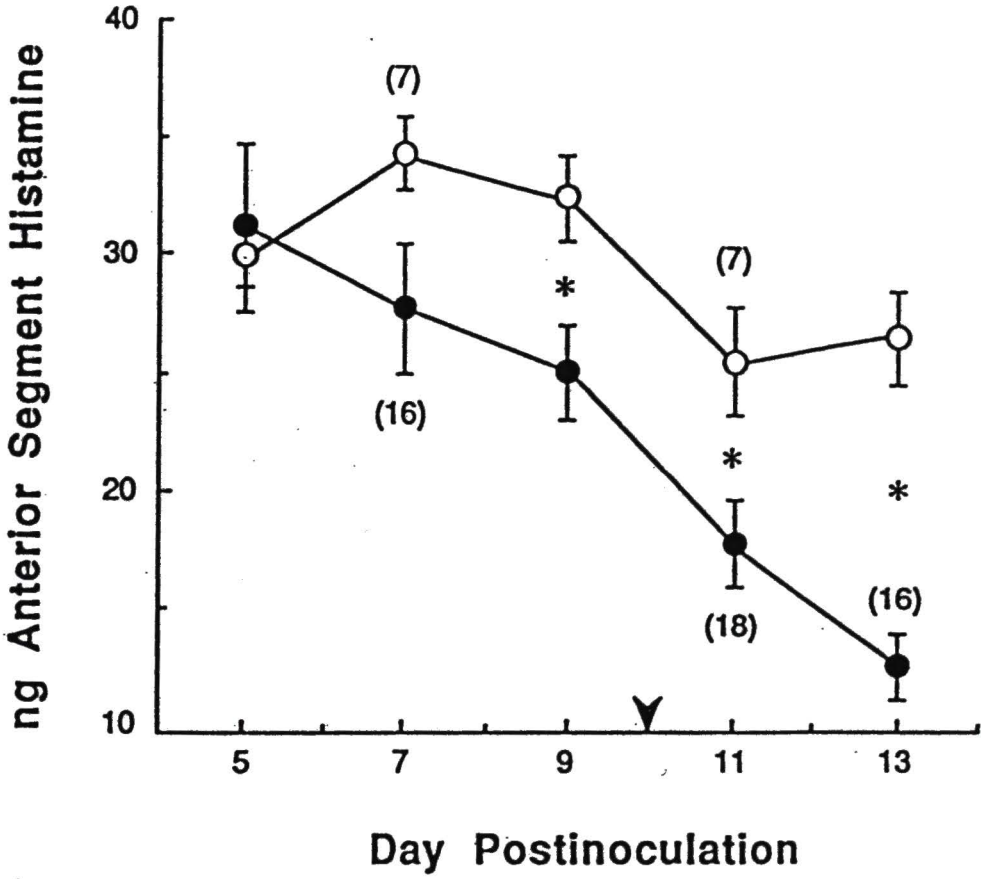
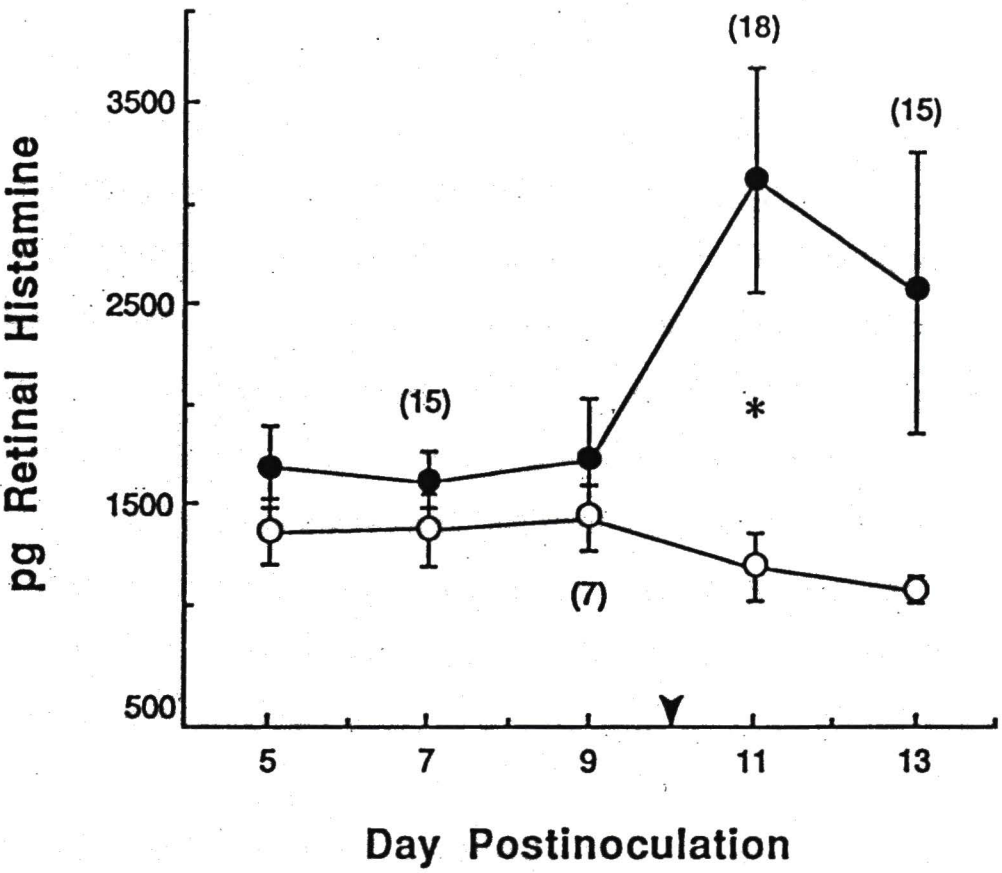

ng Anterior Segment Histamine
40
30
20
10
(7)
(16)
(7)
(18)
(16)
*
*
*
5
7
9
11
13
Day Postinoculation

Figure 11. Changes in histamine content of retinas from Sag/EAU eyes (●) during the course of EAU. Values for saline/sCFA control eyes (O) are shown for corresponding days. Error bars represent SEM; $n = 8$, except as indicated by numbers in parentheses. The arrowhead indicates time of onset of clinical symptoms. * indicates that $p < 0.05$ as compared to the corresponding control group.


pg Retinal Histamine
3500
2500
1500
500
(18)
(15)
(15)
(7)
*
5
7
9
11
13
Day Postinoculation

induced in the retina when histamine levels rise, HMT levels in the retina were determined before (day 9 pi) and during (days 11 and 13 pi) Sag/EAU (Fig. 12). A significant increase in HMT was found ($p < 0.001$), and may account for the decrease in retinal histamine observed on day 13 pi (Fig. 11).

Histamine concentrations of aqueous fluid from the eyes of Sag/EAU rats did not deviate significantly from those of saline/sCFA controls (Fig. 13). Scleral histamine values for both control and Sag/EAU eyes were highly variable, as shown in Figure 14. Histamine levels (Figs. 9, 10, 11, 13, and 14) did not change significantly over time in saline/sCFA control eyes.

Wet weights of tissues in Sag/EAU. Wet weights of all of the tissues (edema indicator) evaluated increased during the intense inflammation of Sag/EAU (Fig. 15), and all except the wet weight of the anterior segment had increased significantly by day 13 pi. The large initial weight of the lens (about 70 percent of the wet weight of the anterior segment, data not shown) may have obscured some of the weight increase in this tissue due to Sag/EAU. No changes in wet weights of tissues from saline/sCFA eyes were observed for days 5 through 13 pi (data not shown).

Leflunomide Treatment of Rats with Sag/EAU.

Rats inoculated with Sag/sCFA and treated with leflunomide did not develop Sag/EAU. Sag/EAU did occur in untreated controls inoculated with Sag/sCFA. Saline/sCFA control rats treated with leflunomide did not develop Sag/EAU.

The direction of the changes in histamine levels for untreated Sag/EAU rats in the leflunomide study (Figs. 16, 17, and 18) were very similar to those

Figure 12. Changes in histamine methyltransferase (HMT) levels in retinas from eyes with Sag/EAU (●), expressed as nmol 1-[^{3}H] methylhistamine formed/g/30 min. Error bars represent SEM; n = 10, except as indicated by number in parenthesis. * indicates that $p < 0.05$ as compared to the value for day 9 pi.

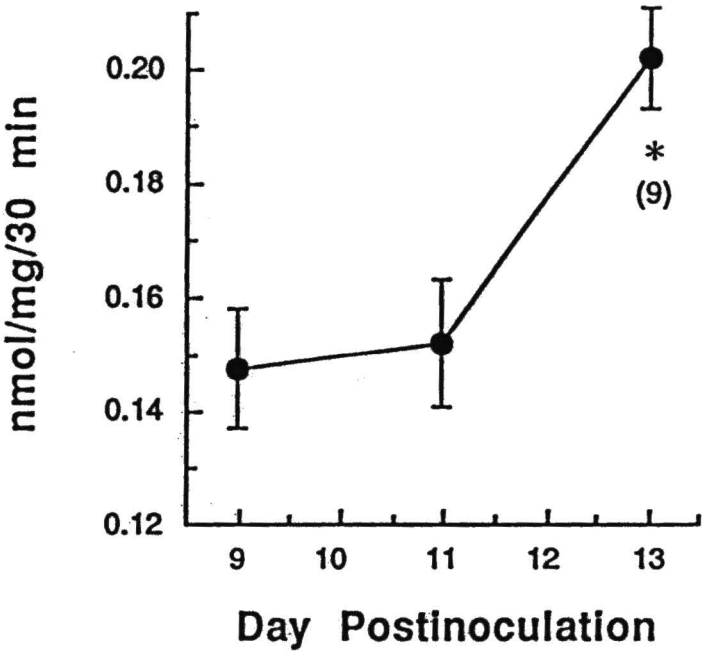

nmol/mg/30 min
0.20
0.18
0.16
0.14
0.12
*
(9)
9
10
11
12
13
Day Postinoculation

Figure 13. Changes in histamine concentration of aqueous humor from EAU eyes (●) during the course of Sag/EAU. Values for saline/sCFA control eyes (O) are shown for corresponding days. Error bars represent SEM; $n = 8$, except as indicated by numbers in parentheses. The arrowhead indicates time of onset of clinical symptoms.

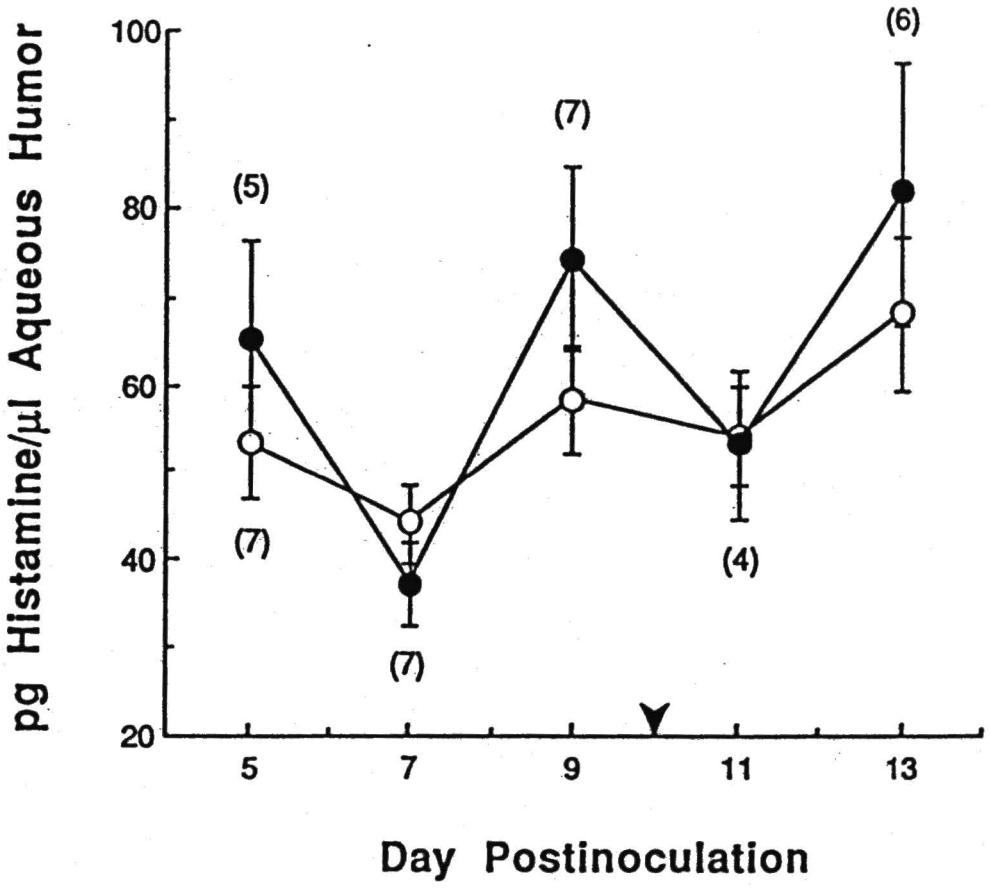

pg Histamine/µl Aqueous Humor
100
80
60
40
20
(5)
(7)
(7)
(7)
(4)
(6)
5
7
9
11
13
Day Postinoculation

Figure 14. Changes in histamine content of sclera from Sag/EAU eyes (●) during the course of EAU. Values for saline/sCFA control eyes (O) are shown for corresponding days. Error bars represent SEM; $n = 8$, except as indicated by numbers in parentheses. The arrowhead indicates time of onset of clinical symptoms.

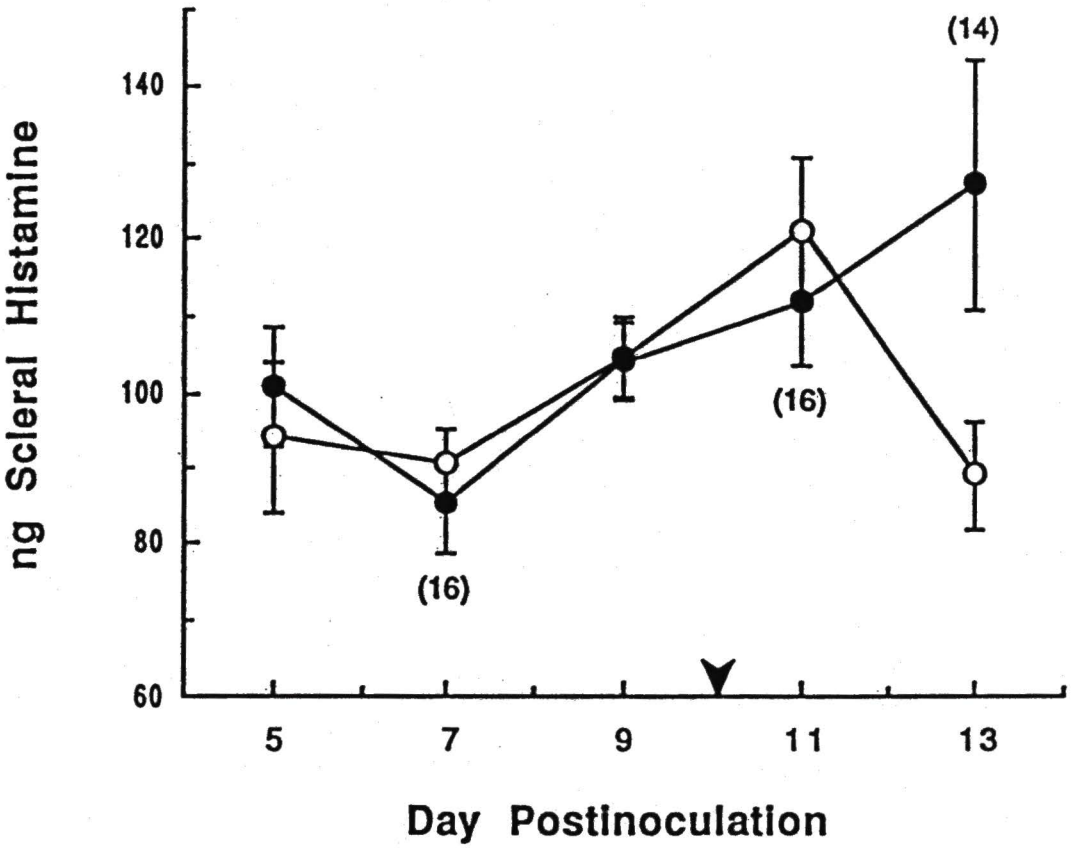

ng Scleral Histamine
140
120
100
80
60
(14)
(16)
(16)
5
7
9
11
13
Day Postinoculation

Figure 15. Wet weights of anterior segments (O), choroids (●), scleras (△) and retinas (▲) from Sag/EAU eyes. Error bars represent SEM. * indicates that $p < 0.05$ as compared to the value for day 9 pi. *n* are as follows, except as indicated by numbers in parentheses: day 5 pi, 8; day 7 pi, 16; day 9 pi, 8; day 11 pi, 18; day 13 pi, 14.

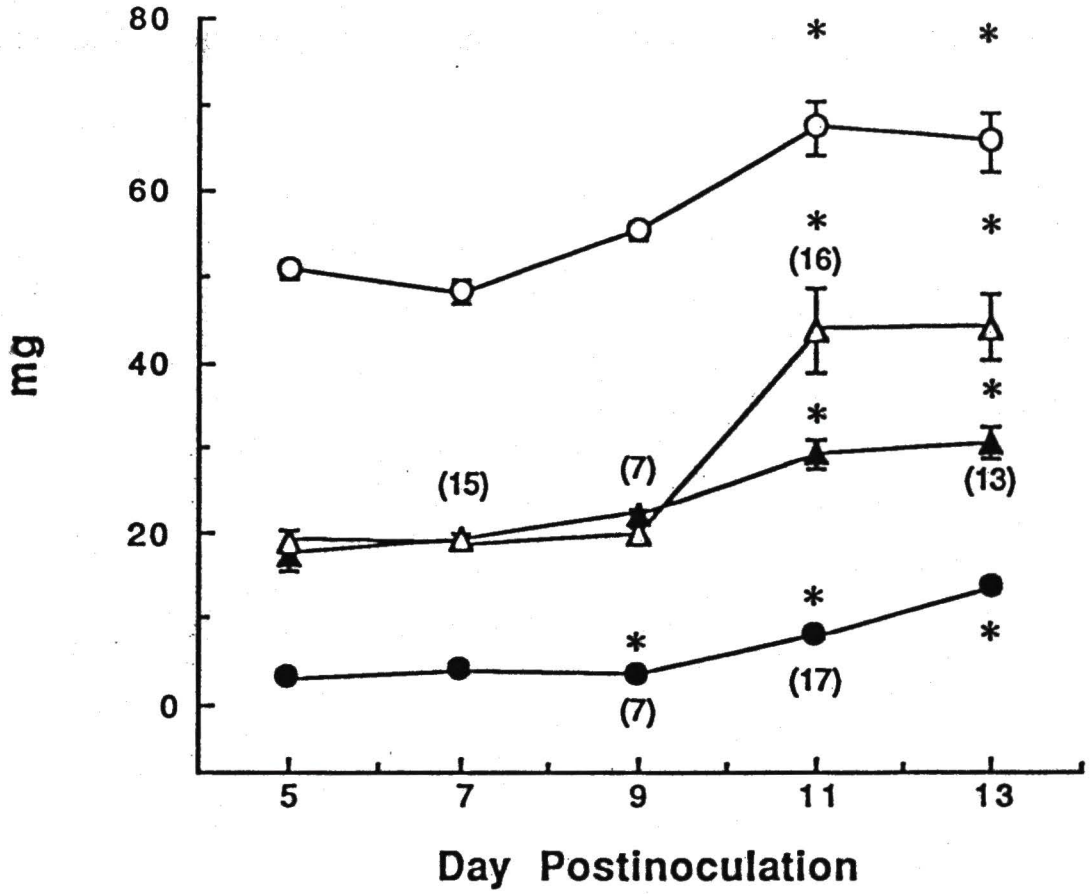

80
60
40
20
0
mg
5
7
9
11
13
Day Postinoculation
(15)
(7)
(16)
(13)
(7)
(17)
*

Figure 16. Histamine content of anterior segments of Sag/sCFA-inoculated rats treated with leflunomide (LEF/Sag/sCFA) (○), Sag/sCFA alone (●), and saline/sCFA rats treated with leflunomide (LEF/saline/sCFA) (□). Error bars represent SEM. The *n* for each group is 8. # indicates that the LEF/Sag/sCFA group is significantly different from the LEF/saline/sCFA group. + indicates that the Sag/sCFA group is significantly different from the LEF/Sag/sCFA group.

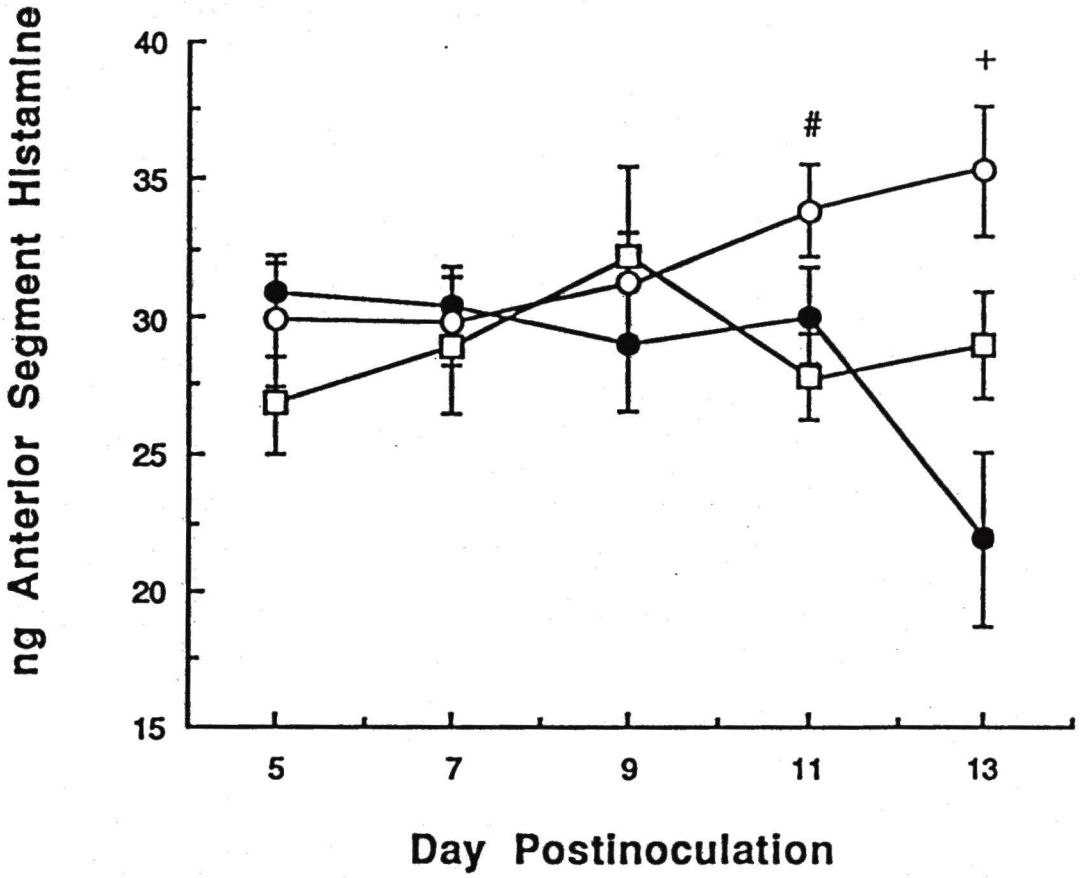

ng Anterior Segment Histamine
40
35
30
25
20
15
5
7
9
11
13
Day Postinoculation
#
+

Figure 17. Histamine content of choroids of Sag/sCFA-inoculated rats treated with leflunomide (O), Sag/sCFA alone (●), and saline/sCFA rats treated with leflunomide (□). Error bars represent SEM. The *n* for each group is 8. * indicates that the Sag/sCFA group is significantly different from the other groups; + indicates that the Sag/sCFA group is significantly different from the Sag/sCFA group treated with leflunomide.

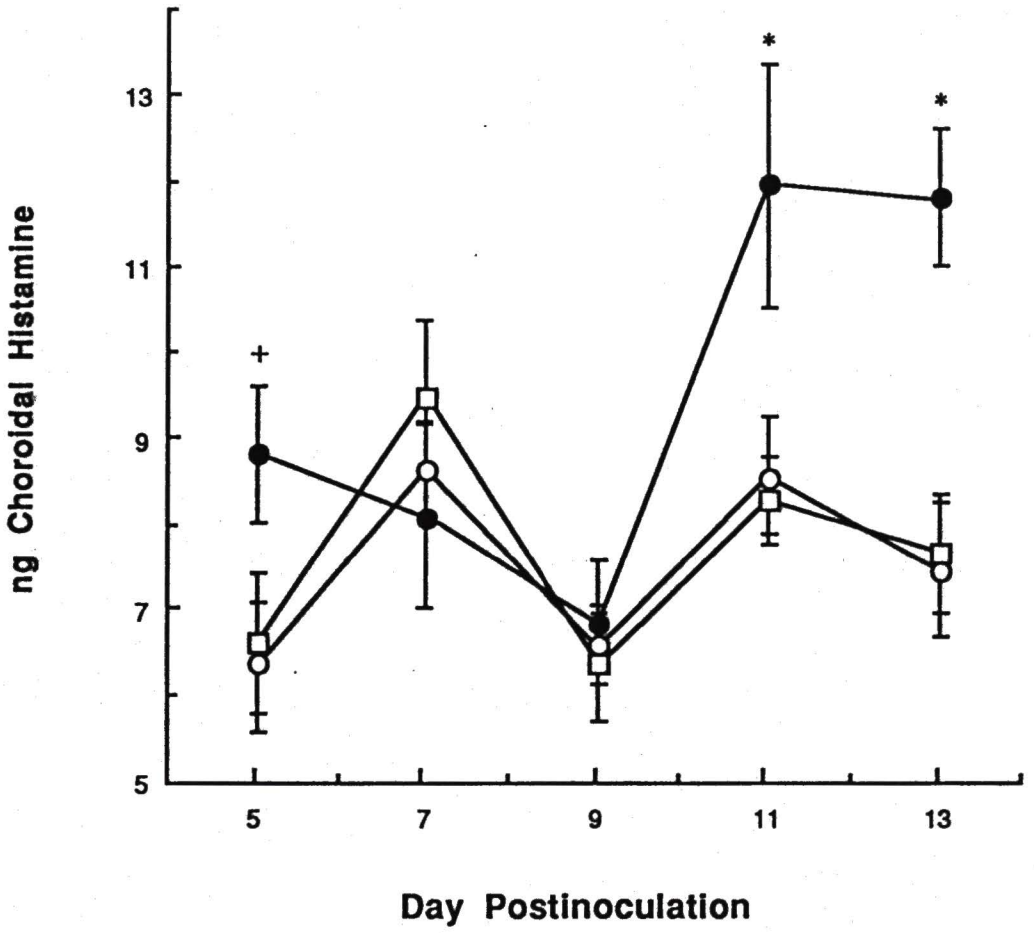
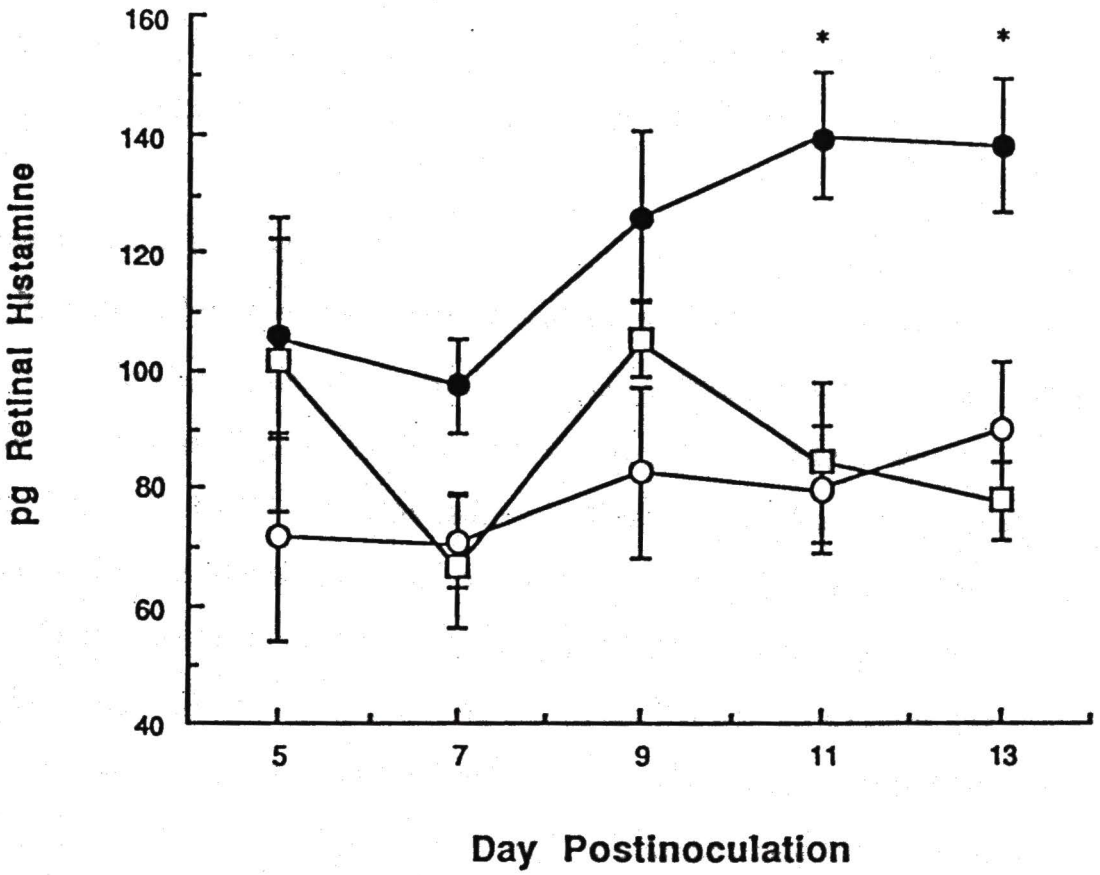

ng Choroidal Histamine
13
11
9
7
5
+
*
*
5
7
9
11
13
Day Postinoculation

Figure 18. Histamine content of retinas of Sag/sCFA-inoculated rats treated with leflunomide (O), Sag/sCFA alone (●), and saline/sCFA rats treated with leflunomide (□). Error bars represent SEM. The *n* for each group is 8. * indicates that the Sag/sCFA group is significantly different from the other groups.


160
140
120
100
80
60
40
pg Retinal Histamine
*
*
5
7
9
11
13
Day Postinoculation

observed earlier for Sag/EAU (Figs. 9, 10, and 11). Anterior segment histamine (Fig. 16) decreased significantly, and in the choroid (Fig. 17) and the retina (Fig. 18), levels of histamine increased significantly. In addition, no significant changes in histaminelevels occurred in ocular tissues from leflunomide treated, saline/sCFA controls when compared to leflunomide treated Sag/sCFA animals (Figs. 16, 17, and 18). In addition, leflunomide prevented tissue edema, as demonstrated by the wet weight increases observed in anterior segments, choroids, and retinas of untreated Sag/sCFA rats (Fig. 19).

In vitro Histamine Release

Release of histamine by exposure of mast cells to S-antigen. When PMC, harvested from rats with Sag/EAU or from saline/sCFA control rats, were exposed to various dilutions of Sag, very little histamine release was observed (Tables 1 and 2). Histamine release from PMCs was not affected by the day postinoculation on which cells were harvested (Tables 1 and 2). In all cases, percent histamine released was close to that released on exposure to buffer alone for PMC from both Sag/EAU and saline/sCFA rats. In addition, the number of cells exposed to Sag did not affect results. When 250 or 2000 cells (Table 2) were exposed to Sag, results were similar to those for 1000 cells (Table 1). In addition, no increase in histamine release was observed when unpurified populations of cells were used (BSA gradient purification was omitted, results not shown).

Percent histamine release resulting from exposure of mast cells to a histamine releasing agent, Compound 48/80, was also determined, as a

Figure 19. Wet weights of anterior segments (●), choroids (■), and retinas (▲) from Sag/EAU eyes. (O), (□), and (△) represent the same tissues, respectively, from eyes of Sag/sCFA-inoculated rats treated with leflunomide. Error bars represent SEM (most error bars are obscured by symbols); the *n* for each group is 8, except as indicated by numbers in parentheses. * indicates that $p < 0.05$ when treated and untreated groups are compared for each day.

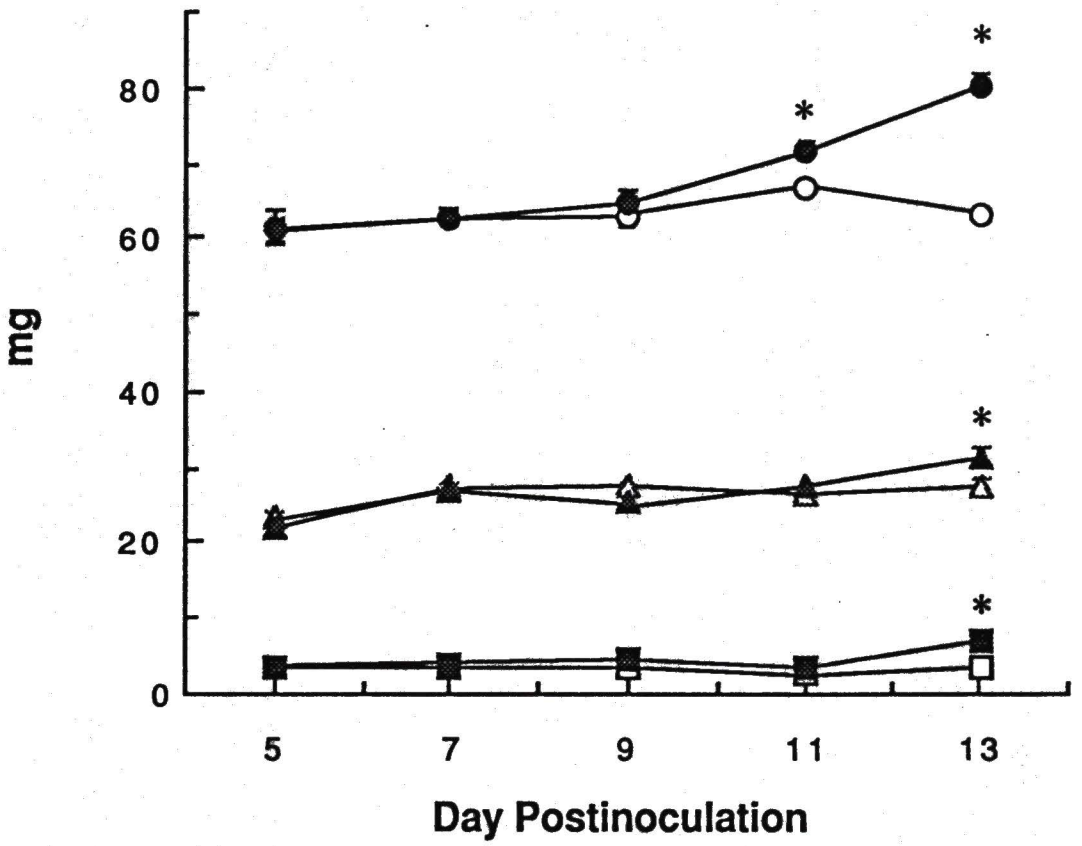

mg
80
60
40
20
0
5
7
9
11
13
Day Postinoculation
*
*
*
*

Table 1.

Percent Histamine Released from 1000 Peritoneal Mast Cells Exposed to S-Antigen

Day pi, Sample Type, and *n*	Sag Concentration (μg/ml)					
	100	10	1	0.1	0.01	0.001
Day 5 pi						
Saline/CFA, *n* = 2	1.2±1.2*	0.8±0.8	2.0±2.0	0.4±0.4	0	1.2±1.2
Sag/EAU, *n* = 2	1.6±1.6	2.1±1.4	0	1.4±0.8	1.8±0.7	3.3±3.1
Day 7 pi						
Saline/CFA, *n* = 2	1.0±0.2	3.0±1.8	2.4±2.2	1.0±0.1	0	1.2±1.2
Sag/EAU, *n* = 2	0	0.6±0.6	1.0±1.0	0	0	0
Day 9 pi						
Saline/CFA, *n* = 2	1.3±1.3	0.4±0.4	0.2±0.2	0	0.6±0.6	0
Sag/EAU, *n* = 3	0	0	0	0.1±0.1	0	0
Day 11 pi						
Saline/CFA, *n* = 2	0.2±0.2	0.6±0.6	2.3±2.3	0.4±0.4	0	0.2±0.2
Sag/EAU**	2.8±1.8	2.0±1.1	0.8±0.7	1.2±1.2	0.1±0.1(2)	0(2)
Day 13 pi						
Saline/CFA, *n* = 2	2.6±1.8	0.1±0.1	0.5±0.5	0.3±0.3	0.2±0.5	0.5±0.5
Sag/EAU**	2.0±0.8	0.2±0.2	3.1±1.7	1.2±1.2	0.8±0.8(2)	0.4±0.2(2)

* Values are means±SEM; ** *n* = 3, except as indicated in parentheses.

Table 2.

Percent Histamine Released from 250 and 2000 Peritoneal Mast Cells Exposed to S-Antigen

Number of Cells, Day pi, and *n*	Sag Concentration (μg/ml)					
	100	10	1	0.1	0.01	0.001
250 PMC						
Day 13 pi, *n* =1	0	0.7	0	0	0	0
2000 PMC						
Day 11 pi, *n* = 1	0.2	0.9	1.8	1.8	0.5	2.7
Day 13 pi, *n* = 3	1.2±0.4*	1.7±1.1	2.5±1.6	0.4±0.4	1.4±1.2	1.6±1.0

* Values are means±SEM

positive control. As shown in Figure 20, Compound 48/80 dose-dependently induced release of histamine from PMC. In contrast, maximum histamine release from choroidal tissue occurred between one and 50 µg/ml Compound 48/80.

Sections of limbal and choroidal tissue from Sag/EAU animals were also exposed to Sag, at two concentrations (0.1 and 1µg/ml), to evoke mast cell degranulation (Table 3a). PMC (1000 cells), also from Sag/EAU animals, were exposed to Sag for comparison. Background release of histamine was higher for limbal and choroidal tissue than for PMC, but no increase in percent histamine release from ocular tissues or PMC was observed in the presence of Sag.

Passive sensitization of mast cells by S-antigen-sensitized serum. PMC, limbi, or choroids from naive rats were incubated with possible IgE-containing serum from Sag/EAU rats to allow absorption of IgE to the mast cell membrane. When these PMC or tissues were subsequently exposed to Sag at two concentrations (0.01 and 0.1 µg/ml), no histamine release above background was observed. Results are shown in Table 3b.

Activation of ocular mast cells in Sag/EAU by histamine releasing factors. Aqueous humor from rats with Sag/EAU was incubated with PMC obtained from naive rats to detect HRF activity (Table 4). Release of histamine was not significantly higher than that for control values for the various dilutions of aqueous humor used, nor did undiluted aqueous humor elicit histamine release from PMC.

Figure 20. Percent release of histamine from peritoneal mast cells (PMC) (▨) and choroidal tissue (■) exposed to Compound 48/80. 2000 PMC were used, in duplicate, at each concentration of 48/80. Error bars represent SEM. For choroidal tissue, each point represents results for one choroid.

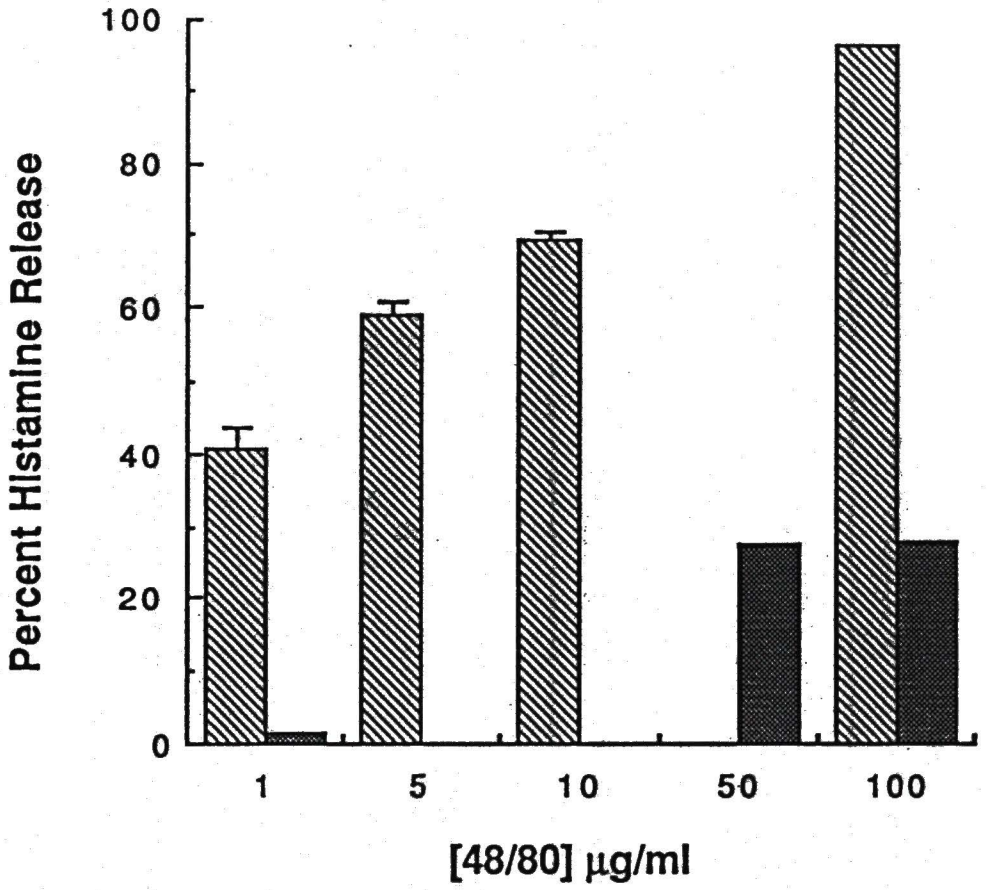

Percent Histamine Release
100
80
60
40
20
0
1
5
10
50
100
[48/80] µg/ml

Table 3.

Percent Histamine Released from Peritoneal Mast Cells and Ocular Tissues Exposed to S-Antigen

Treatment	PMC*	Choroid	Limbus
a. Exposure of ocular tissues from EAU rats to S-antigen			
Buffer only	9.4 %	35.6±4.1 (4) %**	31.3±2.5 (4) %
0.1 µg/ml Sag	7.0	29.8±0.7 (2)	30.1±0.1 (2)
1.0 µg/ml Sag	4.4	34.7±5.4 (2)	31.4±3.0 (2)
b. Passive sensitization of mast cells by day 11 pi serum from EAU rats			
Buffer only	16.0 %	38.4 %	12.2±0.1 (2) %
0.01 µg/ml Sag	8.2	18.9	10.1
0.1 µg/ml Sag	17.8	ND***	12.5

* 1000 PMC were used; ** Values are means±SEM, *n* are shown in parentheses, except where *n* = 1; *** Not Done

Table 4.

Percent Histamine Released from 1000 Peritoneal Mast Cells Exposed to Aqueous Humor from Sag/EAU rats

Aqueous Used and Sample Number	Aqueous Dilution and Incubation Time								
	Not Diluted 10 min.	Not Diluted 30 min.	Dilution in Buffer (30 min. Incubation)						
			1/2	1/10	1/20	1/50	1/100	1/1000	1/10000
Naive Aqueous									
1		0.8							
2		2.1		2.2		1.1	1.4		
3		3.2		0.0		2.1	8.7		
4	14.9								
5	0.0								
mean±SD	7.5±10.5	2.0±1.2		1.1±1.6		1.6±0.7	5.1±5.2		
Day 9 pi Aqueous									
1		2.6		0.8		0.2	0.7		
Day 11 pi Aqueous									
1	9.7								
2	3.9								
3		4.3	4.3		0.0		8.2	2.6	3.9
mean±SD	6.8±4.1								
Day 13 pi Aqueous									
1	8.0								
2		4.0		0.0		0.6	3.6		

M-peptide-Induced EAU

Activation of ocular mast cells in an M-peptide-induced EAU (Mpep/EAU) was assessed using the same approach as for the study of mast cell activation in Sag/EAU. The number of mast cells in limbal and choroidal tissue and the histamine content of anterior segments (which includes limbal mast cells), choroids, and retinas were determined over the course of the disease. Results for saline/sCFA eyes were consistently unchanged in the Sag/EAU and leflunomide studies. Therefore, to reduce the number of animals in the study, data from Mpep/sCFA-inoculated rats that were sacrificed on pre-disease day 9 pi were used for comparison with the results for diseased eyes, instead of using saline/sCFA control eyes for reference values. A similar comparison of Sag/EAU histamine data to results for pre-disease day 5 pi is used in discussing differences in the two forms of EAU.

Induction of Mpep/EAU. The Mpep preparation employed by Smith et al. (97) was used in the present study, and it produced ocular disease symptoms (Figs. 4, 21a, and 22) similar to those described previously (97). The outer segments and associated cell bodies of photoreceptor cells were destroyed (Fig. 22a). Disruption of the retinal architecture in Mpep/EAU was limited to the outer cell bodies and their processes (Fig. 22a) in contrast to the extensive destruction, involving the entire neural retina, that was observed in Sag/EAU (Fig. 5a), and described for Mpep/EAU induced with *B. pertussis*/sCFA (28). Little inflammation or edema was observed in the anterior portion of the eye (Fig. 22b). This difference in anterior segment involvement is evident when Figures 5b and 22b are compared. Disease scoring for Mpep/EAU (Figs. 4 and 21a) also reflected the mild disease.

Figure 21. Regional disease scores for a) Mpep/EAU and b) Sag/EAU for the four days of most intense disease (z axis). The scoring method is described in Chapter 2. The maximum score for region 1 (limbal vessels) is 3; for regions 2-5, the maximum score is 4. Values are means ± SEM. $n = 12$ eyes for Mpep/EAU; $n = 6$ eyes for Sag/EAU.

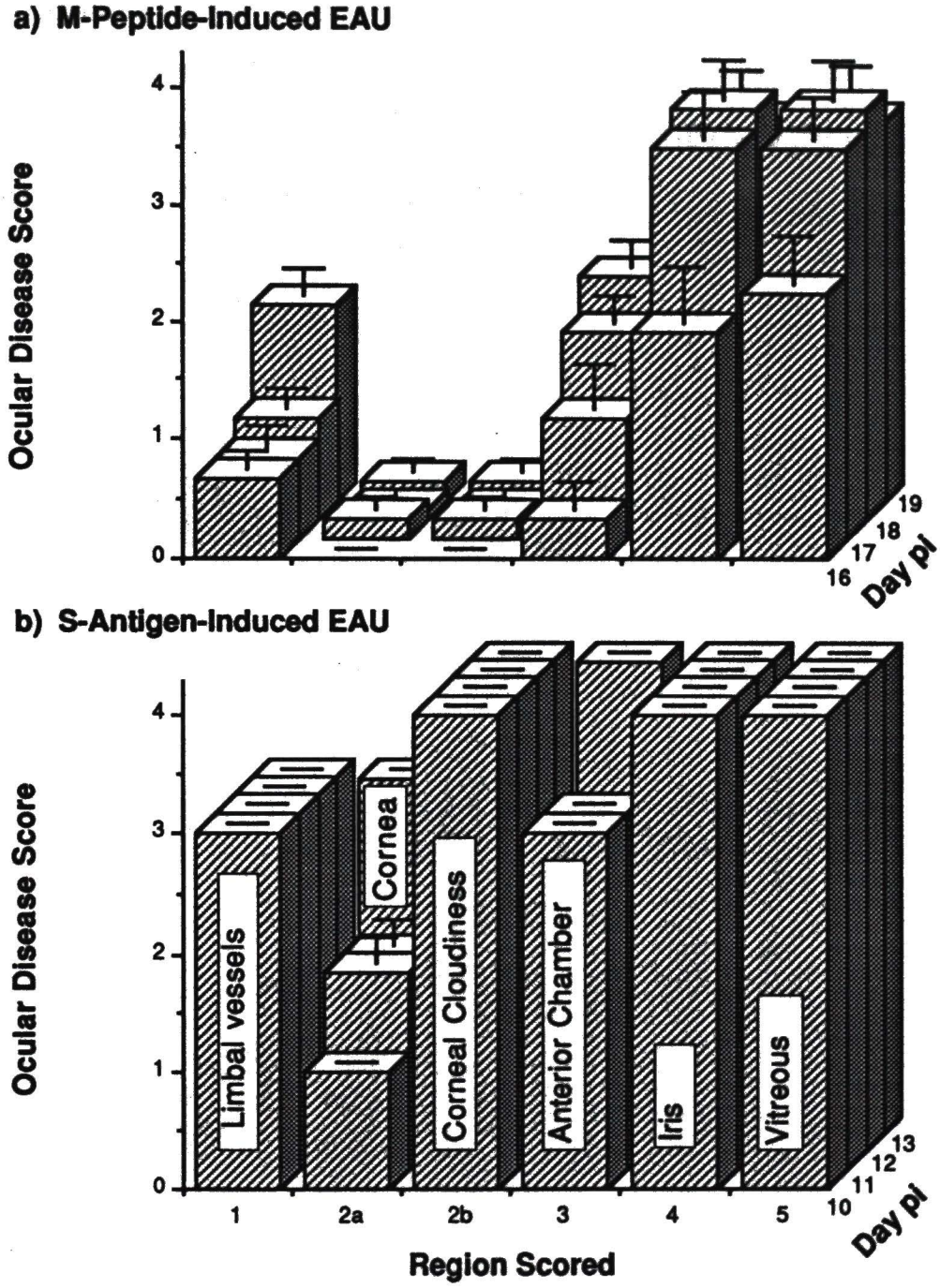
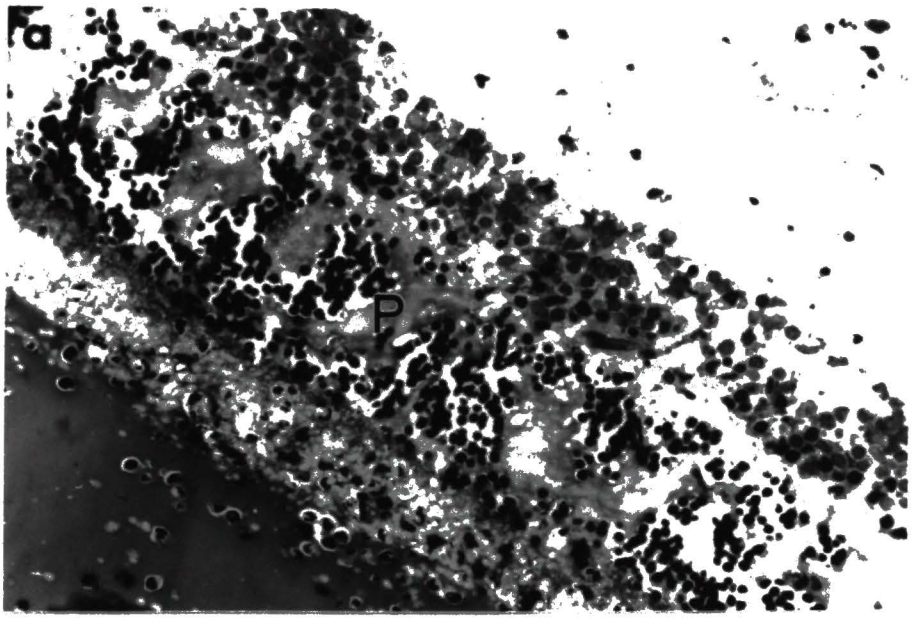

a) M-Peptide-Induced EAU
Ocular Disease Score
0
1
2
3
4
16
17
18
19
Day pi
b) S-Antigen-Induced EAU
Ocular Disease Score
Limbal vessels
Cornea
Corneal Cloudiness
Anterior Chamber
Iris
Vitreous
1
2a
2b
3
4
5
Region Scored
10
11
12
13
Day pi

Figure 22. Micrographs of a) retina and b) anterior segment from eye with Mpep/EAU obtained on day 24 pi (ocular disease score, 9). The choroid is labeled (**C**). In micrograph b), from the same eye, limbal vessels (**L**) are located to the left of the micrograph and the ciliary body (**CB**) is to the right. Stained with H & E. Original magnification X250.


Fa
P

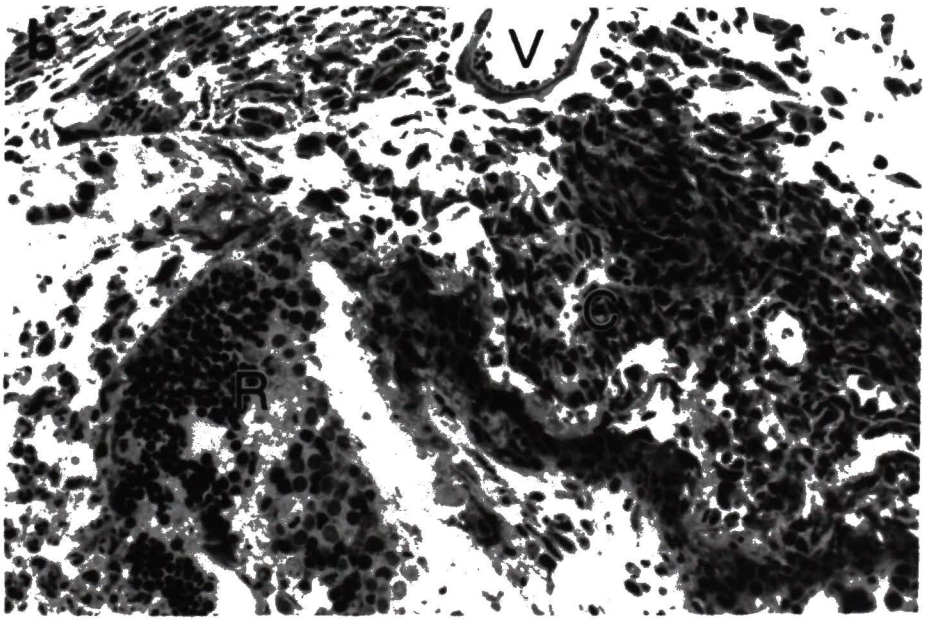

b
V
tl
C
R

Figure 21a shows clinical scores for each eye region evaluated on the four most severe days of Mpep/EAU. This data also illustrates the relatively minor involvement of the anterior portion of the eye in Mpep/EAU (represented by score regions 1
through 4), compared to that observed in Sag/EAU (Fig. 21b). In Mpep/EAU, clinical disease scores increased very slowly, and the maximum total disease score attained (day 19 pi) was approximately 58 percent of the maximum for Sag/EAU eyes (day 11 pi) (Fig. 4).

The day of onset for Mpep/EAU ranged widely, from day 11 to day 16 pi. On day 11 pi, only three of the 48 eyes in this study exhibited a positive score (8.3 percent). This is in contrast to the onset of Sag/EAU, in which clinical disease symptoms were observed in 98 to 100 percent of the animals on day 10 pi.

Ocular mast cell numbers in Mpep/EAU. For both choroidal and limbal mast cells, when numbers of mast cells for eyes with positive disease scores were compared to numbers of mast cells for eyes from pre-disease day 9 pi, no significant changes were noted (Fig. 23). This contrasts with Sag/EAU, in which significant decreases in ocular mast cell numbers occurred when disease symptoms were severe (at day 11 pi for the limbus, day 13 pi for the choroid) (Figs. 7 and 8).

Histamine levels in ocular tissues in Mpep/EAU. In contrast, significant increases in histamine levels occurred in choroid, anterior segment, and retina tissues (Figs. 24, 25, and 26, respectively) during the period of most intense disease in Mpep/EAU (Figs. 4 and 21a). Choroidal histamine was

Figure 23. Mast cells counted in limbal (3.2 mm^2) and choroidal (4.4 mm^2) tissue of eyes from rats inoculated with Mpep/sCFA as described in Chapter 2. (●) represents limbi and (▲) represents choroids from eyes that developed EAU; (O) represents limbi and (△) represents choroids from eyes that did not develop EAU. Values are means ± SEM. $n = 4$, except as indicated by numbers in parentheses.

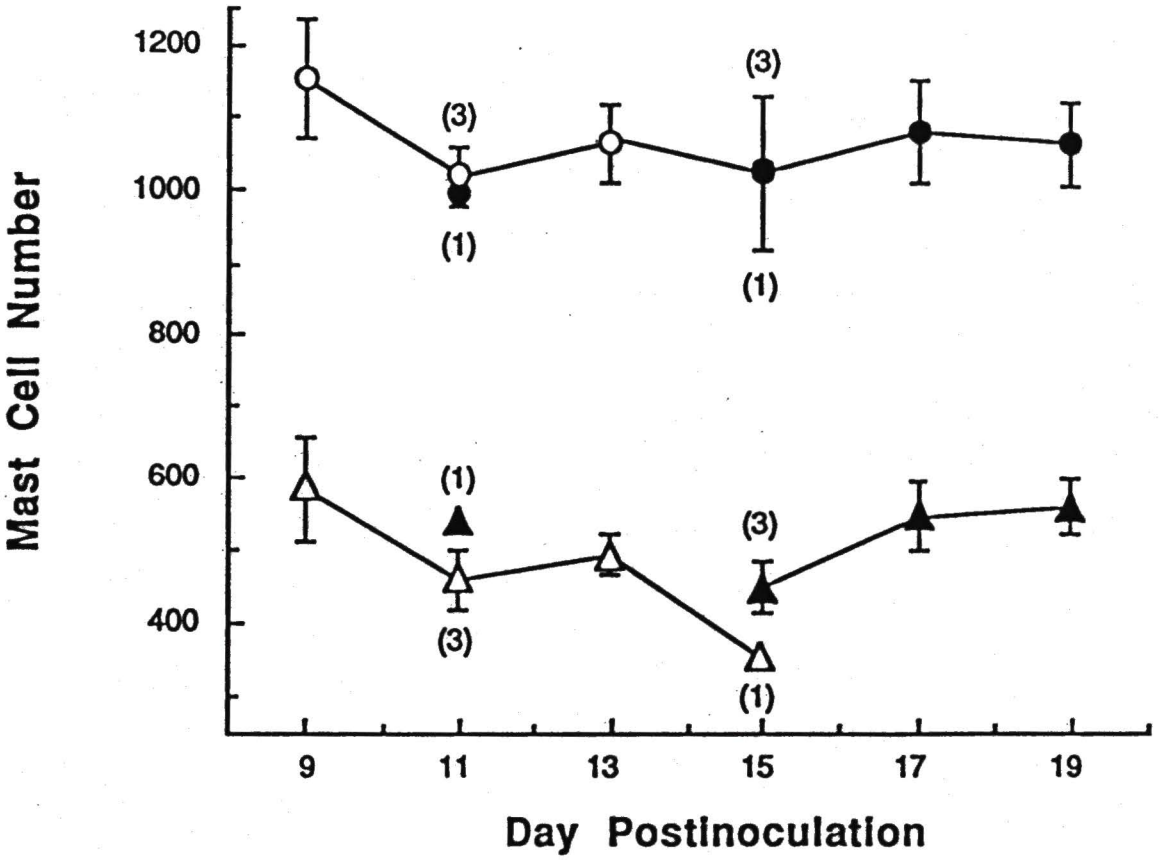

Mast Cell Number
1200
1000
800
600
400
(3)
(1)
(3)
(1)
(1)
(3)
(3)
(1)
9
11
13
15
17
19
Day Postinoculation

Figure 24. Changes in choroidal histamine for eyes of rats inoculated with M-peptide. (●) represents eyes that developed EAU; (O) represents eyes that did not develop EAU. Values are means ± SEM. $n = 8$, except as indicated by numbers in parentheses. * indicates that $p < 0.05$ as compared to histamine values for day 9 pi eyes.

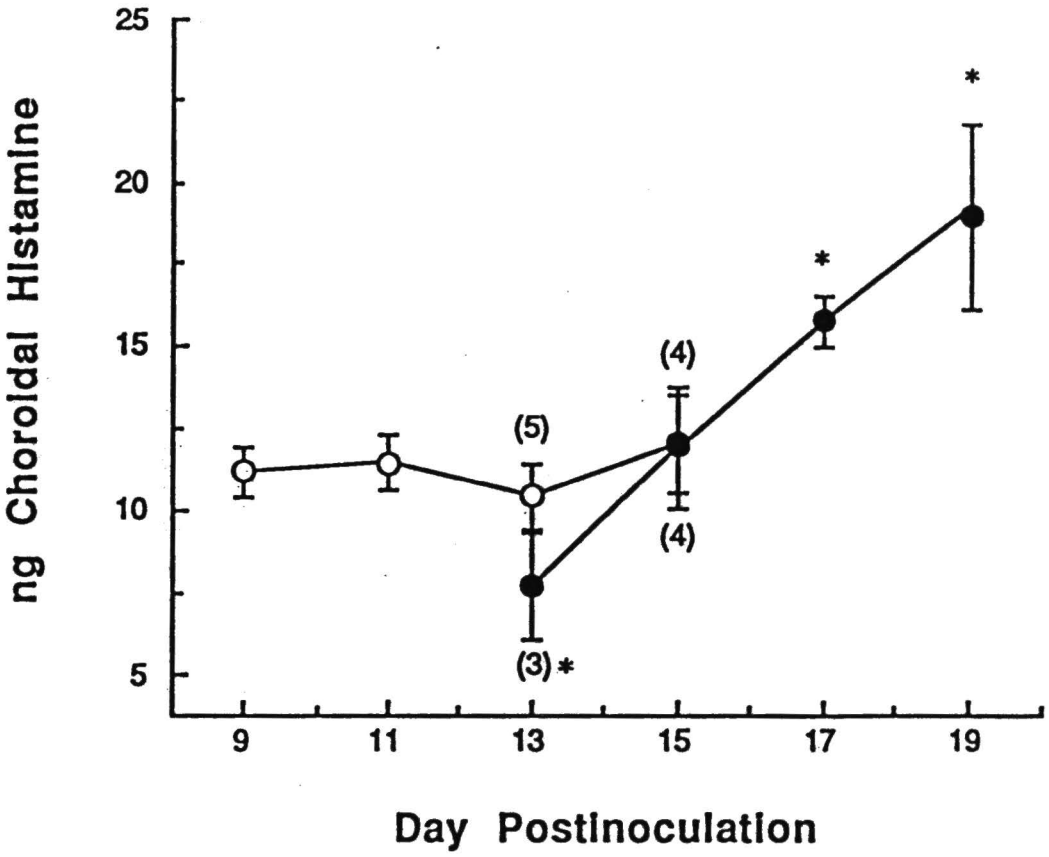

ng Choroidal Histamine
25
20
15
10
5
9
11
13
15
17
19
Day Postinoculation
(5)
(4)
(4)
(3)*
*
*

Figure 25. Changes in anterior segment histamine content for eyes of rats inoculated with M-pep/sCFA. (●) represents eyes that developed EAU; (O) represents eyes that did not develop EAU. Values are means ± SEM. $n = 8$, except as indicated by numbers in parentheses. * indicates that $p < 0.05$ as compared to histamine values for day 9 pi eyes.

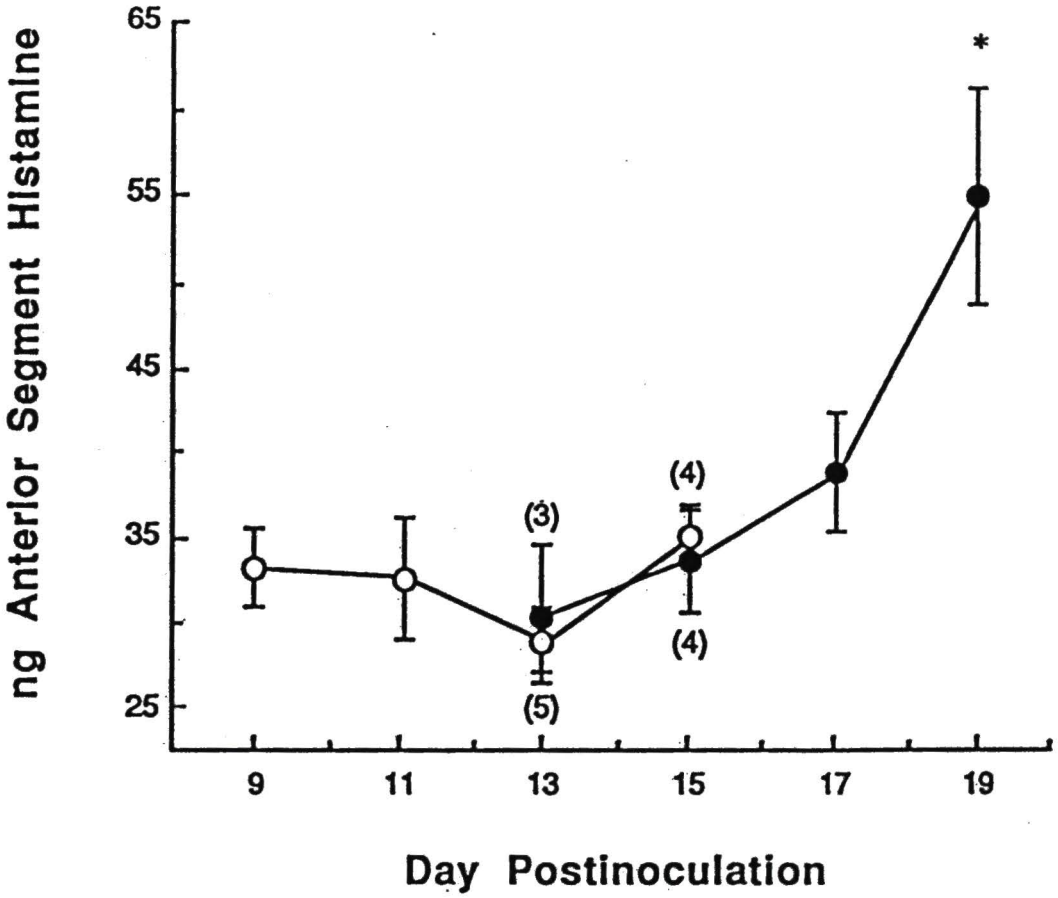
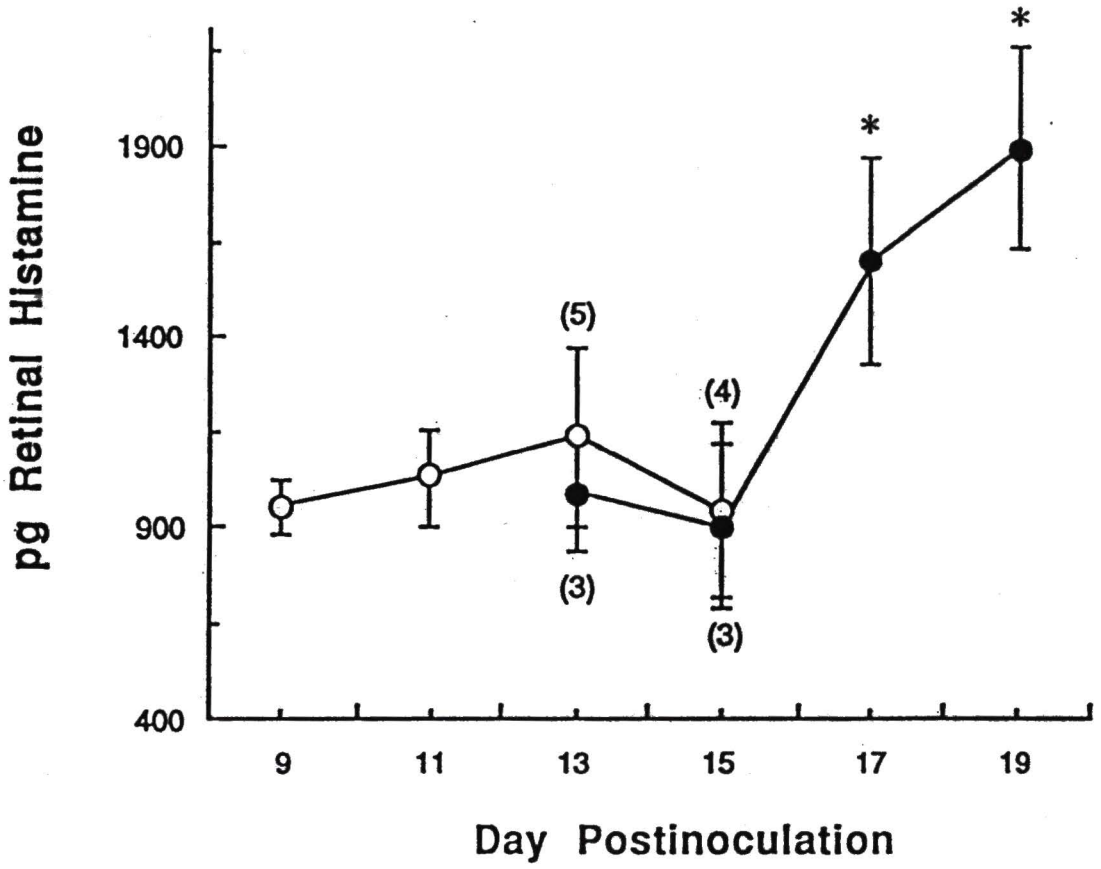

ng Anterior Segment Histamine
65
55
45
35
25
9
11
13
15
17
19
(3)
(4)
(4)
(5)
*
Day Postinoculation

Figure 26. Changes in retinal histamine content for eyes of rats inoculated with Mpep/sCFA. (●) represents eyes that developed EAU; (O) represents eyes that did not develop EAU. Values are means ± SEM. $n = 8$, except as indicated by numbers in parentheses. * indicates that $p < 0.05$ as compared to histamine values for day 9 pi eyes.


pg Retinal Histamine
1900
1400
900
400
9
11
13
15
17
19
Day Postinoculation
(5)
(4)
(3)
(3)
*
*

increased significantly on days 17 pi and 19 pi (Fig. 24). The difference in histamine values for pre-disease and diseased eyes on day 13 pi was also statistically significant. However, only three eyes had manifested positive disease scores on day 13 pi, and no difference in histamine values was observed on the following sacrifice day, day 15 pi. The increase in anteriorsegment histamine was not statistically significant until day 19 pi (Fig. 25). In the retina, histamine levels were increased significantly by day 17 pi, and continued to exhibit a significant increase on day 19 pi (Fig. 26). The increases in histamine levels in the choroid and the retina in Mpep/EAU were very similar to those for the same tissues in Sag/EAU (Fig. 27).

Wet weights of tissues in Mpep/EAU. Figure 28 shows wet weights for ocular tissues from rats that were inoculated with Mpep/sCFA. Weights for tissues from eyes that developed EAU were compared with weights for tissues from pre-disease day 9 pi eyes. Only anterior segment wet weight increased, to marginal significance by day 19 pi. This increase in wet weight of anterior segment tissue occurred much later postinoculation than the increases observed in all of the tissues from Sag/EAU eyes (Fig. 15), consistent with the later development of Mpep/EAU.

Figure 27. Percent change in histamine content of anterior segments, choroids, and retinas during Sag/EAU (■) and Mpep/EAU (▨) on the day of the greatest change from pre-disease values. This occurred on day 19 pi for Mpep/EAU for all ocular tissues assessed, day 11 for Sag/EAU retinas, and day 13 pi for Sag/EAU anterior segments and choroids. All of the values shown are significantly different ($p < 0.05$) from the corresponding pre-disease groups (Sag/EAU, day 5 pi; Mpep/EAU, day 9 pi). Error bars represent SEM. $n = 16$ for Sag/EAU anterior segments and choroids; for Sag/EAU retinas $n = 15$. $n = 8$ for all Mpep/EAU tissues.

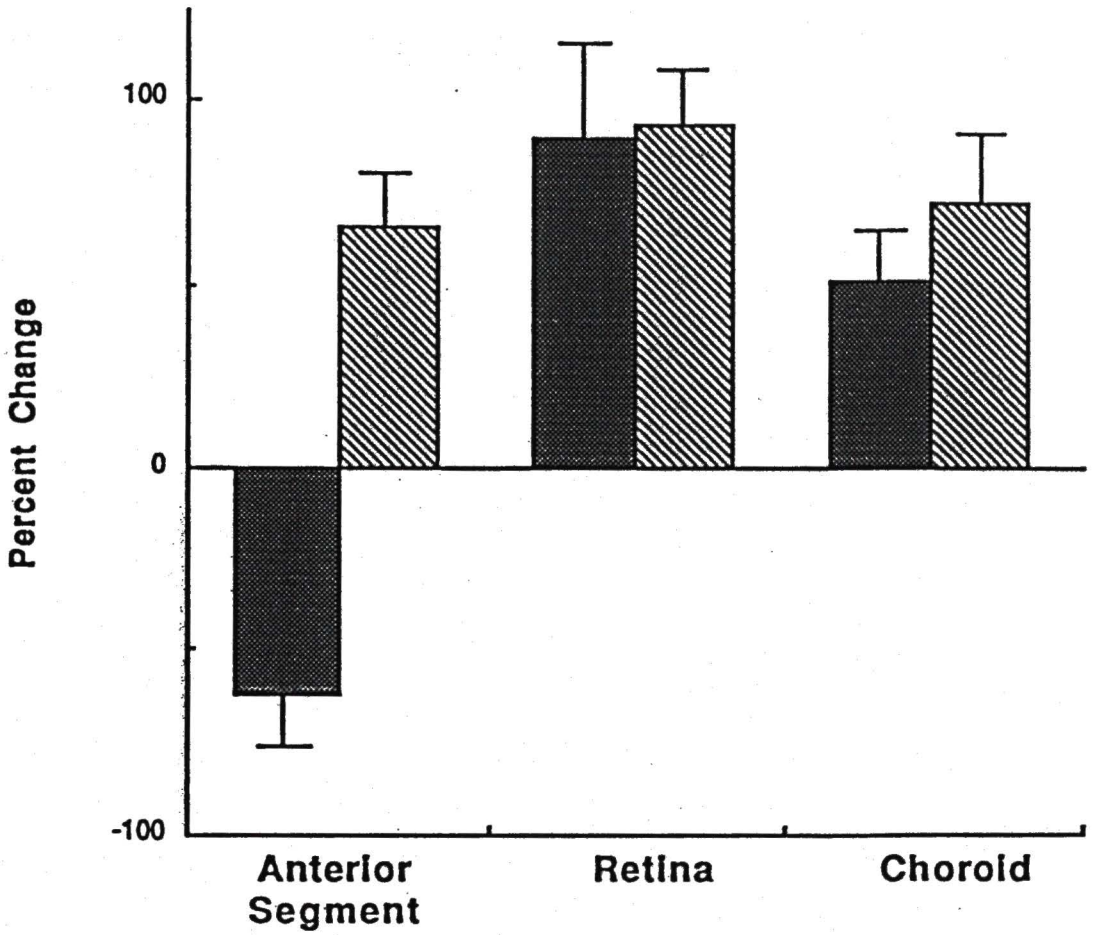
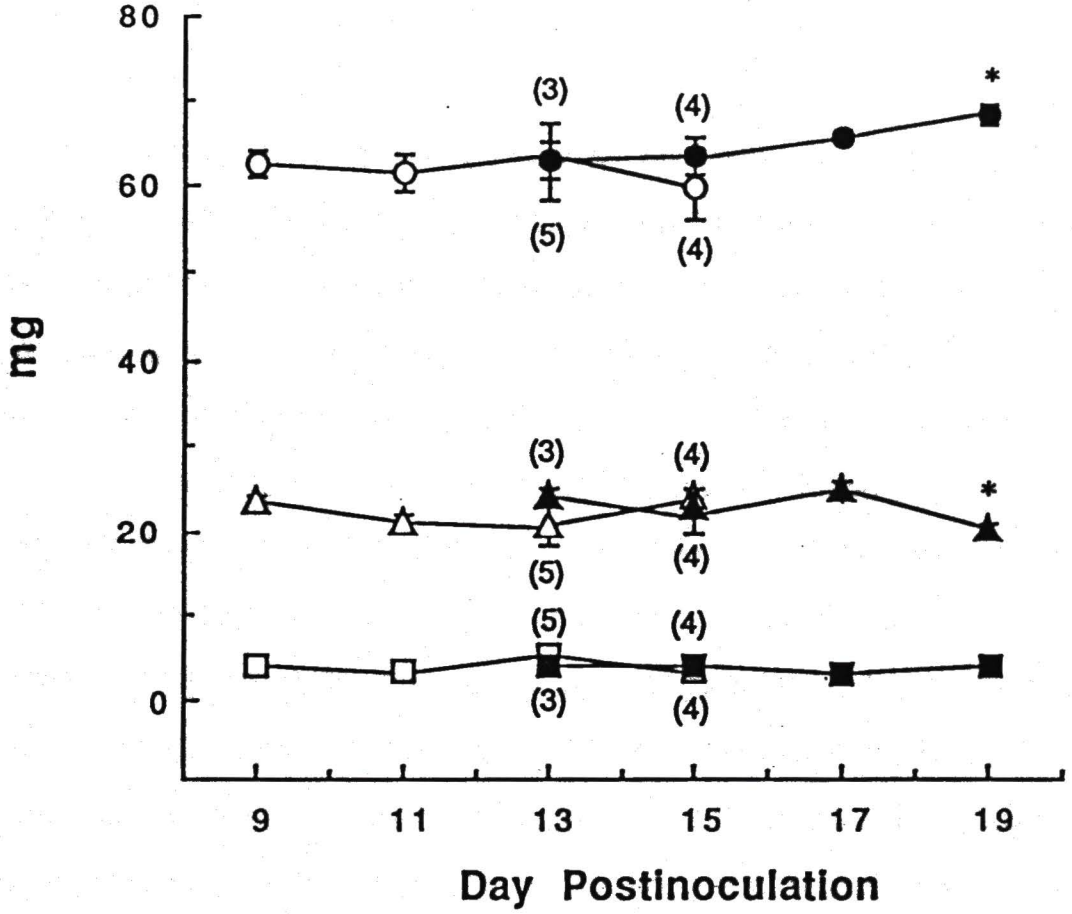

100
0
-100
Percent Change
Anterior Segment
Retina
Choroid

Figure 28. Wet weights of anterior segments (●), choroids (■), and retinas (▲) from eyes that developed Mpep/EAU. (O), (□), and (△) represent the same tissues, respectively, from eyes that did not develop Mpep/EAU. Error bars represent SEM (most error bars are obscured by symbols); the *n* for each group is 8, except as indicated by numbers in parentheses. * indicates that $p < 0.05$ as compared to the value for day 9 pi.


80
60
40
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mg
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9
11
13
15
17
19
Day Postinoculation

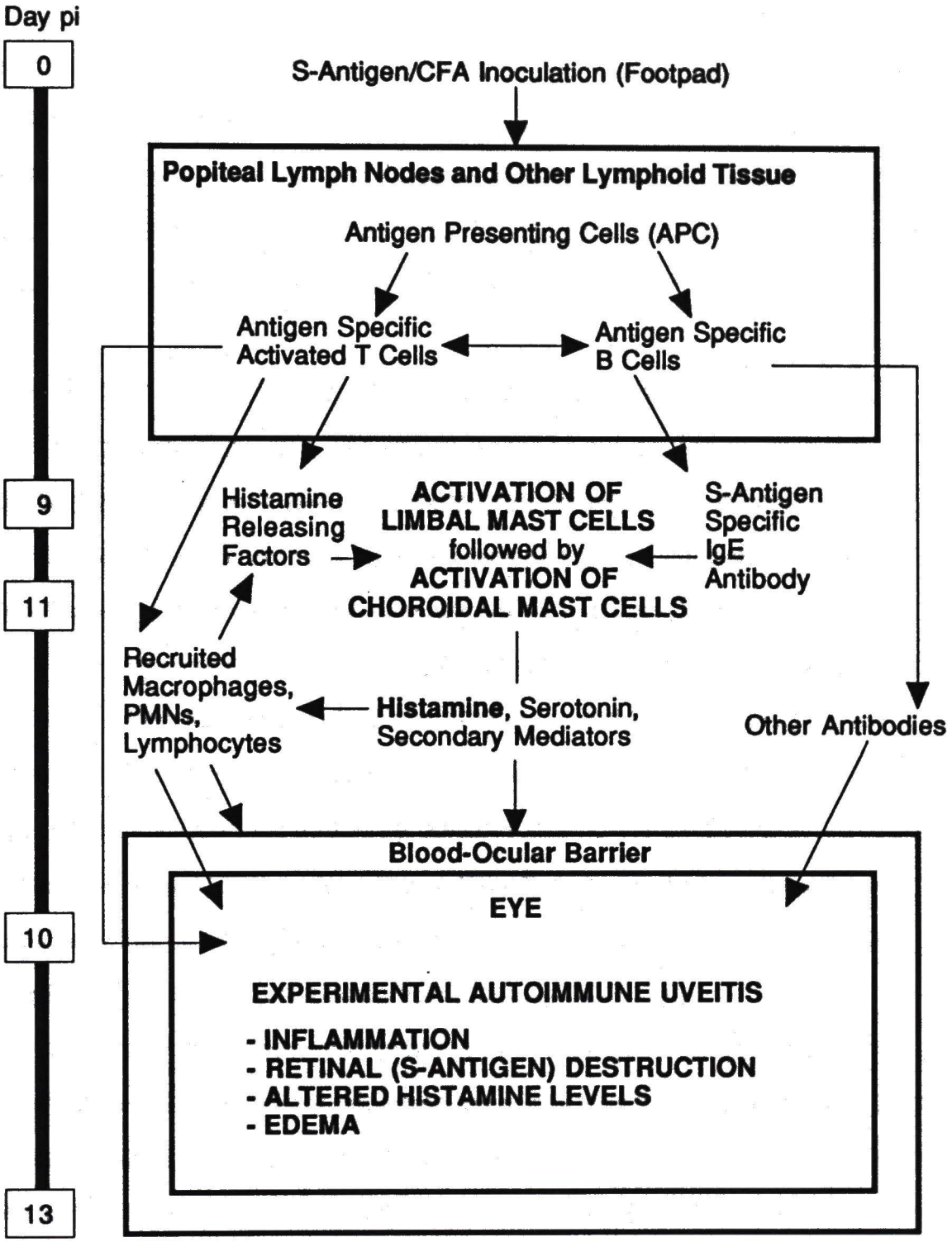
CHAPTER 4

DISCUSSION

The results of this study strongly support involvement of ocular mast cells in the pathogenesis of Sag/EAU. Specifically, significant decreases in numbers of limbal and choroidal mast cells and significant changes in histamine levels of ocular tissues confirm that activation of ocular mast cells occurs during Sag/EAU. These results also show, for the first time, that ocular histamine levels vary in a regionally and temporally specific manner during Sag/EAU, and that limbal mast cells, as well as choroidal mast cells, are involved in Sag/EAU. In addition, prevention of Sag/EAU by leflunomide, a drug that is known to inhibit mast cell degranulation, lends support to the hypothesis that mast cells contribute to the development of Sag/EAU. The results of an *in vitro* study of ocular mast cell activation in Sag/EAU suggest that a mechanism other than IgE/S-antigen crosslinkage on the mast cell membrane is responsible for activation of ocular mast cells when sCFA is used as the adjuvant. Lastly, significant changes in histamine levels of ocular tissues during a mild form of EAU induced by M-peptide, a peptide unit of S-antigen, also suggest a mast cell involvement in EAU.

The schematic in Figure 29 outlines events in the induction and establishment of Sag/EAU, and the parameters considered in this study relative to ocular mast cell activation in Sag/EAU are integrated into the chart as well.

Figure 29. Diagram of events that result in the establishment of Sag/EAU. The time course at the left denotes activation of mast cells, onset of Sag/EAU, and day of peak disease as determined by clinical disease scores.


Day pi
0
9
11
10
13
S-Antigen/CFA Inoculation (Footpad)
Popiteal Lymph Nodes and Other Lymphoid Tissue
Antigen Presenting Cells (APC)
Antigen Specific Activated T Cells
Antigen Specific B Cells
Histamine Releasing Factors
ACTIVATION OF LIMBAL MAST CELLS followed by ACTIVATION OF CHOROIDAL MAST CELLS
S-Antigen Specific IgE Antibody
Recruited Macrophages, PMNs, Lymphocytes
Histamine, Serotonin, Secondary Mediators
Other Antibodies
Blood-Ocular Barrier
EYE
EXPERIMENTAL AUTOIMMUNE UVEITIS
- INFLAMMATION
- RETINAL (S-ANTIGEN) DESTRUCTION
- ALTERED HISTAMINE LEVELS
- EDEMA

In addition, the diagram shows development of the immune response in the lymphatic tissues as described for delayed hypersensitivity-type reactions, including antigen presentation to T cells, B cell activation, antibody synthesis, and recruitment of other cells of the immune system by the activated Sag-specific T cells (54). The resulting pathogenesis of Sag/EAU is shown at the bottom of the diagram. Times observed for activation of ocular mast cells and onset and development of disease are shown on the left of the diagram.

Ocular Mast Cell Activation in S-Antigen-Induced EAU

Statistically significant decreases in limbal and choroidal mast cell numbers occur with the onset and progression of Sag/EAU. In addition, histamine levels in the anterior segment (where limbal mast cells are found), choroid, and retina were significantly altered during Sag/EAU.

The decrease in limbal mast cell numbers that occurred prior to the decrease in choroidal mast cell numbers (Figs. 7 and 8) correlates with the temporal and anatomical sequence of appearance of immune cells in Sag/EAU. Specifically, infiltration by polymorphonuclear cells and T lymphocytes in Sag/EAU is seen first in the ciliary body, then in the anterior choroid and the iris; infiltration of the retina occurs later, is less intense, and peaks later than for the anterior portion of the eye (17).

In addition to those in the limbus and choroid, connective tissue mast cells are present in the ciliary body and iris of the Lewis rat eye ((65); Lee and Orr, unpublished observations), and in the same tissues in human (48) and rabbit eyes (104). Li et al. (65) recently reported that a small population of iris and ciliary body mast cells decreased prior to the onset of Sag/EAU in Lewis

rats (day 6 pi), but returned to normal by day 10 pi, at the end of the induction period. Numbers of these mast cells were not assessed after the onset of Sag/EAU. These results (65) suggest that products from iris and ciliary body mast cells, as well as from the more numerous limbal mast cells (which have decreased in number by day 9 pi, Fig. 8), may be involved in the early stages of Sag/EAU. Because few and highly variable numbers of mast cells were observed in these tissues in the eyes of both Sag/EAU and saline/sCFA control Lewis rats in this study, changes in numbers of ciliary body and iris mast cells were not assessed.

The measurement of histamine in ocular tissues during Sag/EAU afforded another, highly sensitive method for observing mast cell activation. Although total numbers of limbal and choroidal mast cells were not determined, the relative histamine content of these mast cell-rich tissues from saline/sCFA rats (Figs. 9 and 10) was in accord with the relative distribution of mast cells shown in Figs. 7 and 8 and reported by others (2, 96).

Histamine has not been found in large amounts in the retina (75, 91) and has not been identified as a neurotransmitter in the vertebrate retina (75). Thus, the significant increase in retinal histamine on day 11 pi (Fig. 11) may represent an influx of histamine from the choroid occurring after the blood-retina barrier is opened by inflammatory disease in the area. However, although choroidal histamine levels were still increased on day 13 pi (Fig. 9), some decrease in retinal histamine levels was observed on day 13 pi (Fig. 11). The significant increase in histamine methyltransferase in the retina on day 13 pi (Fig. 12) could account for this decrease in retinal histamine in Sag/EAU.

No changes in scleral histamine values were observed in rats with Sag/EAU (Fig. 14), indicating that scleral mast cells play no role in Sag/EAU. Similarly, aqueous humor histamine concentrations did not change during the course of Sag/EAU. The edema that occurred during Sag/EAU, as indicated by the significantly increased wet weights of the ocular tissue (Fig. 15), suggests that the lack of change in histamine concentration for aqueous humor (Fig. 13) might be due to volume changes in aqueous humor that would dilute any histamine increase. Release of histamine (and serotonin in the rat) from mast cells is associated with increased local vasopermeability, which facilitates edema during inflammation (92). In addition, the infiltration of inflammatory cells, aided by the increased vasopermeability, is massive in Sag/EAU (Fig. 5), and likely contributes to the wet weight increases observed (Fig. 15).

As illustrated in Fig. 10, anterior segment histamine levels decreased over time in Sag/EAU, and this decrease occurred concurrently with the decrease in numbers of limbal mast cells (Fig. 8). This indicates that released histamine is rapidly drained from anterior eye tissues, and/or that released histamine is rapidly degraded in anterior eye tissues.

Limbal histamine reaching the posterior eye may contribute to the histamine increase in the choroid (Fig. 9) and to the increase in retinal histamine (Fig. 11) during Sag/EAU. Limbal mast cells are closely associated with the vessels of the episcleral ring (Fig. 6), which branch to form the circle of vessels that serve the ciliary body (19). Histamine could diffuse through the ciliary body into the aqueous humor or into the vitreous. In addition, the drainage system for aqueous humor, i.e., the angular aqueous plexus (analogous to Schlemm's canal (19)), is located in the limbal area. Limbal

histamine could wash out of the anterior chamber in the aqueous humor into the blood through the aqueous veins and, to a lesser extent, into the posterior portion of the eye through intrascleral drainage (19, 55). Conjunctiva and lids are rich in mast cells (2), and histamine is found in tears in allergic reactions (1). Whether conjunctival histamine diffuses into the eye from tears or limbal histamine diffuses out into tears has not been determined.

Enzymatic degradation of histamine in the anterior eye might account for decreases in anterior segment histamine and lack of change in aqueous humor histamine during Sag/EAU. However, levels of the histamine degrading enzymes, histamine methyltransferase and diamine oxidase, have not been determined for either aqueous humor or for anterior eye tissues during Sag/EAU.

The increase in choroidal histamine becomes significant by day 11 pi, and remains high on day 13 pi (Fig. 9), whereas the number of stainable mast cells decreases during this time (Figs. 7 and 8). Whether the increased histamine in the choroid is due to increased synthesis and/or to accumulation of histamine from limbal mast cells or blood plasma is unknown and was not addressed in this investigation.

Based on the available stores of histamine represented by the very large numbers of ocular mast cells and on the degranulation of these cells that occurs in Sag/EAU (Figs. 7 and 8 and (20)), the major contributor of histamine in ocular tissues in Sag/EAU would appear to be ocular connective tissue mast cells. However, other cells, such as endothelial cells (49), T lymphocytes (4) and macrophages (79), may also contribute to the increases in histamine levels observed in Sag/EAU in the choroid and the retina (Figs. 9 and 11). Although

the extent of such contributions is unknown, the available amount of histamine stored in ocular mast cells is substantial (sCFA/saline control eyes, Figs. 7 and 8) and adequate to account for the increased levels of histamine observed in the choroid and retina.

Histamine, assessed in this study as an indicator of mast cell activation, is a potent biogenic amine that constitutes the major component (on a molar basis) of mast cell granules (92). The effects of histamine are potentiated through three different types of cell-surface histamine receptors, expressed on a variety of cells. The H_1 receptor, which is inhibited by classical antihistamines such as mepyramine, is present in retina (18, 45). In inflammation, histamine has been described as an "early messenger," regulating cytokine synthesis, and, in turn, regulated by cytokines (31). Histamine has been implicated in both the development and in the resolution of inflammation (31). Histamine released from mast cells effects local vasopermeability, the basis for the suggestion that mast cells may be involved in the initiation of EAU through opening the blood-ocular barrier (20), and the basis for designating mast cells "gatekeepers" for neural-immune system interactions (82).

The extent of the contribution of histamine to the breakdown of the blood-ocular barrier (20), or to the recruitment, attraction, or activation of other immune system cells (9, 31), illustrated in Figure 29, are not specifically addressed in this study. However, alterations in histamine distribution in the eye during Sag/EAU, along with decreases in ocular mast cell numbers would make an "innocent bystander" role for ocular mast cells unlikely. Determination of the concentration and ocular distribution of mast cell products other than

histamine has not been reported. Many of the cytokines found to be associated with mast cells (12, 38) are involved in the pathogenesis of uveitis (22). Tracking of mast cell-synthesized TNFα and PAF during the inflammation of EAU would be useful in determining mast cell contribution to the disease.

These results demonstrate that significant changes in mast cell numbers and histamine levels occur in the eye during Sag/EAU, and they strongly support a mast cell contribution to this disease. In addition, the results of this study suggest a number of areas for further investigation.

Whether there is a strain specificity associated with the number of resident limbal mast cells in rats, as was found for choroidal mast cells (70) and mast cells of the ciliary body and iris (65) is unknown, and might be investigated. Although many histological and immunohistochemical studies of the anterior eye have been reported (13, 37, 48, 67), the exact relationship of the mast cells of the limbal area to the structure of limbal tissue or to other types of cells in the vicinity has not been investigated. For example, Langerhans cells are located in the same limbal area, and, during neovascularization, both mast cells (100) and Langerhans cells (73) migrate onto the cornea with developing blood vessels. Dimlich et al. (25) have shown that mast cells of the dura mater are arranged in linear arrays, and they note that mast cell associations with blood vessels, nerves, and other connective tissue cells have been suggested.

Any mast cell relationship to the pinealitis that is induced along with Sag/EAU has not been investigated. CNS-associated mast cells are found in association with blood vessels located in certain regions of the brain (82) and in the dura mater (25, 82). The pineal gland is surrounded by pia mater, from

which connective tissue trabeculae invaginate into the pineal, carrying blood vessels and nerves (56). Whether mast cells, or mast cell mediators, are involved in pinealitis is not known, although histaminergic nerve fibers have been found in the rat pineal (68).

The reconstitution of mast cell granules to the intact, pre-EAU condition during resolution of the disease has not been followed. In addition, it is possible that synthesis and release of histamine and other mast cell mediators continue throughout EAU, and may contribute to the resolution of the disease. For example, mast cells are involved in angiogenesis (74). Maintenance of vasopermeability in EAU through continued release of mast cell mediators such as histamine and serotonin might facilitate clearance of the debris resulting from the inflammation of EAU, and would be another possible function for mast cells in EAU.

The eye, like the central nervous system (CNS), was once considered an "immune privileged" site. The mechanism for penetration of the blood-ocular barrier is of interest. de Kozak et al. (20) suggested that vasopermeability resulting from release of choroidal mast cell mediators may facilitate entry of activated T-cells into retina, where they initiate the disease process. However, Wekerle et al. (112) reported that activated T cells can enter the CNS randomly independent of antigen specificity. Hickey et al. (44) found that such activated T cells specific to CNS antigens persisted in higher numbers in the CNS than non-specific T cells, and that activated T cells also entered the eye. Kim et al. (57) reported that tritiated, activated Sag-specific T cells injected into the vitreous did not remain in the eye, although EAU did develop. These reports would appear to indicate that there is no "immune privilege," and that the initial

entry of inflammatory cells into the eye in EAU would not require the action of mast cell products. However, the location of mast cells, in close proximity to blood vessels, suggests encounters with activated T cells entering or leaving the eye, and would not preclude activation of mast cells by Sag-specific activated T cells.

Although the amount of histamine contained in limbal and choroidal mast cells is so large that it probably eclipses contributions from any additional sources, the exact source of histamine over the course of EAU should be determined, using tracers or antibody to histamine. Both endogenous tissues and inflammatory cells may synthesize and release histamine (4, 46, 49, 79), but the magnitude of any contribution to histamine distribution in EAU is not known. EAU reportedly can be induced in mast cell deficient mice using IRBP as the inducing antigen (6). Determination of histamine levels in ocular tissues of these mice during EAU would also be of interest, and it could provide insight into any additional sources for histamine in EAU.

Suppression of S-Antigen-Induced EAU with Leflunomide

Treatment with leflunomide, an antiphlogistic, immunomodulating drug, suppressed both disease symptoms of Sag/EAU and changes in mast cell-related parameters in rats inoculated with Sag/sCFA (Figs. 16, 17, 18, and 19). Pathogenic changes that may be attributed to mast cell activation, i.e., changes in histamine content (Figs. 16, 17, and 18) and edema formation (Fig. 19), were significantly attenuated or blocked in the animals treated with leflunomide.

Leflunomide has been shown to prevent the development of both Sag/EAU and another T cell-mediated autoimmune disease, EAE (8), in which

mast cell involvement has been reported (23, 83). Leflunomide inhibits histamine release and synthesis of biologically active metabolites of arachidonic acid from rat PMC, human basophils, and a bone marrow-derived mast cell line (8). Disodium chromoglycate and ketotifen, drugs that also prevent release of mast cell mediators, have also been shown to prevent or attenuate disease symptoms of Sag/EAU (21).

The primary action of leflunomide in suppressing the immune response is the inhibition of activation and proliferation of lymphocytes. Leflunomide inhibits tyrosine kinase activity, which is required for epidermal growth factor-dependent cell proliferation (8). Leflunomide blocks activation of lymphocytes through inhibition of expression of IL-2 and transferrin receptors (115). In addition, leflunomide has some limited, differential effects on arachidonic metabolism. For example, synthesis of 5-hydroxyeicosatetraenoic acid is suppressed by leflunomide, but not synthesis of leukotriene B_4 or prostaglandin E_2 (8). Bartlett et al. (8) suggest that such differential effects on mediators, which act on other immune system cells, may contribute to immunorestoring effects of leflunomide (i.e., restored response of lymphocytes to mitogens), which were demonstrated in rats.

The Sag/EAU induced in the leflunomide study exhibited typical disease symptoms and similar histamine level changes involving both the anterior and posterior eye, but as noted in the Results, maximum clinical scores were slightly lower than for Sag/EAU in earlier studies. The significant changes in histamine levels (Figs. 16, 17, and 18) also occurred later than did histamine changes in the Sag/EAU study (Figs. 9, 10, and 11). Sources for rats and materials were the same, but some variation in animals and/or the adjuvant or

antigen preparations may have occurred that would account for the difference in the disease pathogenesis.

The results establish that mast cell-related changes are suppressed by leflunomide. Anterior segment, choroidal, and retinal histamine levels were not altered, and wet weights did not increase in rats treated with leflunomide (Figs. 16, 17, 18, and 19). Whether this is a direct effect of the drug on mast cells, the result of the action of leflunomide on other elements of the immune system, or a combination of effects is not evident from these results. Although delineation of the specific mechanisms responsible for the effect of leflunomide on Sag/EAU is warranted, the results of this study support the involvement of mast cells in Sag/EAU.

Mechanisms for Activation of Ocular Mast Cells in EAU

The mechanisms eliciting activation of mast cells during Sag/EAU are of interest because of the suggestion that IgE-Sag crosslinkage on choroidal mast cell membranes is responsible for the activation of mast cells during Sag/EAU (20). Because supplemented complete Freund's adjuvant (sCFA), used as the only adjuvant with Sag in this study to induce Sag/EAU, is not conducive to IgE production (53), an investigation of other possible mast cell activation mechanisms was undertaken. de Kozak et al. (20) postulated that choroidal mast cell activation occurs when Sag-specific IgE molecules on ocular mast cell membranes are crosslinked with Sag that has "leaked" into the choroid from photoreceptor cell outer segments--across the retinal pigmented epithelium and Bruch's membrane (part of the blood-ocular barrier) (20). The

resulting action of released mast cell mediators was postulated to facilitate initial access of the immune system to the target retinal antigen (20).

B. pertussis is known to stimulate IgE production (72). Including *B. pertussis* as an additional adjuvant results in a more severe form of Sag/EAU with an earlier onset than Sag/EAU induced using sCFA alone, and also facilitates induction of Sag/EAU in resistant animals (102). *B. pertussis* has a similar effect in induction of other cell-mediated autoimmune diseases (71). The adjuvant effect of *B. pertussis*, which is usually injected at a separate site at the time the disease-inducing inoculum is introduced, is not fully understood (14, 39, 51, 114).

To assess the possibility, albeit remote, that Sag-specific IgE was present when sCFA was used as the only adjuvant, peritoneal mast cells (PMC) and ocular mast cells from rats with Sag/EAU were exposed to Sag *in vitro* using the same concentrations of Sag employed by de Kozak et al. (21) for testing PMC sensitivity to Sag. However, instead of counting numbers of intact mast cells remaining after exposure to Sag as described in (21), mast cell activation was evaluated by the more sensitive method of determining the percent histamine released after exposure to Sag. Because mast cells of Sag/EAU rats did not release histamine in response to exposure to Sag, these results indicate that IgE is not involved in activation of mast cells in Sag/EAU induced with sCFA as adjuvant (Tables 1, 2, and 3a). Direct comparison of histamine release in Sag/EAU induced with sCFA alone with histamine release in Sag/EAU induced with sCFA/*B. pertussis* would have been useful to resolve this difference. However, if IgE had been present, some histamine release would have been observed in the varying conditions tested.

No indication of passive sensitization of the mast cells was found (Table 3b) when naive PMC and mast cell-rich ocular tissues were exposed to Sag *in vitro* after incubation with serum from Sag/EAU rats (passive sensitization) (Table 3b). However, after day 7 pi, de Kozak et al. (21) found IgE antibody in serum from Sag/EAU rats in which disease was induced using *B. pertussis*. In addition, peritoneal mast cells from rats with Sag/EAU were sensitized to Sag as early as day 5 pi and this sensitization continued at least until day 30 pi (20, 21). The results of this study (Tables 1, 2, and 3) establish that IgE/Sag crosslinkage is not the mechanism that activates ocular mast cells when sCFA is used alone as adjuvant.

In an additional *in vitro* study, PMC from naive rats were exposed to aqueous humor from rats with Sag/EAU to assess whether mast cell activation could have resulted from the action of histamine releasing factors (HRFs) (Table 4). However, HRF activity was not detected in this study. HRFs are substances elaborated by cells of the immune system that activate mast cells, without a requirement for antigen-specific antibody. Infiltration of inflammatory cells is a significant feature of the pathogenesis of Sag/EAU (35). Polymorphonuclear leukocytes (PMNs), mononuclear leukocytes (MNLs), macrophages, and lymphocytes are all part of the inflammatory cell population, and all of these cell types have been shown to elaborate histamine releasing factors (HRFs) *in vitro*, which act on mast cells or basophils, by immune specific or immune non-specific methods, to cause degranulation (66, 93, 105, 113).

Aqueous humor from rats with Sag/EAU was used to assess HRF activity (Table 4). However, absence of HRF activity is not conclusive from these results. Samples from earlier days pi should be assessed, and isolated ocular

mast cells might be used instead of PMC for exposure to aqueous humor. There is a continuous flow of aqueous humor (the rate of turnover is about 1.5 percent per min (19), which might remove or dilute any HRF present. Because of this possibility, extracts from inflamed ocular tissues might also be evaluated for HRF activity. It is possible that the half-life for substances such as HRFs may make detection of activity in diseased tissues and fluids difficult. Use of a control consisting of an HRF isolated from one of the systems described in the references would be helpful to verify results.

The diagram in Figure 29 includes two possible mechanisms for activation of ocular mast cells in EAU--crosslinkage of IgE antibody with Sag and the action of HRFs. The results of this study eliminate IgE/Sag crosslinkage as the mechanism for mast cell activation when Sag/EAU is induced using Sag/sCFA alone. Although the results did not establish that HRFs are present, further investigation is needed to rule out HRF activation of ocular mast cells in Sag/EAU.

In vitro observation of interaction of Sag-specific activated T cells that can adoptively induce EAU with ocular mast cells may be a productive approach for further investigation of the mechanism for mast cell activation. Injection of activated T cells bypasses the "priming" activity in lymphoid tissue shown in Figure 29. In addition, close functional and morphological association between mast cells and sensory nerves has been found (3) and activation of mast cells by stimulation of nerves has been demonstrated in rat dura mater and tongue (24). This is another possible mechanism for activation of mast cells in Sag/EAU, and should be investigated.

Mast Cell Activation in M-Peptide-Induced EAU

The results presented here support the hypothesis that mast cells are activated in Mpep/EAU. Although the significant changes in mast cell numbers that occur in the limbus and choroid during Sag/EAU (Figs. 7 and 8) were not observed in Mpep/EAU (Fig. 23), histamine levels significantly increased in all of the ocular tissues tested (Figs. 24, 25, and 26). These results suggest a more limited mast cell activation as a factor in Mpep/EAU.

The inflammation that occurs in Mpep/EAU exhibits an inflammatory cell profile similar to that of Sag/EAU, but disease symptoms are less intense (97). The Mpep preparation that was employed by Smith et al. (97), with sCFA as the adjuvant, was used in this study, and produced similar ocular disease symptoms (Figs. 4, 21a, and 22). The Mpep/EAU described by Donoso (28, 29), enhanced by the use of *B. pertussis* as an additional adjuvant, is an EAU identical in symptoms to Sag/EAU, including severe inflammation of the anterior portion of the eye. Any investigation of mast cell activation in the more severe Mpep/EAU described by Donoso et al. (28, 29) has not been reported.

The lack of change in limbal mast cell numbers (Fig. 23) was not surprising considering the relatively normal appearance of the anterior eye during Mpep/EAU (Fig. 22b). Likewise, the lack of change in choroidal mast cell numbers (Fig. 23) is not inconsistent with the more moderate inflammation in the retinal area (Fig. 22a). Whether the limbal mast cell response in Mpep/EAU differs from that of the choroidal mast cell response in Mpep/EAU is not evident from these results. Use of a larger inoculum (100 mg) of Mpep does not increase disease intensity (60).

In Sag/EAU, significant decreases in mast cell numbers did not occur until disease symptoms were severe (at day 11 pi for the limbus, day 13 pi for the choroid, Figs. 7 and 8). These results suggest that the early activation of only a small number of mast cells may be adequate to "open the gates" for inflammation, or, alternatively, that a small amount of mediator release from a large number of mast cells may occur. Additionally, it is possible that the mast cell activation that occurs in Mpep/EAU is not detectable using the toluidine blue staining technique alone. Ultrastructural evidence has shown that stimulated dura mater mast cells, which are of the same mast cell type as ocular mast cells, secrete granule contents when no degranulation is apparent with toluidine blue staining of mast cells (24).

That mast cell activation does occur in Mpep/EAU is supported by the significant increases in histamine levels during Mpep/EAU in all ocular tissues tested (Figs. 24, 25, and 26). The increases in histamine levels in the choroid and the retina were very similar to those for the same tissues in Sag/EAU (Fig. 27). However, disease scores do not reach maximum values in Mpep/EAU in the posterior segment of the eye (Fig. 21a, regions 4 and 5), reflecting the relative effect of the small amount of histamine released from choroidal mast cells in Mpep/EAU, compared to the large amount of histamine released during Sag/EAU, in which mast cell numbers decrease significantly during the disease.

In the anterior segment a significant increase in histamine was observed in Mpep/EAU (Fig. 25). This increase in histamine in the anterior segment is in direct contrast to the decrease in histamine found during Sag/EAU (Fig. 10). The histamine increase during Mpep/EAU results from a stimulation of

histamine synthesis that does not culminate in release of mast cell granule contents. The increase in histamine levels does not represent recruitment of additional mast cells, as was reported for Sag/EAU (20) because mast cell numbers do not change during Mpep/EAU from day 9 pi through day 19 pi. Although only moderate inflammation of limbal vessels (Fig. 21a, region 1), and minor involvement of the rest of the anterior portion of the eye (Fig. 21a, regions 2-4) was observed in Mpep/EAU, the significant increase in histamine indicates that mast cells are involved. In Sag/EAU, the same regions of the anterior segment are maximally involved from the onset of symptoms (Fig. 15b, regions 1-4), and released histamine is removed from the area, as discussed earlier.

The amount of mast cell histamine present and the mast cell capacity for synthesis of nascent histamine is extremely large (10). Histamine is also synthesized by endogenous cells and by invading inflammatory cells (4, 46, 49, 79). However, non-mast cell histamine synthesis would not appear to account for the increases in histamine in Mpep/EAU. Few inflammatory cells are observed in the anterior portion of the eye during Mpep/EAU, and, therefore, are unlikely to serve as the source of the significant increase in histamine levels. The magnitude of histamine synthesis by endogenous cells is not clear (49). Specific tracing of mast cell histamine would be required to eliminate possible contributions from endogenous cells. The minimal inflammation of the anterior eye in Mpep/EAU suggests that stimulation of endogenous cells to synthesize histamine would not be significant.

Disease scores for the days of most intense disease indicate that only a moderate inflammation of limbal vessels occurs during Mpep/EAU (Fig. 21a, region 1). In contrast, in Sag/EAU the limbal vessels are maximally involved

from the onset of disease symptoms (Fig. 21b, region 1). It is possible that the lack of intensity of the inflammation in Mpep/EAU only initiates an increase in histamine synthesis that does not culminate in degranulation. An increase in wet weight (Fig. 28), an indication of edema, reached significance only in the anterior segment, and occurred late in the course of the disease when compared to the significant increases found in Sag/EAU in all of the ocular tissues (Fig. 15).

The reason for the relative lack of inflammation in the anterior portion of the eye in the Mpep/EAU described by Smith et al. (97) has not been fully investigated. A very low dosage of Sag (1 μg) also induces an EAU that is restricted to the posterior eye (107). The Mpep peptide fraction of Sag corresponds to only one of several uveitogenic sites that have been identified for bovine and human Sag (26). Some monoclonal antibodies to Sag recognize a protein in the anterior portion of the eye, i.e., the iris and ciliary body (50). The presence of this protein, which is not uveitogenic, might account for the intense involvement of the anterior eye in Sag/EAU that is not seen in Mpep/EAU, if this protein has no commonality with the Mpep sequence. However, this would not explain the uveitis that is induced only in the posterior eye by a very low dose of Sag.

Mast cell activation in forms of EAU induced by other antigens is also of interest. Interphotoreceptor retinoid binding protein (IRBP) induces a severe EAU with some features that are different from Sag/EAU (32). In addition, a protein from the retinal pigmented epithelium (15), induces a form of EAU involving only the anterior eye (experimental anterior autoimmune uveoretinitis (EAAU)). Variations in immune cell populations and pathogenesis have also

been observed when the dosage of Sag is varied (107), and the pathogenesis of EAU is different when different animal species are used (35). The patterns of mast cell activation in these forms of EAU have not been reported.

Summary

This study was undertaken to investigate the hypothesis that choroidal mast cells contribute to the pathogenesis of EAU. The results presented here support and extend this hypothesis. In addition to confirming statistically a report that choroidal mast cells are activated during Sag/EAU, the study was extended to include the mast cells concentrated in the limbus of the eye. The limbal mast cells were also found to be activated in Sag/EAU, earlier than the choroidal mast cells (62, 63, 64), which suggests that any mast cell participation in the initiation of Sag/EAU is by limbal mast cells, not choroidal mast cells.

To consider mast cell related changes that occur during EAU, the distribution of histamine was determined in ocular tissues at intervals during the development of EAU. Wet weights of the same tissues were also recorded to observe increases indicative of edema, one of the effects of mast cell vasoactive amines, such as histamine and serotonin. Both histamine levels and wet weights were altered during EAU, suggesting that mast cells contribute to the pathogenesis of the disease (61, 62, 63, 64).

The same mast cell related parameters, histamine levels and wet weights, were suppressed by an immunomodulating drug, leflunomide, that suppresses EAU. These results provide further support for mast cell involvement in EAU.

The question of the mechanism(s) involved in activation of ocular mast cells was not resolved by the study. However, results indicate that IgE involvement appears to be unlikely when sCFA alone is used as the adjuvant for inducing EAU.

Determination of ocular mast cell numbers and histamine levels in a milder form of EAU, Mpep/EAU, showed that although massive mast cell activation resulting in decreases in mast cell numbers did not occur, the mast cell-related parameters--histamine levels and wet weights--were altered, suggesting a different mast cell involvement from that seen in Sag/EAU (63).

APPENDIX A

OCULAR DISEASE SCORING PARAMETERS

Table 5.

Ocular Disease Scoring Parameters

Region	Score	Description
1. Limbal Vessels	0	Normal amount of vessels visible, no dilation but secondary and tertiary vessels are barely visible.
	1	Primary limbal vessels dilated or engorged.
	2	Secondary and tertiary vessels dilated.
	3	Hemorrhage or petechia of secondary and tertiary vessels.
2a. Cornea	0	Clear, all structures are visible, smooth surface.
	1	Cloudy, underlying structures are still visible, but obscured.
	2	Extremely cloudy, underlying structures are not visible.
	3	Neovascularization of 25% of corneal surface.
	4	Perforated.

Ocular Disease Scoring, continued:

Region	Score	Description
2b. Corneal Cloudiness Area	0	0% of corneal area cloudy.
	1	25% of corneal area cloudy.
	2	50% of corneal area cloudy.
	3	75% of corneal area cloudy.
	4	100% of corneal area cloudy.
3. Anterior Chamber	0	Normal structure. No cells or debris visible.
	1	Fibrin deposits visible, usually on lens. Some material may be visible on pupil margins.
	2	Faint trace of hypopyon. Usually some white material visible behind iris.
	3	Hypopyon obvious, fibrin and cellular deposits may be visible behind iris.
	4	Blood in anterior chamber.

Ocular Disease Scoring, continued:

Region	Score	Description
4. Iris Response	0	Pupil responds to mydriatic agent, full (100%) dilation. Very little iris visible in anterior chamber. Clear view to posterior portion of eye.
	1	Pupil dilates 75% in response to mydriatic agent.
	2	Pupil dilates 50% in response to mydriatic agent.
	3	Pupil dilates only 25% in response to mydriatic agent.
	4	Pupil fails to dilate in response to mydriatic agent.
5. Vitreous	0	Clear view of retinal vessels and optic nerve head.
	1	Hazy view of retinal vessels. Cloudy corneal channel in vitreous (hyaloid or Cloquet's canal) running from center of lens to retina, visible with small amount of cells.
	2	Some areas of retina not visible, possible clumps of cells. Cloquet's canal filled with enough cells to form a triangle figure.
	3	Blood in vitreous.
	4	Score arbitrarily assigned if pupil is closed to the degree that the vitreous cannot be viewed.

APPENDIX B

DISTRIBUTION OF HISTAMINE IN CONTROL EYES

Table 6.

Distribution of Histamine in Control Eyes (Data from Saline/sCFA-Inoculated Rats)

Tissue	Histamine (pg)	Wet Weight (mg)	Hm/Wet Weight (pg/mg)
Anterior Segment (n = 39)	29277 ± 1011*	53.3 ± 0.61	552 ± 20
-- without lens		16.0 ± 0.18 (est)	29261 ± 1011
Sclera (n = 40)	100056 ± 3728	18.8 ± 0.38	5322 ± 164
Choroid (n = 40)	10377 ± 464	2.5 ± 0.12	4478 ± 326
Retina (n = 40)	1348 ± 69	20.1 ± 0.64	73 ± 6

* Values are means±SEM.

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