Interleukin 4 and T Helper Type 2 Cells Are Required for Development of Experimental Onchocercal Keratitis (River Blindness)

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Summary

Inflammation of the corneal stroma (stromal keratitis) is a serious complication of infection with the nematode parasite Onchocerca volvulus. Because stromal keratitis is believed to be immunologically mediated in humans, we used a murine model to examine the role of T cells and T helper cell cytokines in the immunopathogenesis of these eye lesions. BALB/c mice immunized subcutaneously and injected intrastromally with soluble O. volvulus antigens (OvAg) developed pronounced corneal opacification and neovascularization. The corneal stroma was edematous and contained numerous eosinophils and mononuclear cells. Stromal keratitis in immunized mice was determined to be T cell dependent based on the following observations: (a) T cell–deficient nude mice immunized and injected intrastromally with OvAg fail to develop corneal pathology; and (b) adoptive transfer of spleen cells from OvAg-immunized BALB/c mice to naive nude mice before intrastromal injection of OvAg results in development of keratitis. OvAg-stimulated lymph node and spleen cell cytokine production was dependent on CD4 cells and included interleukin (IL)-4 and IL-5, but not interferon γ, indicating a predominant T helper type 2 cell–like response. Inflamed corneas from immunized BALB/c mice and from reconstituted nude mice had greatly elevated CD4 and IL-4 gene expression compared with interferon γ. Mice in which the IL-4 gene was disrupted failed to develop corneal disease, demonstrating that IL-4 is essential in the immunopathogenesis of O. volvulus–mediated stromal keratitis.

Onchocerciasis is a major cause of blindness in Africa and Latin America. Approximately 20 million persons are infected by the causative nematode, Onchocerca volvulus, and 2% of these individuals are blinded from the disease (1, 2). In the African savanna, blindness due to onchocerciasis is most commonly due to stromal keratitis, whereas blindness in rain forest areas is largely due to retinitis (3, 4). Clinical observations indicate that the sequence of pathologic events in keratitis is initiated when first-stage larvae, microfilariae, migrate through the skin into the cornea. The microfilariae elicit little or no pathologic responses when alive, but an inflammatory reaction develops when the parasites die either by natural attrition or after administration of chemotherapy (5–7). This is initially characterized as a punctate keratitis, in which discrete sites of corneal opacification correlate with the presence of dead and degenerating microfilariae. In the more advanced stage of infection, sclerosing keratitis ultimately leads to permanent loss of vision (5, 8). This condition is manifest as stromal opacification and corneal neovascularization, and histologic examination of corneas and conjunctival tissue from chronically infected individuals shows mononuclear cells, neutrophils, and eosinophils (5, 9). Coupled with the observation that persons with onchocerciasis have elevated Th2 responses (10–15), these histologic and clinical findings provide circumstantial evidence that stromal keratitis is due to host immune–mediated inflammatory reactions. This notion is supported by experimental studies of guinea pigs and mice that show that prior sensitization to live microfilariae or soluble O. volvulus antigens (OvAg)1 exacerbates stromal keratitis after local injection with the same antigen.

1Abbreviations used in this paper: HPRT, hypoxanthine phosphoribosyltransferase; LNC, lymph node cell; mRNA, messenger RNA; OvAg, Onchocerca volvulus Ag; PPD, purified protein derivative; RT, reverse transcription.
(16–19). In the current investigation, we demonstrate that T cells and IL-4 are required for development of onchocercol stromal keratitis in mice.

Materials and Methods

**Animals.** Female BALB/c mice weighing 18–20 g were purchased from Charles River Laboratories (Wilmington, MA). T cell–deficient nude mice on a BALB/c (H-2b) background were obtained from the Ireland Cancer Center (Case Western Reserve University, Cleveland, OH). To maintain a relatively germ-free environment and to minimize eye irritation resulting from waste fumes, nude mice were housed in microisolators at three mice per cage. Cages were changed at least twice per week.

Mice in which the IL-4 gene had been disrupted were produced by insertion of a neor gene into the first exon of the IL-4 gene as described by Kopf et al. (20). These mice were derived from the F2 generation of 129Sv × C57Bl/6 breedings and termed IL-4−/− mice. Control mice for these experiments included: (a) age-matched wild-type (IL-4+/+) female mice from the same breedings; and (b) age-matched female C57Bl/6 mice (Charles River Laboratories). IL-4−/− and IL-4+/+ mice were sent to us by Dr. Ed Pearce (Cornell University, New York) with kind permission from Dr. Manfred Kopf (Basel Institute for Immunology, Basel, Switzerland) and were maintained as breeding pairs in the animal facilities at Case Western Reserve University. The IL-4−/− genotype was confirmed by amplification of genomic DNA using primers specific for the neor and IL-4 genes (20). All of the mice designated IL-4−/− had neor PCR products, whereas no neor PCR products were detected in genomic digests of IL-4−/− or C57Bl/6 mice.

**Ag Preparations.** OvAg was prepared from onchocercal nodules surgically excised from West African patients. To remove human tissue, nodules were digested with 0.4% collagenase (Boehringer Mannheim Biochemical Products, Indianapolis, IN) for 8 h at 37°C and washed repeatedly with PBS, pH 7.0 (18, 19). Worms were then homogenized on ice in a tissue grinder (Ten Broeck; Baxter Healthcare Corp., McGaw Park, IL), and the homogenate was centrifuged at 20,000 × g for 60 min. Supernatants containing soluble OvAg were passed through a 2-μm syringe filter (Amicon, Inc., Beverly, MA), and protein concentration of the supernatant was determined using the bicinchoninic acid assay (Pierce, Rockford, IL) with BSA as standard (Sigma Chemical Co., St. Louis, MO). OvAg concentration was adjusted to 1 mg/ml and stored at −70°C. Parasites and, in some experiments, soluble OvAg were kindly provided by Dr. David Freedman (University of Alabama, Birmingham, AL). *Mycobacterium tuberculosis* purified protein derivative (PPD) was obtained from Dr. Henry Boon (Case Western Reserve University).

**Immunization and Intrastromal Injection.** Mice were given three weekly subcutaneous immunizations with 10 μg OvAg or PPD in 100 μl HBSS (GIBCO BRL, Gaithersburg, MD) mixed with 100 μl adjuvant consisting of squalene, Tween 80, and pluronic acid (21, 22). 1 wk after the final immunization, corneas were scarified using a 30-gauge needle, and 10 μg antigen in 10 μl HBSS was injected into the corneal stroma using a 33-gauge needle attached to a gas-tight syringe (Hamilton Co., Reno, NV). Successful intrastromal injection was confirmed by the appearance of transient corneal clouding followed by reversion to the normal transparent state by the next day. This procedure was highly reproducible, and no more than 3 μl of fluid leaked from the wound. All procedures conformed with resolutions on the use of animals in research determined by the Association for Research in Vision and Ophthalmology.

**Clinical and Histological Evaluation.** Slit lamp microscopy was performed, and keratitis was graded for opacification and neovascularization in a masked fashion as described previously (18, 23, 24). Opacification is used in this context as the degree of corneal clouding based on visibility of iris detail, rather than the strict clinical sense of permanent scarring. Photographs were obtained with a vertical photo-slit lamp microscope (Marco Ophthalmic Inc., Jacksonville, FL) using Ektachrome film (160T; Eastman Kodak Co., Rochester, NY).

For histological examination, eyes were removed at various times after OvAg injection and fixed in 10% neutral buffered formalin. They were then embedded in paraffin, sectioned, and stained with hematoxylin and eosin using standard methods. Reproducibility was attained by aligning the eye such that the cornea was uppermost and sectioning through the center of the cornea and pupil. Eosinophils were identified by the characteristic horseshoe-shaped nuclei and eosin–staining granules.

**Lymph Node and Spleen Cell Preparations.** Cell suspensions from spleens or inguinal lymph nodes (LNC) were prepared by standard aseptic technique. Briefly, erythrocytes were lysed with 0.01 M Tris, pH 7.2, containing 0.75% ammonium chloride, and cells were washed three times in RPMI 1640 and suspended in complete medium (RPMI 1640 containing 1 mM sodium pyruvate, 2 mM l-glutamine, 20 mM Hepes, 100 U/ml penicillin, 100 μg/ml streptomycin, and 10% heat-inactivated FCS). Duplicate wells containing 5 × 10⁶ cells were stimulated with 10 μg/ml antigen in a final volume of 200 μl and incubated at 37°C in 5% CO₂ for 72 h.

**Cell Proliferation and Cytokine and IgE Assays.** After 72 h of incubation, 150 μl culture supernatant was removed for cytokine measurements, and cells were pulsed with 0.1 μCi [3H]thymidine (Amersham Corp., Arlington Heights, IL) for an additional 6 h before harvesting onto glass fiber filters (Cambridge Technology, Inc., Watertown, MA). Filters were dried and added to 5 ml scintillation fluid (EcoScint A; National Diagnostics, Inc., Manville, NJ), and the number of counts per minute was determined using a beta scintillation counter (Beckman Instruments, Fullerton, CA). Stimulation index was calculated as: cpm Ag stimulated/cpm unstimulated (22).

Measurements of IL-4, IL-5, and IFN-γ in culture supernatants were performed by two-site ELISA as described (22, 25) using the following mAbs: For IL-4, BVD6 was the coating and biotinylated BVD4 was the detecting mAb (obtained from Dr. F. Heinzel, Case Western Reserve University); for IL-5, TRFK5 was the trapping and TRFK4 was the detecting mAb (obtained from Dr. R. Coffman, DNAX, Palo Alto, CA); and for IFN-γ, R46A2 was the coating and XMG1.2 was the detecting mAb (obtained from American Type Culture Collection, Rockville, MD). Cytokines were measured after addition of streptavidin–peroxidase and α-phenylenediamine substrate (Sigma Chemical Co.), and absorbance was measured at 492 nm in a reader (Multi-skran Plus MKII; Flow Laboratories, Inc., McLean, VA).

Standards for the IL-4 and IL-5 ELISAs were generated in culture supernatants of Con A–stimulated spleen cells from control mice, and recombinant murine IFN-γ (Genzyme Corp., Cambridge, MA) was used as a standard in the IFN-γ assay. Total serum IgE was detected by two-site ELISA using rat anti–mouse IgE mAbs EM95 and BF815 as described (22, 25).

To deplete CD4 or CD8 cells, 2 × 10⁷ spleen cells were incubated with anti–murine CD4 (GK 1.5) or anti-CD8 (53-6.72) hybridoma supernatants for 45 min at 4°C. Cells were then
washed and incubated at 37°C for 45 min with rabbit complement (Pel-Freez Biologicals, Brown Deer, WI) diluted 1:3. Depletion of CD4+ or CD8+ cells with the corresponding mAb was >90% as determined by fluorescence cytometry. Fewer than 10% of cells were depleted when incubated with rabbit complement alone.

Adoptive Transfer of Spleen Cells to Nude Mice. Spleen cell suspensions from either OvAg-sensitized or naive euthymic BALB/c mice were prepared after lysis of red cells with 0.05 M Tris and 0.75% ammonium chloride as described above. 2 × 10^7 viable cells (determined by trypan blue exclusion) were injected intraperitoneally into nude recipients 1 h before intrastromal injection of 10 μg OvAg.

RNA Extraction from Mouse Corneas. Corneas were dissected from the eyes of five mice per group using fine-point scissors, taking care that no limbus or iris tissue was removed. Corneas were then pooled in 500 μl RNazol (Cinna/Biotecx, Friendswood, TX) and homogenized on ice using micro-tissue grinders (Baxter Healthcare Corp.). RNA was extracted in 10% chloroform, and the aqueous phase was washed once in isopropanol and once in 100% ethanol and suspended in 30 μl diethylpyrocarbonate H2O. The integrity of the RNA was determined by agarose gel electrophoresis and detection of 18 S and 28 S ribosomal RNA, and RNA concentration was estimated by OD260. A pool of five corneas routinely yielded 3-30 μg nucleic acid.

Reverse Transcription (RT)-PCR of Corneal RNA. RNA (1 μg) was incubated at 70°C for 5 min with 2 μl random hexamer primers (Boehringer Mannheim Corp.). RT reactions used 200 U reverse transcriptase with RT buffer (M-MuLV; GIBCO BRL) in 25 μl volume containing 25 mM MgCl₂, 0.1 M dithiothreitol, 2.5 mM dNTPs, and 1,000 U RNAsin (Promega, Madison, WI). The mixture was incubated at 42°C for 1 h, and enzyme was then inactivated by incubating at 95°C for 5 min. cDNA products were diluted 1:6 in sterile H2O.

cDNA was amplified using 1 U Taq DNA polymerase and 5 μl PCR buffer (X10; Promega), 25 mM MgCl₂, 2.5 mM dNTPs, and 10 μM sense and antisense oligonucleotide primers in a final volume of 50 μl. Cycle conditions using a thermal cycler (Omnigene; National Labnet Co., Woodbridge, NJ) were as follows: 1 min at 95°C to denature double-stranded DNA, 1 min at 60°C to anneal primers, and 1 min 45 s at 72°C for primer extension. The optimal cycle number was determined empirically for each set of primers using 1 μg RNA from anti-CD3-stimulated spleen cells (positive control). By plotting cycle number against densitometry measurements, an exponential curve was generated that reached a plateau at higher cycle numbers, thereby indicating saturation. The optimal cycle number was therefore defined as the number of cycles that gave a detectable signal for the positive control and was well below saturation level. Primer sequences and cycle numbers were as follows. Hypoxanthine phosphoribosyl-transferase (HPRT): sense, TGGGATACAGGCGAAGCTTTTGG; antisense, GATTCACCTGGCGCTCATCTTACGCC; internal, GTTGGTAGATGCCCTTGGAC (27 cycles). CD4: sense, TGTTGCGAGGATCCTCTCTTGGAGTTCTTCCAAAGG (33 cycles). CD8 primer sequences were obtained from CLONTECH (Palo Alto, CA); IL-4 antisense and internal sequences were as described by Svetic et al. (26); and other sequences were kindly provided by Dr. Fred Heinzel. All primers were synthesized by Operon Technologies, Inc. (Alameda, CA), and a positive sample (cDNA from anti-CD3–stimulated spleen cells) and a negative sample (distilled H₂O) were included in each experiment.

Southern Hybridization. 15 μl of the final reaction mix was run in a 1.5% agarose gel containing 0.1 μg/ml ethidium bromide for 30 min at 150 V. Products of the positive control were generally visible by UV light examination. Double-stranded DNA was denatured by soaking the gel in 0.5 M NaOH in 1.5 M NaCl for 1 h. NaOH was neutralized in 1 M Tris, pH 8.0, 1.5 M NaCl for 1 h, and PCR products were then transferred overnight to nitrocellulose (HyBond; Amersham Corp.) by capillary activity. Products were baked onto the membrane in a vacuum oven at 80°C for 2 h and then prehybridized for 1 h before addition of the FITC-deoxouridine triphosphate–labeled probe (enhanced chemiluminescence; Amersham Corp.). After overnight incubation, the membrane was blocked, incubated for 1 h with horseradish peroxidase–labeled anti-FITC Ab, washed, and treated with chemiluminescent developer according to the manufacturer's directions. Membranes were exposed to autoradiography film (NEF 496; DuPont, Wilmington, DE). Densitometry was performed using a Scitron densitometer (United States Biochemical Corp., Cleveland, OH), and the fold increase was determined by the ratio of the densitometry measurement for a defined area of PCR product for the experimental cornea divided by the same area for control cornea.

Statistics. Statistical analysis was performed using Student's t test, and a P value of <0.05 was considered to be significant.

### Result

Clinical and Histological Features of OvAg-mediated Keratitis. Eyes of unimmunized BALB/c mice observed 7 d after intrastromal injection of OvAg were similar to those of normal, untreated animals in which no corneal opacities or neovascularization were present (Fig. 1 A). In contrast, severe keratitis developed in mice that had been sensitized by immunization with OvAg before intrastromal injection. In the example shown in Fig. 1 B, a diffuse area of opacification is apparent, and neovascularization extends centrally from the limbus.

Corneas from unimmunized mice recovered 7 d after intrastromal injection of OvAg had histological features similar to those of normal animals with distinct epithelial, stromal, and endothelial layers (Fig. 1 C). Collagen lamellae in the stroma were arranged in the regular, parallel configuration of the normal cornea, and resident keratocytes and occasional mononuclear cells were the only cells observed. In contrast, corneas from mice that had been immunized before intrastromal injection of OvAg developed pronounced stromal inflammation characterized by edema, disruption of collagen lamellae, and a profuse cellular infiltrate that included eosinophils and mononuclear cells (Fig. 1 D). The time course of development of keratitis in five individual OvAg–immunized mice is shown in Fig. 2. Opacification progressed rapidly after intrastromal injection,
reached a peak at 7–10 d, and then gradually resolved. By day 21, corneas of most animals were transparent. Vascularization developed gradually, progressing over 10–14 d to the center of the cornea. Neovascularization persisted after opacification had resolved. Corneas from unimmunized mice developed mild opacification after intrastromal injection of OvAg (stromal disease scores <1.0), which resolved after 3 d. Neovascularization did not develop in unimmunized mice.

**Specificity of OvAg-induced Keratitis.** To determine whether corneal lesions in OvAg-immunized animals were specifically associated with this nematode antigen preparation, BALB/c mice were immunized and challenged intrastromally with an unrelated soluble microbial antigen preparation, mycobacterial PPD, using an identical protocol. 7 d after intrastromal injection, all of the mice treated with OvAg developed keratitis, with the intensity of reactions ranging from mild (1.0–2.0) to severe (>2.0) as determined by both opacification and neovascularization (Fig. 3 A). In contrast, 7 of 15 mice treated with PPD had no detectable keratitis, and the remaining eight mice had mild responses. Stromal keratitis scores were significantly higher for OvAg-treated mice than for PPD-treated mice (*P* <0.001 for both opacification and neovascularization).

**LNC Proliferative and Cytokine Responses.** To characterize the systemic Th cell response associated with stromal keratitis, LNC from OvAg- or PPD-immunized mice were incubated with homologous antigen, and cell proliferation and cytokine production were determined. Antigen-driven LNC proliferation was not significantly different between the two groups. The mean ± SD stimulation index was 8.6 ± 2.0 for OvAg- and 10.1 ± 2.3 for PPD-immunized mice (*P* = 0.30), indicating that the absence of a strong inflammatory response in PPD-immunized mice was not due to failure to sensitize the animals compared with OvAg-treated mice. Indeed, LNC from PPD-treated mice produced significantly more IFN-γ in response to antigen stimulation than did LNC from OvAg-treated mice (means ± SD of five animals per group were 12.2 ± 0.2 ng/ml and 0.6 ± 0.4 ng/ml, respectively, *P* <0.01) (Fig. 3 B). In contrast, LNC from OvAg-immunized mice produced 5.3 ± 1.3 ng/ml IL-4 and 4.6 ± 0.8 ng/ml IL-5, whereas PPD-treated
mice produced <0.5 ng/ml IL-4 and IL-5 (P < 0.01). These data demonstrate a qualitative difference in the Th cell cytokine responses of PPD- and OvAg-treated mice that is consistent with induction of predominantly Th1 and Th2 cells, respectively. Total serum IgE was elevated in OvAg-immunized mice compared with unimmunized mice (7.9 ± 2.1 vs 2.6 ± 1.0 µg/ml, P < 0.01). Immunization with PPD did not increase serum IgE.

Spleen cells from OvAg-treated mice had a cytokine profile similar to LNC, producing 4.9 ng/ml IL-4 and 3.2 ng/ml IL-5 but <0.5 ng/ml IFN-γ. In vitro depletion of CD4 or CD8 cells before OvAg stimulation demonstrated that production of these cytokines was dependent on CD4 cells (Fig. 4).

**T Cell Dependence of OvAg-mediated Keratitis.** To determine if stromal keratitis is T cell dependent, T cell-deficient nude mice were either immunized subcutaneously and injected intrastromally with OvAg as described above or were given spleen cells from OvAg-immunized or naive BALB/c mice before intrastromal injection. As shown in Fig. 5 (left), OvAg-immunized nude mice did not develop opacification or neovascularization after intrastromal injection. Nude mice that received cells from naive donors before intrastromal injection also failed to develop keratitis (Fig. 5, right, open circles). However, adoptive transfer of cells from immunized donors before intrastromal injection led to development of both opacification and neovascularization (Fig. 5, right, closed circles). The mean scores for recipients of cells from immunized donors were significantly higher than for the other groups (P < 0.001 for opacification and neovascularization). Histologically, corneal stromas of nude mice reconstituted with cells from immunized euthymic mice were edematous and contained numerous eosinophils and mononuclear cells, whereas stromas of the other groups had no signs of inflammation (data not shown).
Figure 6. T cell and cytokine gene expression in corneas from BALB/c and nude mice. (A) OvAg-immunized BALB/c mice were injected intrastromally in one eye with OvAg as described in Materials and Methods. Corneas from either uninjected (left lane) or injected (right lane) eyes were removed after 3 d, RNA was extracted from a pool of five corneas, and expression of CD4, CD8, IFN-γ, and IL-4 was determined by RT-PCR. Results are representative of five similar experiments. (B) Athymic nude mice were reconstituted with spleen cells from either naive (left lane) or immune (right lane) BALB/c mice before intrastromal injection of OvAg as described in the legend to Fig. 5. Injected corneas were removed 3 d later, and gene expression was determined by RT-PCR. Results are representative of three similar experiments.

T Cell and Cytokine Messenger RNA (mRNA) Expression in Corneas from BALB/c and Nude Mouse. To ascertain whether CD4 or CD8 cells were present at the site of the inflammatory response and whether Th-associated cytokines were produced, RNA was extracted from corneas of BALB/c and nude mice and reverse transcribed. The resulting cDNA was amplified by PCR and examined by Southern analysis. Equivalent amounts of cDNA were added to each well as determined by Southern hybridization for the housekeeping gene, HPRT (Fig. 6, A and B).

Consistent with the avascular nature of normal, uninflamed corneas, corneas extracted from unimmunized BALB/c mice had no mRNA expression for CD4, CD8, IFN-γ, or IL-4 (data not shown). In OvAg-immunized mice, corneas recovered 3 d after intrastromal injection of antigen had 15-fold elevated mRNA expression of CD4 and a fivefold increase in IL-4 expression compared with uninjected corneas from the same animals (Fig. 6 A). CD8 mRNA was detected in both uninjected and injected corneas (2.1-fold increase in injected corneas), and IFN-γ gene expression was low in both samples (Fig. 6 A). There were no significant differences in relative levels of CD4, CD8, IFN-γ, or IL-4 products between corneas examined 3 d after intrastromal injection and those recovered from mice at 7 or 10 d (data not shown).

Corneas were also recovered from nude mice that developed keratitis after adoptive transfer of spleen cells from immunized BALB/c mice and were compared with corneas from nude mice given naive donor cells. As shown in Fig. 6 B, OvAg-injected corneas from recipients of immune donor cells had markedly elevated CD4 mRNA (15-fold) and IL-4 mRNA (13-fold) compared with corneas from recipients of naive cells. CD8 gene expression was also elevated (eightfold), although IFN-γ mRNA levels were low and not elevated in reconstituted donors that developed keratitis.

IL-4 Requirement for Development of Onchocercal Keratitis. Because IL-4 gene expression was prominent in inflamed corneas during keratitis, we determined whether IL-4 was necessary for development of onchocercal keratitis. Mice in which the gene for IL-4 had been selectively disrupted (IL-4−/−) were immunized, injected with OvAg, and compared with age- and sex-matched wild-type IL-4+/+ mice on the same genetic background (129Sv × C57Bl/6). In some experiments, commercially available C57Bl/6 mice were also included.

IL-4+/+ mice, but not IL-4−− mice, that had been immunized and injected intrastromally developed severe keratitis. IL-4+/+ mice had corneal lesions similar to those of BALB/c mice, in which an area of opacification covered most of the cornea and neovascularization extended centrally from the limbus (Fig. 7 A). C57Bl/6 mice immunized and injected with OvAg developed severe keratitis that was identical to that of IL-4+/+ mice (data not shown). In contrast to both IL-4+/+ and C57Bl/6 mice, IL-4−− mice either failed to develop keratitis or had only mild...
transient opacification around the site of injection. In addition, there was no neovascularization present in corneas of IL-4−/− mice (Fig. 7 B). This observation was highly reproducible, and the difference in scores between IL-4+/+ and IL-4−/− mice was significant for >30 animals per group in four separate experiments (P <0.001, for opacification and neovascularization) (Fig. 8). Unimmunized IL-4+/+ and IL-4−/− mice did not develop keratitis after intrastromal injection of OvAg.

Histological examination of corneas from IL-4+/+ mice showed stromal edema, an infiltrate of eosinophils, mononuclear cells, and the presence of blood vessels, whereas corneas from IL-4−/− mice were not edematous and had only occasional inflammatory cells (data not shown).

Discussion

Transparency of the cornea is dependent on an orderly arrangement of collagen lamellae in the stroma that allows light to pass through undiffracted. Maintenance of this lamellar arrangement depends on a critical level of hydration in the stroma. During acute keratitis, the function of osmoregulatory pumps in the corneal endothelium is disrupted, resulting in stromal edema and alteration of the collagen lamellae (27). Corneal opacification, one of the clinical hallmarks of stromal keratitis, is caused by this change in the macromolecular arrangement of collagen and the resulting diffraction of light. Neovascularization, the other major feature of keratitis, is mediated by angiogenic factors produced by invading inflammatory cells and possibly by resident cells of the stroma, such as keratocytes. In the current study, we examined the immunopathogenesis of keratitis induced by sensitization to antigens of the filarial parasite O. volvulus, the cause of “river blindness” in humans.

We found that mice injected intrastromally with OvAg do not develop clinical or histopathologic changes in the cornea unless they are previously sensitized to the antigen. This requirement for repeated and long-term exposure to OvAg corresponds to the association between ocular disease and chronic infection in human onchocerciasis (8, 12). The pathologic changes in the corneas of mice, which include stromal edema, opacification, neovascularization, and infiltration by eosinophils and mononuclear cells, also resemble those of human disease (5, 9).

The specificity of keratitis induced by OvAg in this model was addressed by immunizing and injecting intrastromally mycobacterial PPD, a soluble antigen preparation from a nonhelminthic pathogen. As judged by the capacity to induce and recall antigen-specific proliferation by LNC, OvAg and PPD were equally effective immunogens. However, the duration and severity of clinical and pathologic reactions induced by PPD were significantly less than those mediated by OvAg. Comparison of the Th cell response elicited by each antigen preparation also showed a difference in the pattern of cytokine production. As we reported previously (28), multiple subcutaneous immunizations with PPD elicit CD4+ T cell responses indicative of a dominance of the Th1 subset; i.e., Ag-driven LNC produce IFN-γ but not IL-4 or IL-5. In contrast, immunization with OvAg induces a CD4 Th cell response that is predominantly Th2-like, with elevated IL-4 and IL-5 but relatively little IFN-γ. This pattern of in vitro cytokine production in OvAg-treated mice is consistent with observed in vivo responses, i.e., elevated serum IgE, which is IL-4 dependent (29, 30), and eosinophil production, which is IL-5 dependent (31, 32). The capacity of O. volvulus antigens to induce a Th2-mediated inflammatory response is not unique to this helminth, as several other parasite and nonparasite antigen preparations have been shown to induce Th2-mediated inflammation (25, 33, 34). It is more likely that the unique capability of O. volvulus parasites to mediate keratitis stems from an inherent predisposition to invade the human cornea, thereby causing an inflammatory reaction in chronically infected and hyperimmunized individuals.

Elevated production of IgE, eosinophils, and Th2-associated cytokines are characteristic features of human onchocerciasis (10–15), indicating that the murine model described in the current study reflects these aspects of human infection. In further support of an association between keratitis and Th2 responses, OvAg-specific Th2 recall responses were recently reported to be elevated in persons with onchocercal eye disease compared with infected individuals without ocular manifestations of infection (12, 35). Although these studies measured PBMC responses, one report examined cytokine gene expression in conjunctival tissue from onchocerciasis patients and concluded that IL-4 is the predominant cytokine expressed in this site (36). Consistent with this observation, we found that IL-4 gene expression was greatly elevated in corneas from mice with keratitis compared with uninfamed corneas. In contrast, IFN-γ gene expression remained low in all corneas tested.

The cellular source of IL-4 transcripts in corneas of mice with OvAg-mediated keratitis has not yet been determined. IL-4 production by OvAg-stimulated spleen cells was CD4 cell dependent, and CD4 transcripts were consis-
tently elevated in corneas isolated from mice with keratitis, indicating that CD4 cells are the likely source of IL-4. However, as CD8 gene expression is observed in inflamed corneas and CD8 cells stimulated in the presence of IL-4 produce Th2 cytokines (37–39), it is possible that CD8 cells contribute to IL-4 production in the current study. In addition, IL-4 is produced by FcεRI+ cells (40–42). In situ immunohistochemical analysis will be necessary to determine the cellular source of IL-4 production at the site of inflammation.

Detection of CD8 transcripts in inflamed corneas of OvAg-treated mice in the current study is in contrast to a previous immunohistologic study, which showed that CD4 and not CD8 cells infiltrate corneas of OvAg-immunized A/J mice (43). The discrepancy between results of this earlier study and the data presented here may be related to differences in CD8 responses determined by the genetic backgrounds of the different strains of mice, mode of immunization (single vs multiple subcutaneous injections), or relative sensitivity of the methods to detect CD8 cells.

As Th2 cells and IL-4 are associated with onchocercal keratitis in both human infection and the murine model presented here, we determined if OvAg-specific T cells and production of IL-4 are required for development of keratitis. The role of T cells was examined using nude mice that do not have functional T lymphocytes and do not develop cellular responses to filarial antigens (44, 45). Previous studies demonstrated that nude mice challenged either with lymphatic filariae (44–46) or injected intravenously with Schistosoma mansoni eggs (34) develop smaller inflammatory reactions and granulomas compared with euthymic mice, indicating that these types of helminth-induced pathology are T cell dependent. In the current study, we observed that sensitized T cells are also required for development of onchocercal keratitis. First, nude mice fail to develop keratitis after repeated subcutaneous immunizations and intrastromal challenge with OvAg. Second, adoptive transfer of OvAg-sensitized spleen cells from euthymic BALB/c mice to nude mice before intrastromal injection results in development of keratitis. Adoptive transfer experiments using OvAg-specific T cell clones will be necessary to determine unequivocally the relative contributions of CD4 and CD8 cells in mediating onchocercal keratitis. Similar studies have been used to determine the role of T cell subsets in development of murine keratitis induced by Herpes simplex virus (24).

To determine if IL-4 is required for development of keratitis in the murine model, mice in which the IL-4 gene was genetically disrupted were studied. These mice are deficient in their ability to produce Th2 cytokines after infection with the nematode Nippostrongylus brasiliensis (20) and have reduced mucosal B cell responses to orally administered antigens (47). In the current study, we demonstrated that IL-4−/− animals either fail to develop OvAg-mediated keratitis or exhibit only mild corneal disease. In contrast, IL-4+/+ and C57Bl/6 mice develop severe corneal opacification and neovascularization after immunization and intrastromal challenge with OvAg.

The mechanism by which IL-4 mediates development of OvAg-induced keratitis is not yet known. IL-4 has pleiotropic effects on diverse cell types, including development of IL-4-secreting CD4 and CD8 cells (37, 39, 48, 49) and B cell isotype switching to IgE (30, 50). In addition, IL-4 increases surface expression of MHC class II and CD23 on B cells (51, 52) and expression of vascular cell adhesion molecule 1 on human vascular endothelial cells (53–55). In this context, IL-4 enhances binding and transmigration of eosinophils by a very late antigen 4–dependent mechanism (56, 57). It is therefore possible that an important role for IL-4 in keratitis is to up-regulate expression of adhesion molecules on the surface of vascular and endothelial cells in the limbus, thereby facilitating migration of eosinophils into the corneal stroma. Release of toxic eosinophil granule proteins (58, 59) may then disrupt the structural integrity of the cornea. Eosinophils have been reported to surround dead and dying O. volvulus microfilariae in the skin of infected individuals and to deposit major basic protein on the parasite surface (60). Although the role of eosinophil-derived or other inflammatory cell products in the pathogenesis of onchocercal keratitis is yet to be determined, demonstration of the requirement for IL-4 suggests that immunological interventions that down-regulate production of this cytokine, such as treatment with IL-12 (61), may ameliorate active disease or be useful in the design of antipathology vaccines.
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