Immunoregulation in Onchocerciasis: Persons with Ocular Inflammatory Disease Produce a Th2-like Response to Onchocerca volvulus Antigen

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To examine the role of specific cytokines in mediating the clinical manifestations of human onchocercal disease, microfilariae-positive Ghanaian subjects with inflammatory ocular disease were compared with microfilariae-positive subjects without ocular disease. Onchocerca volvulus antigen (OvAg)-stimulated peripheral blood mononuclear cells (PBMC) from subjects with disease produced significantly more interleukin (IL)-10 (with disease = 447.34 vs. without disease = 292.22 pg/mL; \( P < .01 \)) and IL-5 (with disease = 33.36 vs. without disease = 27.26 pg/mL; \( P = .02 \)). OvAg-stimulated IL-4 and interferon (IFN)-\( \gamma \) levels were essentially undetectable in either group. When cytokine mRNA levels were measured by reverse transcriptase–polymerase chain reaction ELISA, persons with disease produced significantly more OvAg-stimulated IL-4, IL-5, and IL-10 mRNA (\( P = .03, <.01, .05 \), respectively). No difference in IFN-\( \gamma \) mRNA production by either group was seen. Addition of neutralizing antibody to OvAg-stimulated PBMC increased IFN-\( \gamma \) production to detectable levels in 20 of 24 persons.

Onchocerca volvulus infects ~20 million people in Africa and Latin America; 2% of infected persons have onchocercal-induced blindness [1]. Sclerosing keratitis, the leading cause of blindness in onchocerciasis, is thought to be due to an immunologically mediated reaction to intraocular microfilariae [2–8]. In experimental onchocercal keratitis induced in guinea pigs or mice, corneal damage has been shown to be due to a parasite antigen–specific CD4\( ^+ \) infiltrate, which is accompanied by a local eosinophilic response [9]. A recent study in a murine model demonstrated corneal localization of interleukin (IL)-4 but not interferon (IFN)-\( \gamma \) mRNA and an inability of IL-4 knockout mice to develop keratitis, indicating the necessity for a Th2-like response in the development of onchocercal keratitis [12].

The nature of parasite antigen–induced cytokine responses in O. volvulus–infected humans with sclerosing keratitis has not been characterized. Moreover, in a recent comprehensive review of the immunopathogenesis of human onchocerciasis, no published studies examined the profile of cytokine expression in relation to any of the pathologic manifestations of O. volvulus infection [8]. Available limited cytokine data are restricted to overall findings in groups of infected microfilaremic persons and are not stratified in any way as to the presence or absence of clinical manifestations of disease. In these few studies, however, IL-4, IL-5, and IL-10 (IL-10 in one study) production was demonstrated in O. volvulus antigen (OvAg)–stimulated PBMC from some of these unclassified persons [13–20].

To specifically investigate a role for a Th2-like response in the pathogenesis of human ocular onchocerciasis, we compared parasite antigen–specific cytokine production by peripheral blood mononuclear cells (PBMC) from 2 groups of persons having both dermal and intraocular microfilariae; sclerosing keratitis was present in 1 group (with ocular disease); no manifestation of inflammatory intraocular disease was present in the other group (without ocular disease). In addition, the importance of the key regulatory cytokine IL-10 was investigated using neutralizing antibody.

Despite earlier hopes and ongoing concerted efforts for the widespread distribution of the microfilaricidal agent ivermectin [21], onchocerciasis is not likely to be eradicated in the near future. A better understanding of immunologically relevant Onchocerca-specific responses will be necessary before alternative interventions such as vaccines or immunotherapy can be developed.

Materials and Methods

Study population. Twenty-five previously untreated subjects were recruited from two Onchocerca-hyperendemic rain forest villages, Sabram and Kpomkpa, in the Volta region of Ghana. For each subject, parasitologic and clinical examination included clinical history, quantitation of microfilarial density in 7 separate skin snips [22], and a complete ophthalmologic assessment, including slit lamp examination by a certified ophthalmologist [23].

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All subjects gave written informed consent according to human experimentation guidelines of the US Department of Health and Human Services. The protocol was approved by the Institutional Review Board of the University of Alabama at Birmingham.

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were classified into 2 groups: with disease (presence of both dermal and ocular microfilariae, accompanied by sclerosing keratitis or uveitis, or both) and without disease (presence of both dermal and ocular microfilariae with no evidence of sclerosing keratitis, uveitis, iritis, or chorioretinal changes of onchocerciasis). All study subjects received ivermectin therapy after participation in the study.

**Stimulation of PBMC by parasite antigen.** PBMC were isolated by Ficoll-Paque (Pharmacia, Piscataway, NJ) density gradient centrifugation and cultured at 2 x 10⁶/mL in RPMI 1640 (BioWhittaker, Walkersville, MD) with 10% heat-inactivated fetal calf serum (HyClone, Logan, UT), 2 mM 1-glutamine, 50 U/mL penicillin, and 50 µg/mL streptomycin in 48-well tissue-culture plates at 37°C, 5% CO₂, with or without 10 µg/mL OvAg.

OvAg was prepared as a crude saline extract from adult worms [15], 0.02 units/mL streptolysin-O (SL-O) reagent (Difco, Detroit), or 10 µg/mL phytohemagglutinin (PHA; Life Technologies, Norwalk, CT), and cDNA. The quantity of cDNA used in each PCR reaction contained 0.25 µM dNTP mix, 1X PCR buffer (50 mM TRIS-HCl, 1.5 mM MgCl₂), 0.2 µM sense and antisense primers, 1 U of Taq polymerase (Perkin-Elmer Cetus, Norwalk, CT), and cDNA. The quantity of cDNA used in each cytokine PCR of a patient sample was determined from the amount of specific cytokine mRNA (determined in initial experiments) was amplified, and their PCR products were used to generate a standard curve with arbitrary units assigned to each dilution of the standard. The amount of specific cytokine mRNA for the unknown samples on a plate was interpolated from the linear portion of their respective curves and expressed as cytokine or HPRT ELISA units. The amount of cytokine mRNA for each sample is expressed as the ratio of cytokine ELISA units to HPRT ELISA units for the same sample.

**Statistical analysis.** Differences between the study groups were analyzed using the Mann-Whitney U test. Effects of αIL-10 treatment were analyzed using the Wilcoxon signed rank test. Probability values <.05 were considered significant. All values are expressed as geometric means except as noted.

**Results**

**Study population.** The demographic, clinical, and parasitologic data for the study population are shown in table 1. Persons with disease had an average age of 30.8 years; those without disease were older (average age, 46.3). When averaged over the 7 skin snips/patient, persons with disease had a mean dermal microfilarial density of 62.6/mg of skin versus 62.2/mg for persons without disease, indicating an equivalent microfilarial burden in each group. No difference in mean microfilarial densities of the study subjects by village of residence was found (data not shown). None of the study subjects had received previous chemotherapy for onchocerciasis.

**Patterns of cytokine responses in the 2 patient groups.** In mice, experimental sclerosing keratitis has been shown to be dependent on a Th2-like response to OvAg [12]. To determine a possible pathophysiologic role for differential cytokine production in persons with or without ocular disease, gross levels
Table 1. Characteristics of *O. volvulus*-infected study subjects.

<table>
<thead>
<tr>
<th>Group (n)</th>
<th>Village</th>
<th>Age</th>
<th>Dermal microfilariae/mg skin</th>
<th>Ocular findings*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Sabram</td>
<td></td>
<td></td>
<td>Sclerosing keratitis</td>
</tr>
<tr>
<td>Without ocular disease (10)</td>
<td>5</td>
<td>5</td>
<td>30.8 (18–50)</td>
<td>62.6 (30.2–101.2)</td>
</tr>
<tr>
<td>With ocular disease (15)</td>
<td>10</td>
<td>5</td>
<td>46.3 (22–65)</td>
<td>62.2 (9.6–158.8)</td>
</tr>
</tbody>
</table>

* Values indicate no. of subjects with specific clinical manifestation.

1 Arithmetic mean (range) in years.

2 Geometric mean (range).

of IFN-γ, IL-4, IL-5, and IL-10 were measured by ELISA in supernatants from 24- or 48-h OvAg-stimulated PBMC cultures (figure 1). Compared with the subjects without ocular disease, those with ocular disease produced significantly more IL-10 (with ocular disease, 447.34 pg/mL; without ocular disease, 292.22 pg/mL; *P* < .01) and IL-5 (with ocular disease, 33.36 pg/mL; without ocular disease, 27.26 pg/mL; *P* = .02). Nine of 10 persons with ocular disease and 11 of 15 without ocular disease had undetectable levels of IL-4 in supernatants from OvAg-stimulated PBMC; 8 of 10 persons without ocular disease and all 15 with ocular disease had undetectable levels of IFN-γ.

Cytokine mRNA in OvAg-stimulated PBMC. Because IFN-γ and IL-4 from OvAg-stimulated PBMC are characteristically difficult to detect in supernatants [15, 18, 20], specific cytokine mRNA from PBMC from the same culture wells as above was measured by RT-PCR ELISA to be able to determine differential cytokine expression in PBMC from the 2 groups (figure 2). As with the cytokine levels in the supernatants, IL-5 mRNA levels were significantly higher (*P* < .01) in persons with disease (IL-5 to HPRT ratio = 1.22) than in those without disease (IL-5 to HPRT ratio = 0.37). IL-10 mRNA levels were also higher (*P* = .05) in persons with disease (IL-10 to HPRT ratio = 0.24) than in those without disease (IL-10 to HPRT...
Figure 2. Cytokine mRNA production in *O. volvulus* antigen (OvAg)-stimulated peripheral blood mononuclear cells (PBMC). Patient PBMC were stimulated with OvAg for 24 h, and total RNA was extracted, reverse-transcribed, and amplified by polymerase chain reaction (PCR). PCR products were measured using PCR-ELISA and expressed as ratio of cytokine ELISA units to hypoxanthine–guanine–phosphoribosyl transferase (HPRT) ELISA units for same sample. Limits of detection of each assay are denoted with shading; horizontal bars denote geometric means. Differences between study groups were analyzed using Mann-Whitney *U* test. Persons with ocular disease (Dis+) produced more interleukin (IL)-4 (*P* = .03 [C]), IL-5 (*P* < .01 [D]), and IL-10 (*P* = .05 [B]) mRNA than did persons without ocular disease (Dis-). No significant difference was seen in production of interferon (IFN)-γ mRNA (A) by 2 patient groups.

dis+ = 0.19). Of 25 study subjects, 24 had detectable levels of IL-4 mRNA, with significantly greater production (*P* = .03) in subjects with disease (IL-4 to HPRT ratio = 1.67) than in persons without disease (IL-4 to HPRT ratio = 0.59). In contrast to the differences between the 2 patient groups in production of mRNA for the Th2 cytokines, no significant difference in production of mRNA for the Th1 cytokine IFN-γ was detected between the groups (without disease, IFN-γ to HPRT ratio = 0.12; with disease, IFN-γ to HPRT ratio = 0.13). Also, no differences in production of mRNA for either IL-4 or IFN-γ were detected between the 2 groups when PBMC were stimulated with nonparasite antigen (SL-O) or mitogen (PHA) (table 2).

**IL-10 suppresses OvAg-stimulated production of IFN-γ.** IFN-γ levels in OvAg-stimulated PBMC supernatants were undetectable in 23 of 25 samples. Since IL-10 is known to suppress IFN-γ production in ongoing Th2 responses [28], neutralizing MAb to IL-10 was added to OvAg-stimulated patient PBMC to determine if the defect in IFN-γ production in those cells was being modulated by IL-10. Figure 3 shows IFN-γ production from OvAg-stimulated PBMC run in side-by-side wells, with or without αIL-10 treatment. Addition of αIL-10 resulted in an increase of OvAg-stimulated IFN-γ production in both patient groups. In persons with disease, addition of αIL-10 resulted in detectable IFN-γ production in 10 of 14 persons (overall mean, 36.55 pg/mL) compared with none of 14 initially detectable with OvAg only. In subjects without disease, 10 of 10 had detectable IFN-γ levels after addition of αIL-10 (58.08 pg/mL) compared with only 2 of 10 initially. Control wells with neutralizing MAb to IL-4 (no IL-4 was present in any supernatants) did not

<table>
<thead>
<tr>
<th>Stimulant</th>
<th>Cytokine</th>
<th>Without ocular disease (n = 5)</th>
<th>With ocular disease (n = 6)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PHA</td>
<td>IFN-γ</td>
<td>0.34 ± 0.43</td>
<td>0.32 ± 0.20</td>
</tr>
<tr>
<td></td>
<td>IL-4</td>
<td>0.35 ± 0.40</td>
<td>0.40 ± 0.59</td>
</tr>
<tr>
<td>SL-O</td>
<td>IFN-γ</td>
<td>1.21 ± 1.18</td>
<td>1.49 ± 1.08</td>
</tr>
<tr>
<td></td>
<td>IL-4</td>
<td>1.12 ± 0.77</td>
<td>0.86 ± 0.95</td>
</tr>
</tbody>
</table>

NOTE. Data are arithmetic mean ± SD of cytokine/hypoxanthine–guanine–phosphoribosyl transferase ratio. PHA, phytohemagglutinin; IFN, interferon; IL, interleukin; SL-O, streptolysin O. *P* was not significant by Mann-Whitney *U* test between subjects with or without disease.
show any increase in IFN-γ mRNA production (data not shown).

Discussion

The pathogenesis of sclerosing keratitis, the major cause of blindness resulting from human infection with O. volvulus, is thought to be immunologically mediated. Direct evidence for this exists in rabbit, guinea pig, and mouse models of onchocercal keratitis. In these models, the predominantly lymphocytic inflammatory ocular reaction is parasite antigen–specific [9, 10, 29] and CD4+–dependent [11] and manifests only in animals presensitized with parasite antigen [10, 29]. In a recent study, murine onchocercal keratitis has been shown to be Th2 (IL-4)–dependent [12]. Supporting data included corneal localization of IL-4 (but not IFN-γ) mRNA in both intact immunized mice and in nude mice reconstituted with immune splenocytes and a failure to develop keratitis in IL-4 knockout mice.

Corroborating studies on human tissue have not been done to date due to the unavailability of corneal tissue from infected persons. Data on local ocular immune responses in human onchocerciasis are limited, and only conjunctival and iris, but not corneal, tissue has been examined [4, 6]. A single study, however, indicates a predominance of IL-4 (vs. IL-2)–producing T cells among lymphocyte infiltrates in conjunctival biopsies from persons with ocular onchocerciasis.

Because of the unavailability of human tissue data, investigation of the immunopathogenesis of O. volvulus infection has focused on systemic immune responses using mostly mitogen and, in a few cases, antigen-stimulated PBMC cultures [13–20]. When O. volvulus–infected persons have been compared with exposed but infection-free “putatively immune” [30] persons, an association of Th1 cytokine production with protection has been demonstrated [20]. Only minimal investigation of the logical corollary that a Th2 response is associated with onchocercal pathology in humans has been conducted. Limited studies examining infected microfiladermic persons who were unstratified with respect to any pathologic manifestations of infection have indicated that in some of these unclassified patients, antigen-stimulated PBMC produce Th2 cytokines, including IL-4, IL-5, or IL-10 [13–20]. These data, together with the results from animal studies, suggest an association between Th2 cytokine production and pathologic manifestations of human onchocerciasis. However, no rigorous data from human studies exist correlating a Th2 cytokine pattern with any specific clinical manifestation of onchocercal disease.

Therefore, the purpose of our study was to examine whether there is a relationship between O. volvulus–induced corneal pathology and the production of Th2 cytokines in humans. We compared OvAg-stimulated cytokine production in PBMC from 2 groups of subjects, each with ocular microfilariae but differing as to the presence or absence of onchocercal corneal pathology (with disease and without disease, respectively). Neutralizing antibody to IL-10 was then used to examine the role of the regulatory cytokine IL-10 in mediating the OvAg-stimulated response.

When supernatants from OvAg-stimulated PBMC were examined, subjects with disease had significantly higher levels of IL-5 and IL-10 than did those without disease. Because OvAg-stimulated IL-4 and IFN-γ are characteristically produced by PBMC from O. volvulus–infected persons in amounts difficult to detect in supernatants (figure 1) [18, 20], much of the onchocerca data in the literature is based on mitogen-stimulated production of these cytokines. To more directly assess the issue of OvAg-induced IFN-γ and IL-4 production, we stimulated PBMC with OvAg but examined cytokine mRNA production by RT-PCR ELISA. In this manner, we detected significantly greater amounts of IL-4, IL-5, and IL-10 mRNA in subjects with disease than in those without disease, but only...
very low levels of IFN-γ mRNA were detected in either patient group. Taken together, these data suggest a Th2-like cytokine response in persons with disease but not in those without disease.

A relationship between strong Th2 responses and immunopathologic manifestations of other infections with other helminths, such as Schistosoma mansoni and Nippostrongylus brasiliensis [25, 31–33], are well described. In murine schistosomiasis, Th2 responses to egg deposition and the associated suppression of Th1 responses correlate with increased IL-10 production [34], and IL-4-producing Th2 cells are important for egg granuloma formation [25]. In human schistosomiasis, egg antigens induce elevated IL-4 (but not IFN-γ) mRNA levels in PBMC from patients with hepatosplenic manifestations of schistosome-induced pathology [32].

The subjects with disease were significantly older than those without disease, which may or may not reflect increased exposure to the vector. Nevertheless, the data suggest that a Th2 cytokine profile would still be associated with development of ocular pathology regardless of the extrinsic predisposing factors.

We next investigated the role of the regulatory cytokine IL-10 in favoring the Th2 pattern seen in patients with ocular disease. A major function of IL-10, which was found in high levels in our patients, is the suppression of both IFN-γ production and Th1 responses in general [28, 35–37]. In the present study, addition of αIL-10 to OvAg-stimulated PBMC from persons with or without disease significantly increased the amount of IFN-γ detected in culture supernatants in both groups. This finding suggests a role for IL-10 in maintaining the minimal levels of OvAg-stimulated IFN-γ characteristically seen in almost all patients with generalized onchocerciasis. Our results are consistent with findings in animal models of S. mansoni and Brugia malayi infection, in which addition of αIL-10 to antigen-stimulated PBMC from infected mice resulted in enhanced IFN-γ production [34, 38]. Furthermore, in humans infected with Wuchereria bancrofti, addition of αIL-10 to antigen-stimulated PBMC resulted in marked augmentation of proliferation, though IFN-γ levels were not specifically measured [39].

In summary, onchocerciasis patients with sclerosing keratitis have a stronger Th2-like response (in OvAg-stimulated PBMC) than do equally infected persons without inflammatory eye disease. The results of this study of human onchocercal keratitis agree with those of studies of the murine model of onchocercal keratitis. While systemic immune responses are not necessarily reflective of local immune responses such as would be seen in the eye, parallel responses in the two compartments were clearly demonstrated in mice. In the murine models of onchocercal keratitis, OvAg-stimulated lymph node and spleen cells produced IL-4 and IL-5, but no IFN-γ, in a manner that mirrored corneal findings [12]. The relative contribution of CD4+ and CD8+ T cells in this Th2-like response is currently being investigated.

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