The Role of Eosinophils and Neutrophils in Helminth-Induced Keratitis

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PURPOSE. Intrastromal injection of mice with antigens from the parasitic helminth that causes river blindness (Onchocerca volvulus) induces eosinophil recruitment to the corneal stroma at the time of maximum corneal opacification and neovascularization. The present study was conducted to examine the role of eosinophils and neutrophils in onchocercal keratitis in control C57Bl/6 mice and in interleukin-5 gene knockout (IL-5−/−) mice.

METHODS. C57Bl/6 and IL-5−/− mice were immunized subcutaneously and injected intrastromally with soluble O. volvulus antigens. Mice were killed at various times thereafter. Development of keratitis was assessed by slit lamp examination, and inflammatory cells in the cornea were identified by immunohistochemistry.

RESULTS. A biphasic recruitment of inflammatory cells was observed in C57Bl/6 mice; neutrophils predominated during the first 72 hours after intrastromal injection and subsequently declined, whereas eosinophil recruitment increased as time elapsed and comprised the majority (90%) of cells in the cornea by day 7. In contrast, neutrophils were the predominant inflammatory cells in IL-5−/− mice at early and late time points and were associated with extensive stromal damage and corneal opacification and neovascularization. Eosinophils were not detected in these mice at any time.

CONCLUSIONS. In the absence of eosinophils, neutrophils can mediate keratitis induced by helminth antigens. Together with the early neutrophilic infiltrate in control animals, these observations indicate that neutrophils have an important role in onchocercal keratitis. (Invest Ophthalmol Vis Sci. 1998;39:1176–1182)

An estimated 18 million persons are infected with the parasitic helminth Onchocerca volvulus, which is the second leading cause of infectious blindness worldwide.1 Blindness develops in response to larval penetration from the skin to the cornea. The parasites elicit little or no inflammatory response while alive; however, after larval death and degeneration, a localized inflammation is induced that results in corneal opacification and neovascularization.2,3 A chronic inflammatory response to repeated larval invasion causes sclerosing keratitis, which is the most common form of river blindness.2,3

Because of the paucity of corneas from infected people, animal models for O. volvulus-induced keratitis have been developed.4 Corneal opacification and neovascularization can be induced after intrastromal or subconjunctival injection of live parasites5–8 or soluble O. volvulus antigens.9–11 Using a murine model for O. volvulus–induced keratitis in which helminth antigens are injected intrastromally, we demonstrated in a previous study that development of keratitis is dependent on prior immunization and the presence of sensitized T cells and is associated with a predominant T-helper type 2 (Th2) response (interleukin [IL]-4 and IL-5 > interferon-y [IFN-y]).12 We also showed that eosinophils are the predominant inflammatory cells in the cornea after injection of parasite antigens and that the severity of keratitis is associated with the number of eosinophils in the cornea. For example, IL-4–deficient mice develop less severe keratitis than do control animals, and significantly fewer eosinophils are present in cornneas.12,13 Conversely, systemic administration of recombinant IL-12 results in exacerbated keratitis, which is associated with increased eosinophils in the cornea.14 Furthermore, ultrastructural analysis of these corneas shows extensive infiltration of eosinophils, some of which have the appearance of degranulated cells.15

In the present study, we examined the temporal recruitment of granulocytes to the corneas of control, immunocompetent C57Bl/6 mice and IL-5 gene knockout (IL-5−/−) mice that are deficient in their ability to produce eosinophils.16 We found that neutrophils are prominent cells early in development of onchocercal keratitis and can mediate corneal disease in the absence of eosinophils.
**MATERIALS AND METHODS**

**Animals**

Interleukin-5 gene knockout mice were produced as described by Kopf et al. and were back-crossed to a C57Bl/6 background. Breeding pairs were kindly sent to us by Bruce Blazar and Edward Pearce (Cornell University, Ithaca, NY) with permission from Manfred Kopf (Basel Institute of Immunology, Switzerland). Animals were bred in the specialized animal module in the Animal Resource Center at Case Western Reserve University, and females were immunized when aged 6 weeks. Age and sex-matched C57Bl/6 mice (Charles River Laboratories, Wilmington, MA) were used as control animals. All procedures conformed to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

**Parasite Antigens**

Subcutaneous nodules containing adult *O. volvulus* parasites were surgically removed from patients in Cameroon. The procedure reduces the number of circulating microfilarial larvae, thereby reducing levels of disease in the person and transmission in the community. Nodules were kindly provided by Sara Lustigman at the New York Blood Center (New York, NY), and Thomas Unnasch at the University of Alabama, Birmingham. Adult worms were isolated after collagenase digestion of the nodular material as described. Parasites were homogenized using a tissue grinder and were centrifuged to remove insoluble material. Soluble *O. volvulus* antigens were sterile filtered, and protein concentration was adjusted to 1 mg/ml.

**Immunization and Intrastromal Injections**

Mice were sensitized to parasite antigens by three weekly subcutaneous immunizations with 10 µg *O. volvulus* antigens in Hanks' balanced salt solution (Gibco, Gaithersburg, MD) together with adjuvant containing squalene (Aldrich Chemical, Milwaukee, WI), Tween 20 (Fisher, Fair Lawn, NJ), and pluronic acid (Pluronic L-121; BASF, Parsippany, NJ) at a 1:1 ratio. For intrastromal injections, a 30-gauge needle was used to scarify the cornea, and 10 µg *O. volvulus* antigens in 10 µl was injected into the corneal stroma of the right eye using a 33-gauge needle attached to a gas-tight syringe (Hamilton, Reno, NV). This procedure is highly reproducible with less than 3 µl of fluid leakage from the wound. Clinical disease was quantified by determining corneal opacification and neovascularization using the scoring system described previously. Detection of Eosinophils in Blood

Blood was collected retro-orbitally, and differential staining was performed using a modified Wright-Giemsa Stain (Diff-Quik; Dade Diagnostics, Aguada, PR).

**Immunohistochemistry**

Mouse eyes were removed and fixed in 10% formalin for at least 24 hours, followed by processing in a tissue processor (Tissue-Tek VIP; Sakura Finetechnical, Tokyo, Japan). Preparation of eyes for paraffin embedding and sectioning was carried out according to standard methods. Five-micrometer sections were immunostained with rabbit antisera to murine eosinophil major basic protein (MBP) kindly provided by Kirsten Larson and Gerald Gleich at the Mayo Clinic (Rochester, MN) or rat monoclonal antibody 7/4 (Serotec, Oxford, UK), which reacts with a surface component of murine neutrophils but not with eosinophils. Anti-major basic protein (1:1000) and 7/4 mAb (1:100) were diluted in 1% fetal calf serum in 0.05 M Tris-buffered saline (pH 7.6). Slides were incubated at room temperature in a humid chamber for 2 hours. Biotinylated goat anti-rabbit Ig (Dako, Carpenteria, CA) diluted 1:200, or prediluted biotinylated rabbit anti-rat Ig's (Rat Link; BioGenex, San Ramon, CA) were added for 30 minutes, followed by a similar incubation with prediluted alkaline phosphatase conjugated streptavidin (BioGenex). Positive reactivity was detected using substrate (Vector Red; Sigma, St. Louis, MO) containing 12 mg levamisole (Sigma), followed by counterstaining with modified Harris' hematoxylin (Richard-Allen, Kalamazoo, MI). At least two 5-µm sections per cornea were counted directly for positive reactivity with anti-MBP.

**Cytokine Gene Expression in Mouse Corneas**

The method for extracting corneal RNA and reverse transcription-polymerase chain reaction conditions have been described in detail previously. Briefly, corneas were dissected from four mice with similar clinical responses, and RNA was extracted in 500 µl RNA STAT 60 (Tel-Test; Friendswood, TX) using micro—tissue grinders (Kontes, Vineland, NJ). Complementary DNA (cDNA) was synthesized using Superscript II reverse transcriptase (Gibco), and the polymerase chain reactions were performed with *Taq* DNA polymerase (Gibco) using a thermal cycler (Omnigene, National Labnet, Woodbridge, NJ). Reaction conditions were as follows: 1 minute at 95°C, 1 minute at 62°C, and 1.75 minutes at 72°C. For each set of primers, a positive sample (cDNA from anti-CD3-stimulated spleen cells) and a negative sample (distilled H2O) were run in parallel.

Primers sequences and cycle numbers were as follows: hypoxanthine phosphoribosyl-transferase (HPRT): sense: GTT GAA TAC AGG CCA GAC TTT GTT G; antisense: GAT TCA ACT TGC GCT CAT CTT AGG C; internal: GTT GAT TGA TCT GCC CTT GAC (27 cycles); IL-4: sense: TAC TGG TCA TCC TGC TCT T; antisense: CTC AGT ACT AGG AGT AAT CCA; internal: AGG GCT TAC ACG GTG CTT CCG A (30 cycles); IFN-y: sense: TGT TAC TGT CAC GGC ACA GTC ATT; antisense: GTG GAC CAC TCG GAT GAG CTT TGG TTG AAG TCT TGA (35 cycles); and IL-5: sense: GTG AAA GAG ACC TTG ACA CAG CTG; antisense: CAG ACC AAG CTT CAG GCA CTA T (32 cycles). Polymerase chain reaction products were separated by agarose gel electrophoresis and visualized after Southern hybridization by using fluorescein-deoxyuridine triphosphate-labeled probes (Enhanced Chemiluminescence; Amersham, Arlington Heights, IL).

**Cytokine Production by Splenocytes**

Spleens were removed by standard aseptic technique, and red cells were lysed with 0.01 M Tris (pH 7.2) containing 0.75% ammonium chloride. Splenocytes were then suspended in Rosewell Park Memorial Institute (RPMI) medium containing 1 mM sodium pyruvate, 2 mM l-glutamine, 20 mM HEPES, 100 U/ml penicillin, 100 µg/ml streptomycin, and 10% heat-inactivated fetal calf serum. Duplicate wells containing 5 X 10³ cells were stimulated with 2 µg/ml anti-CD3 (2C11); kindly provided by Thomas Forsthuber, Department of Pathology, Case West-
Eosinophil Recruitment to the Cornea of Control C57Bl/6 Mice

Previous studies demonstrated that immunized mice injected intrastromally with *O. volvulus* antigens exhibit maximum corneal opacification and neovascularization after 4 to 10 days and that eosinophils are prominent in the corneal stroma at this time. To determine the kinetics of granulocyte recruitment to the cornea, immunized C57Bl/6 mice were killed at 12 hours and 1, 3, or 7 days after intrastromal injection of *O. volvulus*, and immunostained with Ab specific for eosinophils and neutrophils. Cells were counted at high magnification.

Few or no eosinophils were detected in the corneal stroma 12 hours and 1 day after intrastromal injection (Fig. 1). However, the number of eosinophils increased to 455 ± 80 cells per 5-μm section by day 3 and 635 ± 106 by day 7. In contrast, 352 ± 36 neutrophils were detected as early as 12 hours after intrastromal injection and increased to 1106 ± 90 by day 1 (90% of total cells in the cornea). By day 3, the number of neutrophils declined to 708 ± 159 and represented 50% of the cell infiltrate. By day 7, the number of neutrophils had further decreased to 17 ± 10 per section, and they were absent in many corneas.

Diminished Blood Eosinophils and Elevated Blood Neutrophils in Interleukin-5 Gene Knockout Mice

As a first step in determining the role of neutrophils in the absence of eosinophils, we examined the effect of IL-5 deficiency on circulating eosinophils and neutrophils after immunization with parasite antigens. C57Bl/6 and IL-5−/− mice were immunized and injected intrastromally with *O. volvulus* antigens, and the percentage of blood eosinophils was determined 7 days later. C57Bl/6 mice had 3.11 ± 1.2% eosinophils compared with 0.17 ± 0.2% in IL-5−/− mice (P < 0.01). Conversely, neutrophils were significantly higher in IL-5−/− mice than in C57Bl/6 mice (34.6 ± 9.3% versus 16.8 ± 3.7%; P < 0.01). The remaining blood leukocytes were mononuclear.

Corneal Opacification and Neovascularization in Interleukin-5 Gene Knockout Mice

To determine whether helminth-mediated keratitis is induced in animals that are deficient in eosinophils, IL-5−/− mice were immunized and injected intrastromally with *O. volvulus* antigens, as described in the Materials and Methods section. Clinical manifestations of onchocercal keratitis were determined in a blinded manner by slit lamp examination. IL-5−/− mice exhibited corneal opacification and neo-
vessels after intrastromal injection of parasite antigens, and clinical scores at day 6 were not significantly different from those in control C57BI/6 mice (Fig. 2; \( P > 0.05 \)). Examples of individual animals are shown in Figure 3 (left panels). No differences in keratitis were noted at any other time point, and no clinical manifestations were detected in nonimmunized mice (data not shown).

**Corneal Disease in Interleukin-5 Gene Knockout Mice**

To characterize the cellular infiltrate in IL-5\(^{-/-} \) mice, animals were killed at day 1 or day 7 after intrastromal injection of *O. volvulus* antigens, and adjacent corneal sections were immunostained with antisera to eosinophil major basic protein (MBP; center panels) or anti-neutrophil monoclonal antibody 7/4 (right panels). Note the presence of eosinophils in the epithelial layer and extracellular MBP (upper center panel) and the presence of neutrophils in the anterior chamber (lower right panel). Original magnifications: left panels \( \times 25 \); all others \( \times 400 \). Sections are representative of two experiments conducted in groups of 15 mice each.

**Figure 2.** Corneal opacification and neovascularization in interleukin (IL)-5 gene knockout (IL-5\(^{-/-} \) ) mice. C57BI/6 and IL-5\(^{-/-} \) mice were immunized and injected intrastromally with *Onchocerca volvulus* antigens. Corneas were examined by slit lamp examination and were scored. Corneal opacification scores were as follows: 0, no opacification; 1+, slight opacity; 2, moderate opacity; 3, severe opacity, and 4, total opacity. Neovascularization scores were determined on the basis of number of vessels and growth from the limbus. Scores are from 6 days after intrastromal injection, and each data point represents an individual animal. No statistical difference in scores was detected between the groups for corneal opacification or neovascularization (\( P > 0.05 \)). Similar results were seen in a repeat experiment.

**Figure 3.** Clinical and histologic appearance of corneas of interleukin (IL)-5 gene knockout (IL-5\(^{-/-} \) ) mice after intrastromal injection of helminth antigens. C57BI/6 and IL-5\(^{-/-} \) mice were immunized and injected intrastromally with *Onchocerca volvulus* antigens as described in the Materials and Methods section. Seven days later, corneal opacification and neovascularization had developed in both mouse strains (left panels). Eyes were fixed in formalin, and adjacent sections were immunostained with antisera to eosinophil major basic protein (MBP; center panels) or anti-neutrophil monoclonal antibody 7/4 (right panels). Note the presence of eosinophils in the epithelial layer and extracellular MBP (upper center panel) and the presence of neutrophils in the anterior chamber (lower right panel). Original magnifications: left panels \( \times 25 \); all others \( \times 400 \). Sections are representative of two experiments conducted in groups of 15 mice each.
FIGURE 4. Expression of T helper cell-associated cytokine mRNA in corneas of C57Bl/6 and interleukin (IL-5) gene knock-out (IL-5 KO) mice after intrastromal injection of *Onchocerca volvulus* antigens. C57Bl/6 and after IL-5-deficient mice were immunized subcutaneously and injected intrastromally with *O. volvulus* antigens. Animals were killed on day 1 or day 8 after injection, and corneas from four mice per group were removed and pooled. Expression of the housekeeping gene HPRT and cytokines interferon (IFN-γ), IL-4, and IL-5 was determined by reverse transcription-polymerase chain reaction as described in the Materials and Methods section. Polymerase chain reaction products were visualized after agarose gel electrophoresis and Southern transfer. Data are representative of two experiments.

Phils mAb 7/4. Neutrophils were the predominant cells in the corneas of IL-5 KO mice at day 1, and cell numbers were not significantly different from C57Bl/6 mice (907 ± 168 versus 790 ± 110, respectively; P > 0.05). However, at day 7, keratitis in C57Bl/6 mice was associated with extensive eosinophil infiltration, whereas neutrophils were the predominant cell type in IL-5 KO mice (Fig. 3). Eosinophils were not detected in the corneas of IL-5 KO mice at any time.

In corneas of C57Bl/6 mice, eosinophils were present throughout the corneal stroma and were occasionally detected in the epithelium (Fig. 3). In addition, extracellular MBP was present, indicative of eosinophil degranulation. In contrast to the distribution of eosinophils in C57Bl/6 mice, neutrophils in corneas of IL-5 KO mice were generally clustered below the epithelium and were often detected in the anterior chamber in close association with corneal endothelial cells.

**Similar Levels of Interleukin-4 and Interferon-γ in Immunocompetent C57Bl/6 Mice and Interleukin-5 Gene Knockout Mice**

Although eosinophil deficiency is the primary defect in IL-5 KO mice, Th cytokines, especially IL-4, are important in development of onchocercal keratitis. We therefore examined the effect of IL-5 deficiency on cytokine production after sensitization to *O. volvulus* antigens.

C57Bl/6 and IL-5 KO mice were immunized systemically and injected intrastromally with *O. volvulus* antigens. Animals were killed 1 day or 8 days later, and corneas were removed and processed for reverse transcription-polymerase chain reaction and Southern blot analysis. One day after injection, C57Bl/6 mice expressed high levels of IFN-γ transcripts, but not IL-4 or IL-5 (Fig. 4). However, after 8 days, IFN-γ expression was diminished and IL-4 and IL-5 were increased, consistent with development of the local Th2-type response described previously. Corneas from IL-5 KO mice showed the same changes in temporal expression of IFN-γ and IL-4 toward a Th2-type response, and transcripts for IL-5 were not detected.

To determine the effect of IL-5 deficiency on systemic production of Th-associated cytokines, animals were killed 8 days after intrastromal injection, and splenocytes from C57Bl/6 and IL-5 KO mice were stimulated in vitro by polyclonal activation with anti-CD3 mAb. C57Bl/6 mice produced IFN-γ, IL-4, and IL-5. Consistent with cytokine expression in the cornea, IL-4 and IFN-γ production by IL-5 KO mice was not significantly different from C57Bl/6 mice (P > 0.05; Fig. 5). Similar results were obtained from animals killed on day 1 (data not shown). Taken together, these results indicate that IL-5 deficiency did not significantly affect production of *O. volvulus*-induced Th-associated cytokines.

**FIGURE 5.** Cytokine production by splenocytes from interleukin (IL-5) gene knockout (IL-5 KO) mice. C57Bl/6 and IL-5 KO mice were immunized and injected intrastromally with *Onchocerca volvulus* antigens. Spleen cells (5 × 10⁶/ml) were stimulated with anti-CD3 monoclonal antibodies 2C11, and cytokines were measured by two-site enzyme-linked immunosorbent assay, as described in the Materials and Methods section. Results are presented as mean ± SD from groups of 10 mice each. Similar results were obtained in a repeat experiment. IFN, interferon.
DISCUSSION

The present study demonstrates that neutrophils comprise an early cellular infiltrate into the cornea and can mediate onchocercal keratitis in the absence of eosinophils. Neutrophils have been implicated in the pathologic course of keratitis caused by *Pseudomonas aeruginosa* and herpes simplex virus. In herpes simplex keratitis, an early phase of neutrophil recruitment occurs after the first 2 to 3 days and is followed by a second, more intense neutrophil infiltrate that is associated with corneal disease. A biphasic infiltrate of granulocyte recruitment was also apparent in murine helminth-mediated keratitis, with an early phase of neutrophil recruitment in C57Bl/6 and IL-5−/− mice. However, in immunocompetent C57Bl/6 mice, the neutrophil infiltrate was replaced by eosinophils, whereas neutrophils were present in IL-5−/− mice at later time points.

The presence of each cell type in the cornea probably reflects a balance between cell recruitment and survival within the tissue. In further studies, the molecular events that regulate infiltration of neutrophils and eosinophils in the cornea will be examined and the role of programmed cell death in development of onchocercal keratitis will be determined. Immune privilege in the cornea is correlated with Fas ligand (CD95) surface expression, especially in the corneal epithelium and endothelium, and contributes to allograft acceptance by inducing apoptosis in inflammatory cells that infiltrate the cornea. This mechanism may also contribute to neutrophil apoptosis observed in herpes simplex keratitis. In helminth-mediated keratitis, CD95-mediated apoptosis of neutrophils may contribute to the temporal shift in C57Bl/6 mice from a predominantly neutrophilic to an eosinophilic infiltrate. Conversely, inhibition of apoptosis may underlie the continued presence of neutrophils in IL-5−/− mice and may relate to differential expression of cytokines that prolong neutrophil survival, including IL-1β, TNF-α, and IL-2.

Temporal recruitment of neutrophils and eosinophils to the corneas of these mice may be related to selective expression of vascular adhesion molecules. For example, vascular cell adhesion molecule-1 (VCAM-1) is important in eosinophil transmigration in vitro, whereas endothelial cell adhesion molecule-1 (PECAM-1) mediates neutrophil adhesion. Cytokine regulation of expression of these adhesion molecules may contribute to selective recruitment of granulocytes to the cornea. Tang and Hendricks showed that PECAM-1 expression on vascular endothelial cells was diminished after in vivo neutralization of IFN-γ, which resulted in selective blockade of neutrophil recruitment to the cornea and reduced severity of herpes simplex keratitis. Conversely, IL-4 selectively upregulates VCAM-1 expression and eosinophil transmigration across vascular endothelial cells in vitro. In helminth-mediated keratitis, the observed early expression of IFN-γ may contribute to neutrophil recruitment through PECAM-1, whereas later expression of IL-4 could upregulate VCAM-1 and enhance eosinophil recruitment.

In addition to vascular cell adhesion molecules, chemotactic cytokines are important in cell recruitment to sites of inflammation. We demonstrated that expression of chemokines is important in eosinophil recruitment to the cornea in helminth-mediated keratitis, because regulated upon activation, normal T-cell expressed and secreted (RANTES), macrophage inflammatory protein (MIP)-1α, and eotaxin are increased in IL-12-treated mice that have exacerbated keratitis, and targeted disruption of the eotaxin gene results in significant reduction in eosinophil recruitment to the cornea. In future studies, the role of chemokines and adhesion molecules in recruitment of neutrophils and eosinophils to the cornea in helminth-mediated keratitis will be examined.

The cytotoxic activity of eosinophils is caused primarily by production of highly cationic granule proteins, notably MBP, which comprises the eosinophil granule core and has been reported in biopsy specimens from patients with allergic conjunctivitis. Major basic protein also inhibits corneal wound healing and has a direct cytotoxic effect on cultured human corneal epithelial cells. In addition, MBP and other eosinophil granule proteins have been detected on *O. volvulus* larvae and surrounding tissues in skin lesions of infected persons after topical or systemic administration of anthelmintics.

The latter study also demonstrated the presence of neutrophils and release of neutrophilic enzymes, including elastase, lysozyme, and myeloperoxidase, in *O. volvulus* skin lesions. These data indicate that worm and host tissue damage under these conditions is mediated by cytotoxic products from neutrophils and eosinophils. Taken together with our current observations in the murine model for onchocercal keratitis, it appears likely that neutrophils and eosinophils contribute to corneal disease in infected people.

In summary, our previous studies demonstrate that T cells and eosinophils are important components in development of helminth-mediated keratitis. In the present study, we found that neutrophils were also involved in corneal disease. They were prominent early in the inflammatory response and mediated keratitis in the absence of eosinophils. An understanding of the molecular interactions underlying these observations may suggest approaches to immunologic intervention relevant to helminthic and atopic disease.

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References


