Immunological Crossreactivity between a Cloned Antigen of *Onchocerca volvulus* and a Component of the Retinal Pigment Epithelium

By Gabriele Braun,* Nicol M. McKechnie,* Vivienne Connor,* Clare E. Gilbert,§ Fred Engelbrecht,* James A. Whitworth,‡ and David W. Taylor*

*From the *Department of Pathology, University of Cambridge, Cambridge CB2 1QP, United Kingdom; ‡MRC Laboratories, Bo, Sierra Leone; the §Department of Preventive Ophthalmology, Institute of Ophthalmology, London EC1V 9EJ, United Kingdom*

Summary

Onchocerciasis (river blindness) is a major blinding disease in Africa, Central America, and South America. Loss of vision can be due to corneal change, optic atrophy, or chorioretinal disease. It has been suggested that autoimmunological reactions resulting from crossreactivity between parasite antigens and components of eye tissues contribute to development of ocular pathology. Using sera collected from onchocerciasis patients as a screening reagent, a cDNA clone (Ov39) has been isolated from a λgt11 expression library of *Onchocerca volvulus*. This antigen exhibits immunological crossreactivity with a component of retinal pigment epithelium cells (RPE). Antiserum raised against this recombinant peptide immunoprecipitates a 22,000 M_r antigen of adult *O. volvulus* and recognizes a 44,000 M_r component of bovine RPE by Western blotting. A 44,000 M_r antigen of cultured human RPE metabolically labeled with ^{35}S-methionine can be immunoprecipitated with the same antiserum. An antigen of the same size is recognized by a rabbit antiserum raised against whole *O. volvulus* extract. Immunocytochemical studies on cryostat sections of the bovine eye using the antirecombinant sera localizes this antigen to the RPE.

Onchocerca volvulus is a filarial nematode and the causative agent of onchocerciasis (river blindness). The clinical manifestations of eye disease in onchocerciasis have been well described (1–4) and include punctate keratitis, sclerosing keratitis, iridocyclitis, optic neuritis, optic nerve atrophy, and chorioretinitis. Inflammatory responses directed against dead microfilariae may explain development of pathological changes in the anterior segment (5, 6), however, the aetiology of lesions in the posterior segment is unclear. In the case of degenerative chorioretinitis, migrating microfilariae (7, 8) and direct interaction of cells of the immune system with microfilariae, accompanied by infiltrate of lymphocytes and plasma cells, have been implicated in pathogenesis (discussed by Donnelly et al. [9]). In addition, lymphocyte-derived chemotactic factors and direct chemotactic effects of microfilarial excretions/secretions may also have a role in pathogenesis (10). More recently, Vingtain et al. (11) have provided evidence to support the idea that autoantibodies may play a significant role in retinal damage. These investigators demonstrated the presence of antibodies with specificity for bovine retinal S antigen and human retinal extract in onchocerciasis patients presenting with chorioretinitis. Van der Lelij et al. (12) extended these observations through experiments using purified human S antigen and interphotoreceptor retinoid binding protein (IRBP).[1] High levels of specific antibody were found in onchocerciasis patients, but these studies failed to reveal an association with chorioretinitis. In addition, Chan et al. (13) demonstrated the presence of autoantibodies in infection sera that could not be absorbed with either S antigen or IRBP; rather, these antibodies were directed against the inner retina, including the nerve fiber layer, ganglion cells, and Müller cells.

It is not clear, however, whether the occurrence of autoantibodies in onchocerciasis patients is a secondary autoimmunization to antigens released from damaged tissue due to an underlying disease process, or whether it might be a primary event that may be explained by crossreactivity between parasite antigens and the retina. To help distinguish between these alternatives, it will be necessary to identify and characterize the crossreacting antigens. In this report, we described the identification of a recombinant antigen of *O. volvulus* that shares immunological crossreactivity with a component of retinal pigment epithelium cells (RPE). The availability of this recombinant antigen and the identification of the cross-

[1] *Abbreviations used in this paper:* IRBP, interphotoreceptor retinoid binding protein; RPE, retinal pigment epithelium.

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reacting target may contribute to the understanding of the pathogenesis of the chorioretinopathy seen in onchocerciasis.

Materials and Methods

Parasites and Eye Tissue Material. Adult female and male *O. volvulus* worms were isolated from excised nodules by collagenase digestion of the encapsulating host tissue (14) and stored in liquid nitrogen.

An aqueous antigen extract was prepared by homogenization of parasites in PBS containing 1 mM PMSF and 1 mM *N*-tosyl-L-phenylalanine chloromethyl ketone (TPCK). The homogenate was centrifuged at 10,000 *g* and the supernatant fluid collected. The insoluble pellet was also recovered and solubilized in 1% SDS.

For SDS/PAGE, these fractions, or whole worm extracts, were resuspended in SDS sample buffer (15) and gel loadings determined empirically.

Bovine eyes were obtained from a local abattoire and transported to the laboratory on ice for immediate processing.

To prepare extracts for PAGE/Western blot experiments, bovine eyes were bisected in the equatorial plane, the vitreous and neuroretina removed, and the eye cup filled with calcium and magnesium-free PBS, pH 7.4, containing 20 mM EDTA (tetra sodium salt). After 1 h at room temperature, cells of the retinal pigment epithelium could be removed from Bruch's membrane by gentle pipetting. Detached cells were washed twice in PBS. Soluble and cytoskeletal fractions were produced by dissolving the cells in PBS containing 1% Triton X-100 and 1 mM PMSF. The resulting fractions were then mixed with SDS sample buffer (15) and boiled for 5 min. Appropriate gel loadings for Western blots were determined empirically.

Cultured human RPE were obtained from the Department of Pathology, Institute of Ophthalmology (London), and maintained and harvested, as described by Boulton et al. (16). Components of RPE cells were sequentially extracted in: (*a*) an aqueous solution of PBS; (*b*) in PBS, 1% Triton X-100; and (*c*) in PBS, 0.1% SDS, for subsequent use in immunoprecipitation experiments.

Source of Antisera. Antisera used in immunological assays came from two sources. First, human onchocerciasis infection sera collected from patients participating in clinical and epidemiological surveys carried out in Sierra Leone by McMahon (17) and Whitworth (18). Second, antisera raised in rabbits after immunization with purified or semi-purified proteins, including recombinant antigens, in the presence of CFA.

Human infection sera were extensively preabsorbed against whole bacterial extracts of *Escherichia coli* and pure β-galactosidase before their subsequent use in immunological screening methods and Western blot technique. The extract was prepared from overnight cultures of *E. coli* PM1090, 50 times concentrated in 10 mM Tris, pH 7.5, saline solution with 1 mM PMSF. The concentrated cells were sonicated and incubated at 37°C for 12 h with 1 mg/ml DNAse. β-galactosidase was added at a concentration of 0.2 mg/ml, and the extract was stored frozen. 200 μl of human infection serum was absorbed against 3 × 1 ml of this extract and subsequently against a 63-cm^2 nitrocellulose filter coated with 1 mg β-galactosidase.

Labeling Procedures and Immunoprecipitations of Antigens. Native antigens were labeled with ^{35}S using ^{35}S labeling reagent (Amersham, Buckinghamshire, UK), according to the manufacturer's instructions.

The mRNA directed in vitro translation products were radiolabeled with ^{35}S-methionine using the rabbit reticulocyte lysate system (19) following the method described by Taylor et al. (20).

Cultured human retinal pigment epithelial cells were maintained in one 75-cm^2 tissue culture flask as described elsewhere (16). For metabolic labeling, the culture medium was supplemented with 500 μCi (1,000 Ci/mmol) ^{35}S-methionine. After several washes in PBS, the cells were harvested and sonicated before extraction as described above.

Immunoprecipitation of radiolabeled antigens was carried out as previously described (20) using 600,000 dpm per immunoprecipitation reaction. NP-40 was added to samples containing SDS in a ratio of 2.5:1 (wt/wt) before addition of the antiserum.

SDS/PAGE and Western Blot Analysis. SDS/PAGE (concentrations of the slab gels are indicated in the figure legends) was performed as described by Laemmli (15). Electrophoretically separated polypeptides were transferred to nitrocellulose paper (BA 85; Schleicher & Schüll GmbH, Dassel, FRG) by overnight blotting at 100 mA (bovine retinal antigens) or 400 mA for 2 h (*O. volvulus* recombinant antigens) following the method of Towbin et al. (21). Immunostaining of bovine retinal extract was carried out according to the method described by Campbell (22). The primary antibody was used at a dilution of 1:300. Goat anti–rabbit peroxidase conjugate (used at 1:50 dilution) was obtained from Sigma Chemical Co. (Poole, Dorset, UK).

After transfer of *O. volvulus* recombinant antigens, the nitrocellulose filter was "blocked" by incubation with PBS containing 1% BSA for 15 min and subsequently incubated with the first antibody (1:400 diluted in washing buffer: PBS containing 0.3% Tween 20) overnight at 4°C. After five washes in washing buffer, the blot was incubated for 1 h at room temperature with an appropriate biotinylated second antibody and again washed with five changes of washing buffer (1 h). The blot was then incubated with streptavidin–horseradish peroxidase. The second antibody and peroxidase complex were purchased from Amersham, and used at a dilution of 1:400 in washing buffer. After five further washes in washing buffer, the blot was developed in Incubation Solution, which was made up fresh mixing 0.6 mg/ml 4-chloro-1-naphthol in methanol with PBS in a ratio of 1:5. 0.06% Hydrogen peroxide was added to the incubation solution shortly before use.

Indirect Immunofluorescence Studies. Cryostat sections of bovine neuroretina and RPE were prepared from fresh bovine eyes. The sections were air dried only and not chemically fixed. Nonspecific binding of Igs was blocked by a 20-min incubation in PBS containing 5% normal goat serum. All antibody dilutions were made up in 1% normal goat serum. Primary antibodies were used at a range of concentrations between 1:50 and 1:200. Sections were incubated with the primary antibody for 2 h. After several washes in PBS, binding of Igs was detected by a 1-h incubation with goat anti–rabbit FITC conjugate (Sigma Chemical Co.), diluted 1:40. After further washes in PBS, sections were mounted on Flurostab (Bio-nuclear Services Ltd., Reading, UK) and examined, and photographed, using a Dialux microscope (Leitz, Wetzlar, FRG) equipped with filters for epi-fluorescent microscopy. All photographic exposures were of the same duration to facilitate comparison. Controls for immunocytochemical specificity were: (*a*) omission of the primary antibody; (*b*) the use of sera obtained from the rabbit before immunization at the same dilution as the test sera; (*c*) the use of sera directed against the carrier peptide of the fusion protein.

Bacterial Strains and Growth Conditions. The phage λgt11 was grown in bacterial strains using standard methods (23). For the production of the Ov39 fusion protein with the β-galactosidase, the temperature-sensitive lysogen of λgt11 recombinant phage in *E. coli* Y1089 was shifted from 30°C to 42°C, induced with isopropyl thio-β-D-galactopyranoside (IPTG) during the exponential growth

phase, and harvested before lysis occurred. The expression vectors pEX34b (23), pGEXI (24), and their derivatives were transformed into *E. coli* 537 and *E. coli* TG2, respectively. For the production and purification of fusion protein in the respective bacterial strains, expression was induced following procedures described by Klinkert et al. (24) and Smith et al. (25).

For the isolation of phage DNA, λgt11 recombinant phage was prepared from a 100-ml culture (luria broth, 50 mg/ml ampicillin, 0.1% glucose, 2 mM $MgSO_4$). *E. coli* Y1088 cells were grown overnight in 3 ml of luria broth, 50 mg/ml ampicillin, 0.2% maltose. The cells were centrifuged, resuspended in 3 ml 10 mM $MgSO_4$, and used for the inoculation of the 100-ml culture. To propagate the phage, this culture was immediately infected with 3×10^6 plaque-forming units and grown overnight at 37°C. After pelleting the remaining bacteria, the supernatant was retained for the recovery of the phage.

Preparation of DNA and RNA. Genomic DNA from *O. volvulus*, *Brugia malayi*, *Acanthocheilonema vitae*, *Loa loa*, and human DNA from a hybrid cell line ESH98 (26), was isolated from homogenized material treated with proteinase K in sarcosyl/EDTA, as described in Maniatis et al. (27). Genomic DNA of *O. gibsoni*, *O. gutturosa*, *O. armillata*, and *B. pahangi*, and cow DNA, was kindly provided by R. Post (University of Salford).

For the preparation of phage DNA, culture supernatant containing λgt11 phage particles was incubated at 37°C for 1 h with 40 μg/ml RNase and 20 μg/ml DNase. The phage was precipitated with 20 ml of 20% polyethylene glycol 8000, 2.5 M NaCl during an incubation period of 2 h on ice, and pelleted at 18,000 rpm (GSA Sorvall rotor; DuPont Co., Wilmington, DE) for 45 min. The phage pellet was carefully resuspended in 2 ml 10 mM Tris, pH 7.5, 1 mM EDTA (TE), and treated for 10 min with 20 mM EDTA. DNA was extracted with 1 ml of phenol. Three further extractions of the aqueous phase with phenol/$CHCl_3$ (27) followed before the DNA was precipitated with 0.3 M NaAc, pH 6, and 2.5-vol ethanol. The DNA was resuspended in TE again, and several more phenol/$CHCl_3$ extractions were performed before the final ethanol precipitation of the DNA.

RNA was prepared from adult female *O. volvulus* worms using either the hot phenol (28) or a combination of the guanidinium/CsCl methods described by Cox (29) and Chirgwin et al. (30), as described by Taylor et al. (20).

cDNA Synthesis. Total RNA of *O. volvulus* was used to prepare cDNA by a combination of the methods by Buell et al. (31) and Wickens et al. (32). The cDNA was methylated, blunt end ligated to 12-bp EcoRI linkers (Boehringer Mannheim GmbH, Mannheim, FRG), and digested with EcoRI. The fragments were size fractionated over a Sepharose 4B column (320 × 2 mm), equilibrated in TE, and 100 mM NaCl.

Cloning Experiments, Screening Methods, and DNA Sequencing. An expression library of *O. volvulus* was constructed by cloning cDNA fragments into the unique EcoRI restriction site of the expression vector λgt11 (33). Phage particles were packaged using commercially prepared extracts by Promega Biotec (Madison, WI). An immunochemical screen to identify antigen-expressing bacteriophage clones was performed as described by Young and Davis (23).

Recombinant clones other than λgt11 were constructed in the high expression vectors pEX34b (24) and pGEXI (25). These vectors were engineered to express antigens in an inducible fashion with the RNA polymerase of bacteriophage MS2 and the glutathione-*S*-transferase of *Schistosoma japonicum* as respective carrier proteins.

Immunological screens of bacterial colonies that express recombinant antigens of *O. volvulus* were performed as described by Hall et al. (34). Fusion proteins, transferred to Nitrocellulose filters, were detected using antibody probes and the developing system as described for Western blots.

DNA sequences were determined using the M13/dideoxy method by Sanger et al. (35). Universal M13 and λgt11 primers were purchased from New England Biolabs (Beverly, MA).

In general, DNA fragments for all subcloning experiments were isolated from DNA prepared from an original λgt11 clone and recovered from agarose gels using NA-45 cellulose membrane (Schleicher & Schüll, GmbH) (36).

Hybridization Reactions. DNA/DNA hybridizations were performed under either high stringency conditions in 50% formamide at 42°C or at lower stringency at 38°C using the radiolabeled cDNA insert fragment as a probe, prepared by the random primer labeling technique (37). This probe was used for hybridization (38) of Southern blots (39) and for bacterial colonies.

Results

Ov39, a cDNA Clone Derived from an Expression Library of O. volvulus. For the preparation of in vitro translation products, RNA was extracted from gravid female *O. volvulus* worms. The amount of RNA recovered from one female worm was ~5.9 μg when prepared by the guanidium/CsCl method. Slightly less RNA was recovered by the hot phenol/SDS protocol. No major qualitative differences could be detected between the two preparations as judged by comparison of the SDS-PAGE profiles of mRNA-directed in vitro translation products (40).

Due to lack of material, and since the amount of cDNAs primed on rRNAs by oligo(dT) seems to be negligible (41), unfractionated RNA rather than poly(A)$^+$ RNA was used to synthesize cDNA for the construction of an expression library in λgt11.

An immunological plaque screening of 80,000 recombinant phages was performed using a pool of human infection sera preabsorbed against *Escherichia coli* lysate and β-galactosidase. These sera were collected from patients living in an endemic area of Sierra Leone and present with high microfilaridermia. Of the clones identified, one designated Ov39 encodes and stably expresses a 128,000 M_r inducible fusion protein with β-galactosidase; the cDNA thus encodes an antigen of ~12,000 M_r (Fig. 1, lane *1*). The complete sequence of the cDNA insert consists of 345 bp (Fig. 2).

The KpnI-SStI fragment of λOv39, carrying the *lacZ* gene and the cDNA insert, was isolated and subcloned into M13mp19. The use of a λgt11-specific primer allowed the determination of the sequence-junction of the *lacZ* gene and the cDNA insert in direction of transcription/translation, and thus revealed the reading frame of the cloned DNA. There is no in-frame stop codon within the cDNA sequence, and the antigen is expressed with the COOH-terminal 16 amino acids of the β-galactosidase encoded by the 3′ end of the *lacZ* gene downstream the EcoRI cloning site. The cDNA insert encodes 115 amino acids with a calculated molecular mass of 13,086 daltons. This is in accordance with the relative molecular mass of the fusion protein established by gel electrophoresis. The cDNA sequence has stop codons in all three reading frames when read in inverse orientation, theoretically allowing the expression of peptides with only 3, 23, or 30 amino acids.

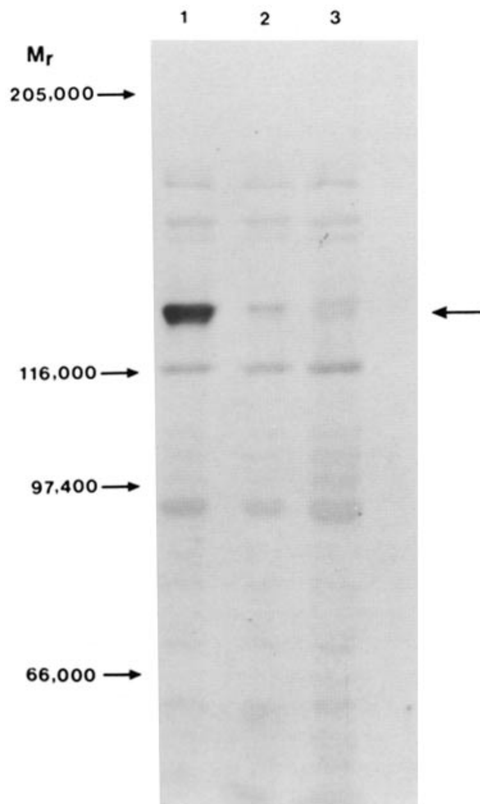

Figure 1. Expression of *O. volvulus* cDNA clone Ov39 as a β-galactosidase fusion protein in an *E. coli*-lysogen. Proteins were electrophoretically separated on an 8% acrylamide/SDS gel under reducing conditions, and the fusion protein was detected by Western blot, using a pool of human infection sera. (Lane *1*) Induced expression of λgt11 Ov39 clone, indicated by arrowhead; (lane *2*) control, uninduced lysogen; (lane *3*) induced expression of λgt11 lysogen as control.

Analysis of the sequence of the cDNA did not reveal a putative polyadenylation signal nor a poly(A)$^+$ tail, and the 3′ end does not agree with the sequence of the EcoRI linker oligo nucleotide that was ligated onto the cDNA. It is assumed that the EcoRI restriction site at the 3′ end constitutes a gene-specific sequence that was not modified by the methylase reaction of the reverse transcript. Therefore, the sequence encoding the COOH terminus of the antigen as well as the poly(A)$^+$ tail might have been cleaved in the cloning process.

The cDNA does not appear to represent the reverse transcript of a full-length message, since DNA sequence analysis did not reveal a poly(A)$^+$ tail nor a sequence that matches the consensus sequence for a ribosome binding site (42). To identify full-length clones encoding the entire antigen, a *O. volvulus* cDNA expression library (no. 37509; American Type Culture Collection, Rockvillc, MD) prepared by Donelson et al. (43) in λgt11 from parasites collected in Central America (Guatemala) was screened by plaque hybridization using the cDNA Ov39 insert as a probe. Three clones have been analyzed that carry an Ov39-like sequence that resides within a single EcoRI cDNA fragment judged by DNA/DNA hybridization of Southern blots and that is longer by 59 bp as established by DNA sequence analysis. The homologous cDNA isolates from independent sources are identical except for one base pair substitution (G → A) in position 176 (Fig. 2), resulting in an arginine residue at position 59 of the antigen in the Sierra Leone isolate in contrast to a glutamine residue in the isolate from Guatemala. All three clones have an additional cDNA fragment of different length at their 5′ end. It has not been established whether they are homologous and derived from the same message, or entirely unrelated.

A search of data bases (EMBL, NBRF, GenBank, PIR, PIR-NEW, SWISS-Prot, and NEWAT) for nucleotide and protein sequence homology failed to identify any similarities with catalogued entries.

Species Specificity of Ov39. The species specificity of clone Ov39 was tested by DNA/DNA hybridization of DNA of *Onchocerca gibsoni*, *O. gutturosa*, *O. armillata*, *Brugia malayi*, *B. pahangi*, *Loa loa*, and *Acanthocheilonema vitae*. Moreover, it was important to establish whether Ov39 shares any sequence homology with human and bovine DNA. Southern blots of EcoRI-digested DNA derived from these sources were hybridized with the cDNA probe of *O. volvulus*. The corre-

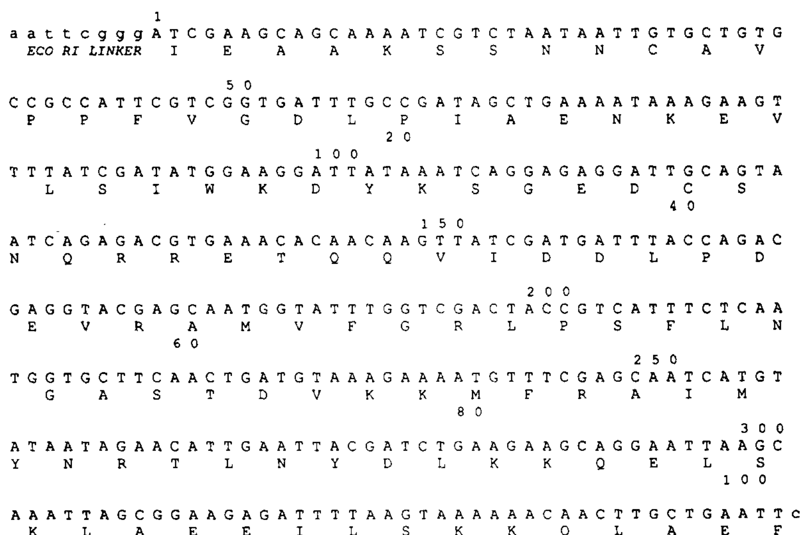

Figure 2. The nucleotide sequence and predicted amino acid sequence of Ov39. The nucleotide residues are numbered with respect to the first nucleotide of the cDNA clone. The single letter amino acid code is used below each codon and numbered below. The sequence of the EcoRI linker is indicated in lower case letters. The gene-specific EcoRI restriction site at the 3′ end of the sequence is underlined.

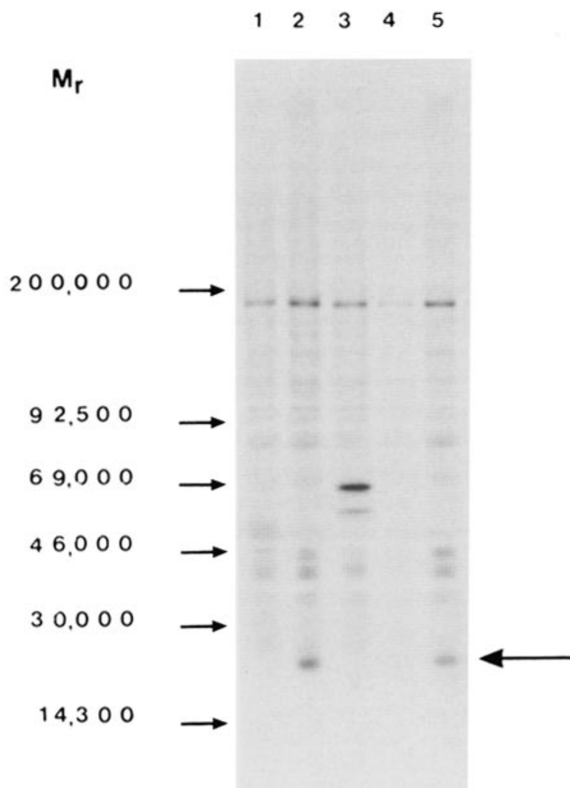

Figure 3. Immunoprecipitation of a 22,000 M_r ^{35}S-labeled *O. volvulus* antigen by rabbit antisera raised against Ov39 recombinant antigen expressed in pEX34b and pGEX1. (Lane *1*) Control experiment performed with serum from rabbit before immunization with fusion protein EX39; (lane *2*) immunoprecipitation performed with serum collected from rabbit immunized with the EX39 fusion protein; (lane *3*) control experiment performed with serum collected from a rabbit immunized with the carrier protein expressed by pEX34b; (lane *4*) control experiment with rabbit serum collected before immunization with fusion protein GEX39; (lane *5*) immunoprecipitation of a 22,000 M_r antigen with rabbit anti-GEX39 serum. The proteins were separated on an 8–20% acrylamide/SDS gel under reducing conditions.

sponding "gene39" or part of the "gene39" is located on a 2.3-kb EcoRI fragment of genomic DNA of *O. volvulus*. All the other *Onchocerca* species tested have a very closely related gene that can be localized on a 2.1-, 2.3-, and 1.8-kb EcoRI fragment of *O. gibsoni*, *O. gutturosa*, and *O. armillata*, respectively. Homologous gene sequences have been identified on an 8-kb fragment of *L. loa* genomic DNA, and on two fragments of 2.3- and 4.2-kb of *B. malayi*. Less stringent hybridization conditions reveal only very weak homologies on a 3.7-kb fragment between *B. pahangi* DNA and Ov39, and entirely failed to identify corresponding sequences in the genome of *A. vitae*, human, and bovine DNA (data not shown).

Subclones of Ov39. Clones expressing the gene of Ov39 in bacteria are a ready source to produce large quantities of recombinant antigen. To obtain fusion proteins with carrier molecules other than β-galactosidase, the cDNA fragment has been subcloned into the high expression vector pGEX1, in which it is expressed as a 35,000 M_r fusion protein (GEX39) with glutathione-*S*-transferase of *S. japonicum*, and pEX34b, where it is expressed as a fusion protein of 27,000 M_r (EX39) with a 15,000 M_r fragment of the RNA polymerase of phage MS2. The capacity of human infection sera to recognize the fusion protein was tested by either ELISA or Western blot analysis. In the case of the GEX39 antigen, it was found that background reactivity with the carrier protein precluded meaningful interpretation of the results (none of the patients donating sera for these experiments were infected with any species of schistosome). However, 1 of 16 human infection sera tested in Western blot showed very strong reactivity against the EX39 recombinant protein (data not shown) and had no background reactivity against the carrier peptide of this recombinant protein.

The Native Antigen Ov39. Rabbit antisera were raised against both the EX39 and GEX39 fusion protein as well as against the respective carrier peptides for sera controls. Both the anti-EX39 and the anti-GEX39 serum recognize the Ov39 antigen of the hybrid molecules and were used in immunoprecipitation experiments to identify the corresponding native antigen. An aqueous extract of antigens labeled with ^{35}SLR was reacted with these sera. Both antisera recognize a water-soluble antigen of 22,000 M_r (Fig. 3).

Immunological Crossreactivity of Ov39 with RPE. The retinal pigment epithelium is formed by a cell monolayer, located immediately under the neurosensory retina. The RPE cells are attached to the Bruch's membrane, from which they can be detached and isolated. Components soluble in PBS, 1% Triton X-100 were analyzed on a Western blot (Fig. 4). The extracted components were probed with rabbit hyperimmune

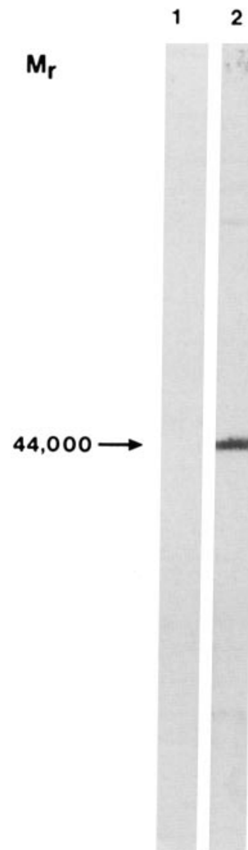

Figure 4. Identification of a 44,000 M_r component of RPE, using rabbit serum raised against the recombinant *O. volvulus* clone Ov39. Components of the PBS, 1% Triton X-100–soluble extract were separated on a 12% acrylamide/SDS gel under reducing condition, transferred on nitrocellulose filter, and probed with: (lane *1*) a control serum, collected from a rabbit before immunization with the EX39 recombinant antigen; (lane *2*) with test serum collected from the rabbit after immunization with the EX39 fusion protein.

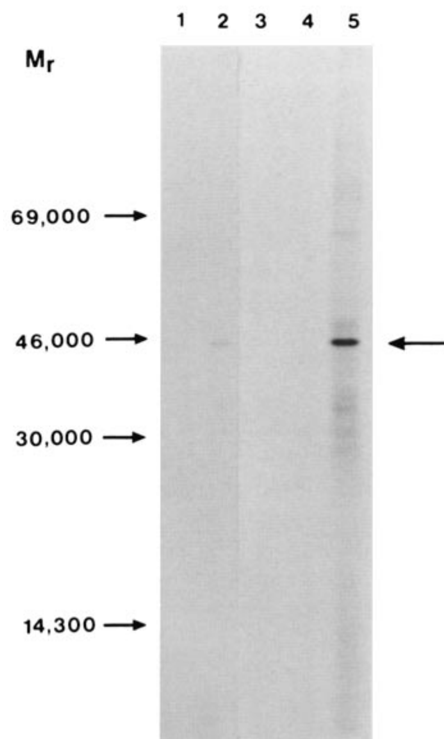

Figure 5. Immunoprecipitation of extracts of cultured human RPE metabolically labeled with ^{35}S-methionine. (Lane *1*) Control, serum of rabbit before immunization with a total extract of *O. volvulus*; (lane *2*) serum of rabbit immunized against a total extract of *O. volvulus*; (lane *3*) control, serum of rabbit immunized with glutathione S-transferase; (lane *4*) control, serum of rabbit before immunization with the fusion protein GEX39; (lane *5*) rabbit serum raised against the recombinant fusion protein GEX39. The proteins were separated on an 8–20% polyacrylamide/SDS gel under reducing conditions.

serum raised against the EX39 fusion protein (lane *2*), and with the rabbit serum that was taken from the rabbit before immunization (lane *1*). Neither the sera collected from the rabbit before immunization with the fusion protein EX39, nor the serum raised against the carrier protein (RNA polymerase of phage MS2) recognized any component of the extract (data not shown). However, the anti-EX39 serum reacts strongly with a 44,000 M_r molecule of the bovine RPE cell extract. No reactivity could be seen with components of the 1% Triton X-100–insoluble cytoskeletal fraction (data not shown).

Shortage of fresh human RPE precludes experiments similar to those described above. To circumvent this problem, human RPE cells were cultured and metabolically labeled with ^{35}S-methionine. Sequential extracts of these cells in PBS, PBS containing 1% Triton X-100, and SDS were immunoprecipitated and subsequently analyzed by SDS-PAGE (Fig. 5). In this experiment, a molecule of 44,000 M_r is recognized in the 1% Triton X-100 fraction by rabbit serum raised against the GEX39 fusion protein (lane *5*). Again, the control serum taken from this rabbit before inoculation with GEX39, and the rabbit serum raised against the carrier protein (glutathione *S*-transferase), failed to react with any RPE component (lanes *4* and *3*, respectively). The anti-GEX39 serum did not react with components of the PBS nor the SDS extract. In addition, a 1% Triton X-100–soluble component of the same size was recognized by a rabbit serum that was raised against a total extract of female *O. volvulus* worms (lane *2*). Homology between these two molecules is not proven.

Histological Localization of Target Antigen. Immunocytochemical studies for the histological localization of the respective molecule were carried out on cryostat sections of bovine retina by the indirect immunofluorescence technique using the rabbit anti-EX39 serum. Fig. 6 *A* illustrates a light micrograph of a section stained with hematoxylin and eosin. The RPE cell monolayer is indicated by the arrowhead. The choroid is located below this cell layer and the neuroretina above. Fig. 6, *B* and *C*, present immunofluorescence micrographs of similar sections, where Fig. 6 *B* shows the immunoreactivity obtained with the serum taken before immunization. Fig. 6 *C* demonstrates the immunostaining of a section obtained with the anti-Ov39 recombinant serum. The crossimmunoreactivity of this serum is clearly directed against the RPE cell layer. Slight staining can be seen in the neuroretina.

Discussion

Hissette (44), Bryant (45), and Ridely (46), described ocular fundus changes in onchocerciasis patients that were characterized by atrophy of the pigment epithelium and underlying choroid. They suggested an association between these lesions and infection with *O. volvulus*. Epidemiological and histopathological evidence supports the causal nature of this association (47–50).

Bird et al. (4) have described posterior segment changes associated with onchocerciasis. Mild or severe pigment epithelial atrophy was present in >50% of individuals studied with associated choroidal atrophy in more advanced cases. Although the precise mechanism is unknown, the role of immune processes in the pathogenesis of some of the complications of onchocerciasis has been established (51–53).

Recently, there has been considerable interest in the role of antibody responses directed against retinal S antigen and IRBP, both of which can induce a chorioretinopathy in experimental animals (54). It has also been suggested that antibodies with specificity for these proteins may have a role in the pathogenesis of the chorioretinopathy seen in individuals infected with *O. volvulus* (55). A significantly higher level of S antigen autoantibodies has been found in patients with posterior pole involvement (11). However, in other studies, no correlation between the levels of antibodies against retinal proteins and the occurrence of chorioretinitis could be found (12, 56).

The formation of anti-S and anti-IRBP antibodies may be a secondary phenomenon resulting from exposure of antigens by an underlying disease process. The occurrence of antibodies against human S antigen and human IRBP may not be sufficient to cause chorioretinopathy in onchocerciasis. The observation that experimental autoimmune uveoretinitis can not be induced by passive transfer of antiretinal antibodies supports this idea (57, 58).

Microfilariae occur in the vitreous and choroid, and have been identified in the retina in vivo (59) and in histological

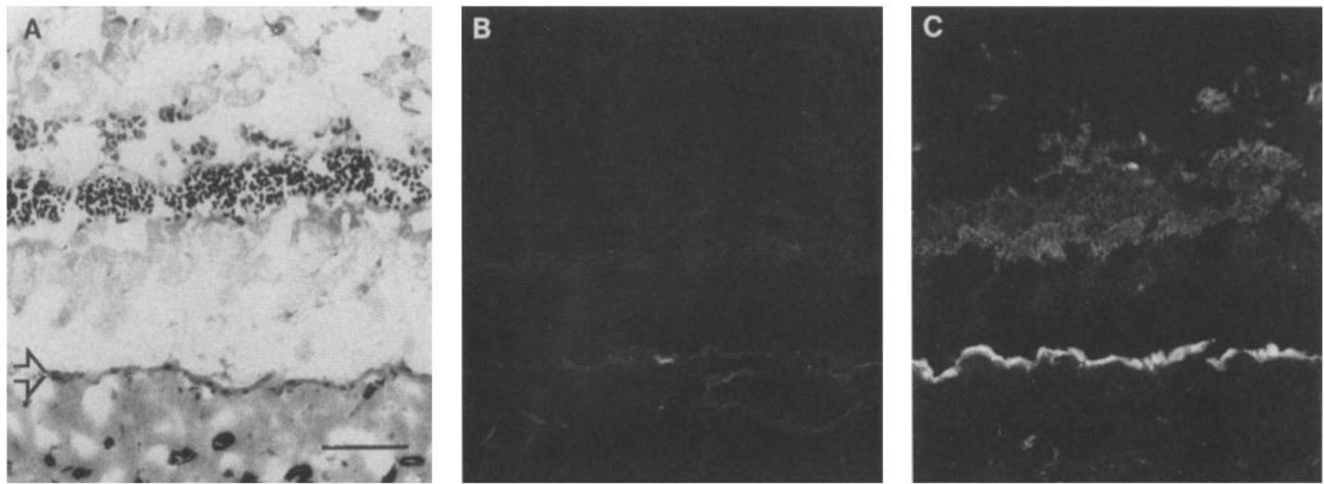

Figure 6. (*A*) Light micrograph of a cryostat section of bovine retina and choroid stained with hematoxylin and eosin. The arrowhead indicates the RPE. The choroid is below and the neuroretina above. (*B*) Immunofluorescence micrograph of a similar section probed with serum from a rabbit before immunization with the recombinant fusion protein EX39 (serum dilution 1:100). (*C*) Immunofluorescence micrograph illustrating the staining obtained using serum collected from the rabbit after immunization with the EX39 fusion protein (serum dilution 1:100). There is specific staining of RPE cells (calibration bar = 100 μm).

sections (7). However, the numbers found in the retina are apparently lower than found in other regions of the eye. Mechanical damage to the retina by microfilariae might therefore contribute less to pathology than indirect inflammatory processes or direct autoantibody reactions based on immunological crossreactivity of retinal and parasitic molecules. The idea that autoimmunity may play a role in pathogenesis can find additional support from the observation that even after drug treatment, the degenerative ocular processes continue (60, 61).

There is no indication that the occurrence of anti-S antigen and anti-IRBP-antibodies in onchocerciasis patients may be based on crossreactivity with parasitic antigens. However, the results presented here demonstrate a specific crossreactivity between a component of the RPE and a characterized recombinant antigen of *O. volvulus*.

Anti-Ov39 reacts with a 44,000 M_r component of the RPE, however, this molecule has not been characterized in detail. IRBP as possible target antigen for the anti-Ov39 can almost certainly be excluded, since IRBP is a high molecular weight glycoprotein of 140,000 M_r. S antigen is also precluded as a target: this antigen has an apparent molecular weight of 48,000 M_r and is localized in the photoreceptor cells, particularly the outer segment. However, the indirect immunofluorescence studies performed with the anti-Ov39 serum localized the target antigen in the RPE. No reactivity could be detected in the photoreceptor outer segments, and only faint reactivity with the remainder of the neuroretina, nor was any staining observed on the interphotoreceptor matrix. If the rabbit anti-Ov39 serum contained antibodies with specificity for S antigen or IRBP, the expected staining pattern should extend to these retinal layers.

S antigen and IRPB, as well as the components of the neural retina, are located in a immunologically privileged site protected by the RPE and endothelial cells of the retinal vasculature. These two cell types form the blood-retinal barrier (62). Antibodies directed against the recombinant peptide Ov39 crossreact with the retinal pigment epithelium and thus do not have to cross this barrier. Indeed, they are directed against a component of the cells that form the blood-retinal barrier.

It was important to establish Ov39 as an authentic parasite antigen. Results of Southern blot experiments did reveal similar DNA sequences in other filarial nematodes with varying degree in human and bovine DNA. This excludes the possibility that Ov39 might be a human-derived cloning artefact. The recombinant antigen Ov39 represents ~60% of the total antigen, with the crossreactive determinant. Since the RNA was extracted from gravid female *O. volvulus* worms, it is to be assumed that the preparation, therefore, contained RNA from female somatic tissue as well as RNA derived from embryonic stages and uterine microfilariae. However, if the anti-Ov39 antibody reactivity should be causative for any pathological changes in the eye, the source and location of the parasitic antigen would be irrelevant.

The Ov39 fusion protein is recognized by 1 out of 16 human infection sera so far tested. Whether crossreactivity of this antigen with the RPE contributes to the pathogenesis of chorioretinitis or choroidal atrophy will have to be established. Recognition of this antigen by human infection sera may correlate with chorioretinal changes. In this regard, we have collected, at six monthly intervals over 3 yr, a bank of sera from ~1,200 patient participants in a community-based trial of ivermectin in Sierra Leone (18). These sera together with the purified recombinant will provide the basis for experiments designed to test this possibility.

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Address correspondence to G. Braun, Department of Pathology, University of Cambridge, Tennis Court Road, Cambridge CB2 1QP, UK.

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