Absence of Cellular Responses to a Putative Autoantigen in Onchocercal Chorioretinopathy

Cellular Autoimmunity in Onchocercal Chorioretinopathy

Philip J. Cooper,*†  Ronald H. Guderian,*  Roberto Proano,*  and David W. Taylor†

Purpose. Onchocerciasis is a major cause of blindness in the developing world. An autoimmune pathogenesis for onchocercal chorioretinopathy was proposed after the identification of a recombinant Onchocerca volvulus antigen (designated Ov39) demonstrated immunologic cross-reactivity with a component of the retinal pigment epithelium and other ocular tissues. The aim of this study was to determine whether patients with onchocercal chorioretinopathy have enhanced lymphoproliferative responses to Ov39 compared to those without chorioretinal disease.

Methods. Lymphocyte blastogenic assays were performed using peripheral blood mononuclear cells (PBMCs) from patients with and without evidence of chorioretinopathy. PBMCs were cultured with Ov39, and supernatant fluids from Ov39-stimulated PBMCs were used to determine levels of the cytokines, interferon-γ, and interleukin-5.

Results. Lymphoproliferative responses to Ov39 were not enhanced in patients with onchocercal chorioretinopathy compared to those without clinical evidence of chorioretinal disease.


Onchocerciasis is a major cause of blindness in parts of tropical Africa and Central and South America. The parasite, Onchocerca volvulus, is a filarial nematode transmitted by black flies of the genus Simulium, and it infects an estimated 18 million people worldwide, of which approximately 350,000 are blind and a similar number are visually impaired.1

Blinding lesions may affect the anterior and posterior segments and cause sclerosing keratitis, iridocyclitis, optic atrophy, and chorioretinopathy.2 Posterior segment lesions, particularly chorioretinopathy, account for most visual loss in this disease.3-5

The pathogenesis of chorioretinopathy is poorly understood. Possible mechanisms include inflammatory reactions to dead microfilariae,6,7 eosinophil-derived toxic effector molecules,8,9 immune complex disease,10 toxic secretory-excretory products of microfilariae,11,12 and autoimmunity.13-16

Numerous observations lend support for an autoimmune role in the pathogenesis of chorioretinopathy in onchocerciasis. First, the epidemiologic relationship between chorioretinopathy and ocular and dermal microfilarial burdens is equivocal,17-20 unlike other ocular lesions for which clear associations have been demonstrated.17-21 Second, chorioretinopathy, unlike other ocular conditions, tends to deteriorate despite effective reductions in microfilarial burdens by chemotherapy or vector control.3,22-28 Further studies have demonstrated the presence of antiretinal autoantibodies in human infection sera, though only two of these were able to demonstrate a relationship between the presence of antiretinal antibodies and chorioretinopathy.13,15

A mechanism by which infection with O. volvulus might lead to the induction of autoimmunity is molecular mimicry. It has been suggested that foreign antigens that share antigenic determinants with host mole-
MATERIALS AND METHODS

Patient and Control Groups

Patients were recruited from a hyperendemic area in the rain forest focus of onchocerciasis in the Santiago River Basin of Esmeraldas Province in Ecuador. The communities living in this area included both indigenous Amerindian (Chachi tribe) and Ecuadorians of African descent (Afro-Ecuadorians). Ethnographic features of these communities have been described elsewhere. Patients were selected from 35 villages. Patients were brought to a central examination area for bleeding and ocular examination.

One hundred seventeen persons infected with *O. volvulus* were recruited (INF group). Selection was based on data compiled as part of a community-based study of the efficacy of the macrofilaricidal drug amoxicarzine. Detailed parasitologic and ophthalmologic information was available for each patient for a period of at least 3 years before the start of this study. All had received 1 to 3 annual doses of oral amoxcarzine, with the last dose administered at least 6 months before bleeding. Patients who were parasitologically positive were selected on the basis of the presence or absence of chorioretinopathy.

In addition, 36 controls not resident in the onchocerciasis focus were selected. They were comprised of two groups: Quito controls (group Q, *n* = 12; all worked at the Hospital Vozandes in Quito and had never visited an endemic area) and San Lorenzo controls (group SL, *n* = 24; all inhabited a small town in Esmeraldas Province where there was no transmission of onchocerciasis). Skin snips were taken from both iliac crests of each individual to confirm the absence of infection. This study followed the tenets of the Declaration of Helsinki, and informed consent was obtained from all participants.

Ocular Examination

Slit lamp examination of the anterior segment and measurement of intraocular pressures was performed as previously described. After pupil dilatation with 1% tropicamide and 10% phenylephrine, the ocular fundus was examined with a Keeler (Windsor, UK) indirect ophthalmoscope. The optic disc was examined for the presence of atrophy, papillitis, increased pigmentation, and sheathing of the central retinal vessels. Abnormalities of the retina and choroid were noted. Particular attention was paid to the presence of mottling or confluent atrophy of the retinal pigment epithelium and chorioretinal scarring with retinal pigment epithelial atrophy, pigment clumping, and/or choriocapillary atrophy.

Antigen Preparation

Female *O. volvulus* adult worms and bovine neuroretinas, obtained from a local abattoir, were ground to powder in liquid nitrogen and resuspended in phosphate-buffered saline. After centrifugation at 55,000*g* for 20 minutes, the supernatants were passed through a 0.2-µm filter, and protein concentrations determined using a commercial kit (Pierce, Rockford, IL). Aliquots of antigens were stored in liquid nitrogen and used in peripheral blood mononuclear cell (PBMC) cultures at concentrations of 10 and 50 µg/ml for *O. volvulus* antigen (Ovag) and bovine retinal extract, respectively.

Isolation of the recombinant antigen Ov39 has already been described. Recombinant clones were constructed in the expression vector pTrcHISB (Invitrogen Corporation, San Diego, CA) and transformed into *Escherichia coli* NM552. Expression and affinity purification of the fusion protein were performed according to the manufacturer’s instructions. Aliquots of purified Ov39 were stored in liquid nitrogen and used in PBMC cultures in a range of concentrations from 0.2 to 200 µg/ml. Concanavalin A (Sigma, Poole, UK) and *Candida albicans* (Allergon, Stockholm, Sweden) were used at concentrations of 5 and 10 µg/ml, respectively.

Lymphocyte Proliferation and Cytokine Assays

Fifty milliliters of venous blood was drawn from the antecubital fossa into heparinized syringes. Peripheral blood mononuclear cells were isolated by centrifugation on lymphoprep (Pharmacia, Uppsala, Sweden). Cells were washed twice with Iscove’s modified Dulbecco’s culture medium (IMDM) and plated onto 96-well, flat-bottomed tissue culture plates (Nunc, Roskilde, Denmark) at a density of 2 × 10⁵ cells/ml in a volume of 100 µl of IMDM culture medium (supplemented with 10% heat-inactivated human serum from nontransfused white male donors and 2% penicillin–streptomycin (Flow, Paisley, UK)). A further 100 µl of medium containing antigen was added. Plates were incubated at 37°C and 5% CO₂ for 3 days (for mitogen) or 6 days (for antigen). After this time, the cultures were pulsed with 0.5 µCi per well of tritiated...
Onchocercal Chorioretinopathy

TABLE 1. Age, Sex, and Race Characteristics of Study Groups

<table>
<thead>
<tr>
<th>Group</th>
<th>Age (years) [median (range)]</th>
<th>Sex</th>
<th>Race</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Male</td>
<td>Female</td>
</tr>
<tr>
<td>Q (n = 12)</td>
<td>35 (23–58)</td>
<td>6</td>
<td>6</td>
</tr>
<tr>
<td>SL (n = 24)</td>
<td>28 (18–60)</td>
<td>12</td>
<td>12</td>
</tr>
<tr>
<td>INF (n = 117)</td>
<td>41 (15–82)</td>
<td>66</td>
<td>51</td>
</tr>
</tbody>
</table>

Q = Quito controls; SL = San Lorenzo controls; and INF = infected with Onchocerca volvulus.

Lymphoproliferative Responses to Mitogen, C. albicans, and Ovag
Blastogenic responses to mitogen (concanavalin A), Candida albicans, and Ovag are shown in Table 3. Significant responses to mitogen, nonparasite (C. albicans), and crude adult O. volvulus antigens were seen in all groups.

Lymphoproliferative Responses to Ov39
Blastogenic responses to Ov39 in each group are shown in Table 3. There was significant intergroup variation in responses (P < 0.001). There was no difference between the proliferation of the two control groups. Responses of the two control groups combined were greater than those in all infected patients (P < 0.001). There was no evidence of antigen recognition in the INF group overall (SI = 1.1). A similar known values were interpolated from standard curves prepared using recombinant standards.

Statistical Analysis
Skin infection intensities are expressed as the geometric mean number of microfilariae per milligram of skin. Comparison of proportions were analyzed using $\chi^2$ and of means using Student's t-test. Comparison of means of more than two independent groups was performed using one-way analysis of variance. Multiple comparisons of group differences revealed by analysis of variance were tested using a modified Student's t-test with the Bonferroni adjustment. Bivariate analysis was performed by calculation of Spearman’s rank correlation coefficients.

RESULTS
Characteristics of Patient Groups
Age, sex, and race distributions of the three study groups are shown in Table 1. In group INF, the median ages of those with and without chorioretinopathy were 44 and 40 years, respectively. Geometric mean infection intensity of the INF group was 6.3 mf/mg. Chorioretinal disease status of subjects in the three groups is shown in Table 2.

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TABLE 3. Peripheral Blood Mononuclear Cell Responses

<table>
<thead>
<tr>
<th>Group</th>
<th>ConA</th>
<th>C. Albicans</th>
<th>Ovag</th>
<th>OV39</th>
<th>Retina</th>
</tr>
</thead>
<tbody>
<tr>
<td>Q</td>
<td>65 (42–99)</td>
<td>7 (4–14)</td>
<td>4 (2–7)</td>
<td>2.6 (1.6–4.2)</td>
<td>1.7 (1.2–2.4)</td>
</tr>
<tr>
<td>SL</td>
<td>70 (45–109)</td>
<td>20 (14–28)</td>
<td>11 (7–17)</td>
<td>1.9 (1.4–2.6)</td>
<td>2.0 (1.6–2.4)</td>
</tr>
<tr>
<td>INF</td>
<td>62 (53–73)</td>
<td>12 (8–19)</td>
<td>17 (14–20)</td>
<td>1.1 (1.0–1.3)</td>
<td>1.7 (1.5–1.9)</td>
</tr>
</tbody>
</table>

Responses are to concanavalin A (ConA), Candida albicans, Onchocerca volvulus adult worm antigen (Ovag), the cross-reactive recombinant antigen OV39, and retinal extract (Retina) in each group. The geometric mean stimulation index and 95% confidence intervals (in brackets) are shown. Q = Quito controls; SL = San Lorenzo controls; and INF = infected with O. volvulus.

pattern was seen using a range of concentrations of OV39 from 0.2 µg/ml to 200 µg/ml (data not shown). Data were analyzed also using a responder–nonresponder dichotomy, with a responder defined as a subject with a stimulation index to OV39 greater than 2. The proportion of responders to OV39 in each group were: Q group, 67%; SL group, 46%; and INF group, 18%.

No relationship was seen between the presence and severity of chorioretinal disease in infected subjects and blastogenic responses to OV39 (Fig. 1). There were no statistically significant intergroup differences. Chorioretinal disease was seen in eight responders, which, as a proportion of all those with chorioretinal disease (8/60, or 13.3%), did not differ from those without (12/57, or 21%).

Longitudinal ophthalmologic follow-up data were available for 41 of the 117 INF group subjects during the previous 3 years. Subjects were defined according to evidence for development or deterioration of chorioretinopathy and were divided into three groups:

FIGURE 1. Relationship between peripheral blood mononuclear cell proliferation to OV39 and retinal extract and chorioretinopathy in subjects infected with Onchocerca volvulus (n = 117). Clear columns denote proliferative responses to OV39, and shaded columns denote responses to retinal extract. Columns represent geometric mean stimulation indices, and bars represent 95% confidence intervals.

no clinical evidence of the development of new lesions or deterioration of existing chorioretinopathy in either eye (type 0); evidence of development of new chorioretinal lesions in previously unaffected individuals (type 1); and evidence of extension of existing disease (type 2). The proportions belonging to each category were: type 0, 37% (15/41); type 1, 37% (15/41); and type 2, 26% (11/41). No significant intergroup proliferative responses were seen (Fig. 2). The relationships between proliferative responses to OV39 and other covariates of interest are shown in Table 4. No significant correlations were seen. No differences in proliferative responses to OV39 were seen between patients with and without chorioretinopathy.

FIGURE 2. Relationship between peripheral blood mononuclear cell responses to OV39 and retinal extract and the development of new chorioretinal lesions and extension of existing lesions in subjects infected with Onchocerca volvulus (n = 41). Types of lesions are: type 0 = no evidence of development or deterioration of chorioretinopathy in either eye; type 1 = development of chorioretinal lesion in previously unaffected person; type 2 = development of new lesion in other eye or extension of chorioretinopathy in affected eye in person with previous evidence of disease. Clear columns denote proliferative responses to OV39, and shaded columns denote responses to retinal extract. Columns represent geometric mean stimulation indices, and bars represent 95% confidence intervals.
Onchocercal Chorioretinopathy

Microfilarial intensity (mf/mg)

FIGURE 3. Relationship between peripheral blood mononuclear cell responses to Ov39, chorioretinopathy, and microfilarial intensity. Clear columns represent proliferative responses to Ov39 in subjects infected with Onchocerca volvulus without chorioretinopathy, and shaded columns denote responses in subjects infected with Onchocerca volvulus with chorioretinopathy. Responses in both groups are stratified according to microfilarial intensity (mf/mg). Columns represent geometric mean stimulation indices, and bars represent 95% confidence intervals.

at different levels of microfilarial intensity in the skin (Fig. 3).

Lymphoproliferative Responses to Retinal Extract

Blastogenic responses to bovine retinal extract are shown in Table 3. There were no significant intergroup differences in proliferation. There was no relationship between blastogenic responses to this antigen and either chorioretinal disease status (Fig. 1) or the development and deterioration of new lesions (Fig. 2). A positive correlation was seen with black race (Table 4).

Cytokine Production by Ov39-Stimulated Peripheral Blood Mononuclear Cells

Production of IFNg and IL-5 were negligible in Ov39-stimulated cultures.

DISCUSSION

There is no evidence in this study to support the hypothesis that cellular autoimmune mechanisms are involved in the pathogenesis of chorioretinopathy in onchocerciasis. There was no evidence of increased cellular responsiveness to either “self” (crude retinal antigen) or the putative parasite autoantigen (Ov39) in subjects infected with O. volvulus with chorioretinopathy compared to those without this lesion. Furthermore, Ov39 was recognized (stimulation index greater than 2) by PBMCs from only a small proportion of subjects infected with O. volvulus, and recognition was more frequent in those without the infection.

Several factors might limit the validity and generalizability of these study findings. First, amocarzine treatment may have prevented disease evolution through reduction of parasite burdens. This is unlikely because a large proportion of the amocarzine-treated sample (26/41, or 63.4%) for which longitudinal ophthalmologic data were available showed evidence of evolving chorioretinopathy. Second, the pathophysiology of chorioretinopathy might vary between disease foci. However, the pattern of ocular onchocerciasis and the clinical appearance and distribution of chorioretinal lesions seen in this focus are identical to those described in West African foci, particularly in rain forest areas. Third, it could be argued that autoreactive T cells are sequestered in ocular tissues and cannot be detected in assays using peripheral blood. This seems unlikely because onchocerciasis is a systemic infection and sensitization to the parasite-derived antigen Ov39 would occur peripherally long before and during the development of posterior segment disease.

The absence of a relationship between cellular responses to Ov39 (and retinal antigen) and onchocercal chorioretinopathy is significant because cellular immune mechanisms (here measured as PBMC blastogenesis) are thought to be of central importance in the pathogenesis of autoimmune inflammatory diseases of the uvea in humans and in experimental autoimmune uveoretinitis in animal models. All current evidence in favor of autoreactivity in onchocercal chorioretinopathy is derived from the detection of retina-specific autoantibodies in human infection sera.

The finding of an association between cellular and humoral responses to the two uveitopathogenic peptides, retinal Santigen and interphotoreceptor retinoid binding protein, and clinical disease in human uveitis has led to the suggestion of a role for these proteins in the pathogenesis of chorioretinopathy in onchocerciasis. Though Vingtain and co-

TABLE 4. Correlations Between Ov39 and Retinal Antigen and Covariates of Interest in Subjects Infected With Onchocerca volvulus

<table>
<thead>
<tr>
<th>Antigen</th>
<th>Race</th>
<th>Sex</th>
<th>Age</th>
<th>MfS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ov39 20</td>
<td>0.154</td>
<td>0.108</td>
<td>-0.168</td>
<td>-0.116</td>
</tr>
<tr>
<td>Retinal antigen</td>
<td>-0.569*</td>
<td>0.119</td>
<td>-0.019</td>
<td>-0.108</td>
</tr>
</tbody>
</table>

n = 117. Test statistics are Spearman’s rank correlation coefficient. MfS = skin microfilarial intensity. *P < 0.05.
workers were able to demonstrate the presence of significantly higher levels of S-antigen antibodies in patients with onchocerciasis with posterior pole involvement, subsequent studies have been unable to demonstrate a similar relationship with either S-antigen or interphotoreceptor retinoid binding protein. Further, cellular responses to these peptides were similar in patients with onchocerciasis with and without chorioretinal diseases.

Autoantibodies with specificity for a number of different tissues have been identified in sera from patients with onchocerciasis. These include nonorgan-specific cytoplasmic and nuclear antigens, the calcium binding protein calreticulin, elastin and collagen, and immunoglobulin M rheumatoid factor. The clinical significance of these findings is uncertain. Detection of autoantibodies is a common finding in healthy populations of tropical regions and in a number of tropical infections. Autoantibodies may result from polyclonal activation of T and B cells, which is a feature of infection with parasites. Hypogammaglobulinemia is a feature of O.volvulus infection and, because 10% to 30% of B cells produce antibodies that are able to bind autoantigens, self-reactivity is not an unexpected finding.

DNA:DNA hybridization studies have demonstrated Ov39-homologous sequences in other nematodes, including other Onchocerca spp, Loa loa, and Brugia malayi. It is likely that other nematodes, including intestinal parasites, share homologous sequences. This might explain the apparent recognition of Ov39 in the control groups.

The mechanism most likely to account for the initiation of retinal pathology is bystander damage resulting from inflammation directed against dead and degenerating microfilariae. Microfilariae have been demonstrated in the retina in vivo and histologically. The relationship between treatment with microfilaricides and the induction of characteristic retinal pathology is strong evidence in support. However, the incidence of new lesions after chemotherapy is extremely low, whereas the extension of existing disease is common. Further, the development of new lesions is correlated with microfilarial burdens in the Ecuadorian focus, whereas the extension of existing disease is not (Cooper et al, unpublished data, 1995). These observations suggest that, though chorioretinopathy is likely to be initiated by the presence of microfilariae, disease extension no longer requires their presence. The induction of pathology might set off self-perpetuating autoimmune processes that no longer require the presence of microfilariae.

Key Words
autoimmunity, chorioretinopathy, Ecuador, filariasis, onchocerciasis

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Onchocercal Chorioretinopathy


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