

Molecular and Immunological Characterization of hr44, a Human Ocular Component Immunologically Cross-reactive with Antigen Ov39 of *Onchocerca volvulus*

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Summary

Structural similarities between host self-antigens and infectious organisms may be involved in the expression of autoimmune reactivity and development of autoimmune disease. The unique eye pathology associated with *Onchocerca volvulus* infection, particularly the development of posterior segment lesions, may be promoted by such autoreactive responses. Ov39 is a parasite-derived antigen that has been shown previously to be antigenically cross-reactive with a 44,000- M_r host ocular component. A clone, designated hr44, was isolated from a cDNA library of human retina by immunoscreen using serum to Ov39. A monoclonal antibody raised to Ov39 also reacted with hr44 and gave evidence for a shared conformational epitope. The primary structure analysis showed that identities between the antigens are limited and confined to small peptides. The cross-reactivity between the antigens appears to involve T cells, since Ov39-specific T cells can be stimulated by hr44, a neural-specific antigen. Based on secondary structure prediction, hr44 has the typical features of a membrane-associated type I antigen with an amino-terminal extracellular domain. mAbs and antisera localized the antigen in the optic nerve, neural retina, retinal pigment epithelium, as well as the epithelial layers of ciliary body and iris.

Onchocerciasis (river blindness) is a chronic parasitic disease caused by the filarial nematode *Onchocerca volvulus*. The clinical manifestations have been well described (1–3), and sclerosing keratitis, iridocyclitis, optic neuritis, and chorioretinitis account for significant visual morbidity (4). Anterior segment disease has been directly associated with intensity of infection as measured by microfilarial load (5–7). The inflammatory responses directed against dying microfilariae are considered the main cause of these pathological changes (8, 9). However, much of the blindness is caused by disease of the posterior segment, namely, inflammation of the choroid, retina, and optic nerve, as well as atrophy of these tissues (3, 10, 11). The pathogenesis is poorly understood and an association with microfilarial burden is equivocal (5–7, 12). Involvement of autoimmune reactivity in the development of ocular onchocerciasis is one of several mechanisms that have been suggested (13).

Evidence in support of a role for autoimmune mechanisms in the pathogenesis of chorioretinopathy comes from several observations. A longitudinal study of patients showed progression of chorioretinal lesions, predominantly at the level of the retinal pigment epithelium, despite the reduction of microfilarial burden by chemotherapy (11). We have previously described Ab cross-reactivity between the recombinant antigen Ov39 of *O. volvulus* and a 44,000- M_r component of bovine and human retinal pigment epithelial

cells (14) that was subsequently shown to be neurospecific and present in those tissues affected by ocular onchocerciasis (15). Here, we report cross-reactivity between the parasite and host ocular antigen (designated hr44) on the Ab and T cell level, describe its molecular organization, and discuss it as a target molecule for antiparasite-directed immune responses.

Materials and Methods

Cloning Experiments, Screening Methods, and DNA Sequencing. Clone hr44 was isolated from a λ gt11 expression library of human retinal tissue (Clontech Laboratories, Inc., Palo Alto, CA). To identify the antigen-expressing bacteriophage clone, the library was screened as described before using rabbit serum raised to the recombinant parasite antigen Ov39 expressed in pEX34b (14). For this purpose, the serum was extensively preabsorbed against whole bacterial extracts of *Escherichia coli* PM1090 and pure β -galactosidase (14). Recombinant-phage DNA was prepared as previously described (14). The cDNA insert was obtained by EcoRI digest and purified using the QIAEX Gel Extraction system (Qiagen, Hilden, Germany). The sequence of both cDNA strands, cloned into pBluescript II SK⁺ (Stratagene, La Jolla, USA), was determined using the Taq DNA Polymerase Sequencing System (United States Biochemical Corp., Cleveland, OH). The primers used were both specific for pSK⁺ and internal

sequences of the cDNA. DNA and amino acid sequence analysis was performed using the programs of R. Staden and of the Genetics Computer Group [GCG] (Madison, WI), which included programs for the secondary structure analysis of amino acid sequences based on various methods (16–18). The FASTA program by Pearson and Lipman (19) was used for nucleotide sequence comparisons with entries in EMBL and NEWEMBL. Protein sequences were compared with entries in the SwissProt library.

The cDNA of hr44 and Ov39 were both cloned into the expression vector pTrcHisB (Invitrogen, San Diego, CA). The cDNA encoding Ov39 was derived from the construct in pEX34b (14) by EcoRI digest and purification with the QIAEX gel extraction system. Two truncated versions of hr44 were also constructed for expression in pTrcHisB. One truncated molecule, designated hr44-Sal, was expressed after restriction digest of the plasmid with SalI (single restriction site at position 820 of the cDNA) and HindIII (single restriction site in the vector downstream of the cDNA insert) to remove 362 nucleotides from the 3' end of the cDNA. The plasmid was religated after fill in reaction with Klenow fragment (20). For the expression of a second truncated molecule, designated hr44-3, a PCR product was generated from part of the cDNA using the primers 5'GGATCGTTA-GGATTGTT and 5'ACGACGATGACGATAA, complementary to position 641–657 of the coding strand of the cDNA insert and complementary to pTrcHisB position 493–508 upstream to the multiple cloning region, respectively. The PCR fragment was cloned into pCRTMII (Invitrogen) and subsequently isolated from the plasmid after digest with XhoI (restriction site in the multiple cloning region of pTrcHisB) and HindIII (restriction site in pCRTMII). This allowed the directional reinsertion of this fragment into XhoI- and HindIII-digested pTrcHisB for in-frame expression. An irrelevant antigen derived from *O. volvulus* and designated Ov3.11 (Braun, G., unpublished data) was also expressed in pTrcHisB and used for control experiments.

Production and Purification of Recombinant Antigens. All recombinant antigens used in this study were expressed from pTrcHisB in *E. coli* NM522 upon induction with 0.75 mM isopropyl β -D-thiogalactoside at a density of $2\text{--}3 \times 10^8$ cells \cdot ml⁻¹. After 4 h, the cells were harvested from the LB culture medium (20) and lysed using a French press. The antigens were purified from the cytosolic fraction by column metal chelate chromatography using Pro-Bond resin (Invitrogen), as recommended by the supplier. A second purification step was conducted by gel filtration using Sephacryl S-200 (Pharmacia, Uppsala, Sweden) and PBS for elution. The purified antigens were used for immunizations to produce B cell hybridomas and reactive T cells and were used in ELISA and T cell response assays.

Antisera, SDS-PAGE, and Western Blot Analysis. Production of rabbit sera to native 44,000-M_r antigens of bovine optic nerve and to Ov39, expressed as fusion protein from pEX34b and pGEXI, was described elsewhere (14, 15). Hr44, immobilized onto EMphaze biosupport material (Pierce, Amsterdam, The Netherlands), was used to affinity purify specific Abs from serum to native ocular antigens. The Abs were eluted with 0.1 M glycine-HCl, pH 2.8, and neutralized with Tris-HCl, pH 8.8. Eluted Abs were made up to the original volume of serum. SDS-PAGE and Western blot analyses were performed as described previously (14).

B Cell Hybridomas, ELISA Assays, and Synthetic Peptides. mAbs were produced against Ov39 and hr44, both expressed in pTrcHisB. 6-wk-old BALB/c mice received two injections of the respective antigen. The first injection of 10 μ g antigen in FCA was administered intraperitoneally. The second immunization with 20 μ g of

the antigen resuspended in PBS followed 3 wk later by the same route. The spleen was removed 4 d after the second immunization. B cell hybridomas were produced using the P3 X63 Ag8.653 cell line (21) as a fusion partner for the sensitized spleen cells following the protocol by Galfre and Milstein (22). Cell culture supernatants were tested by ELISA for reactivity against Ov39, hr44, the control antigen Ov3.11, and the synthetic peptide MGGSHHHHHHGMASMTGGQQMGRDLYDDDD-KDPSSRSAAGTIW, which represents the carrier peptide of pTrcHisB. This peptide was HPLC purified and supplied by Dr. L. Packman (Department of Biochemistry, University of Cambridge). For ELISA, the peptide was resuspended in PBS (0.1 μ g \cdot ml⁻¹), and Ov39 and Ov3.11 were resuspended in 0.05 M carbonate buffer at a concentration of 1 μ g \cdot ml⁻¹ and 0.5 μ g \cdot ml⁻¹, respectively. ELISA plates were coated overnight. hr44 was resuspended in distilled water at a concentration of 5 μ g \cdot ml⁻¹ and dried onto the plate by evaporation. Ab binding was detected by using peroxidase-labeled goat anti-mouse γ chain-specific Ab (Sigma, Dorset, UK) and ABTS (2,2'-azino-bis (3-ethylbenzthiazoline 6-sulfonic acid) diammonium; Sigma). The mAbs to Ov39 are 39/24C5 and 39/21A1. mAb 44/72C2 was obtained after immunization with hr44. All cell lines were cloned at least three times by the limiting dilution method. The mAbs used in the study were of the IgG1 subclass, as determined by the red cell agglutination assay (Serotec, Oxford, UK), and they were obtained from serum-free tissue culture supernatants that had been concentrated 50 \times by ammonium sulfate precipitation.

Based on the primary structure of Ov39 (14), a set of 36 peptides was purchased from Cambridge Research Biochemicals (Cambridge, UK). Each of these peptides was immobilized on "pins" and consisted of 10 amino acids overlapping by 7 residues. These peptides were used in an ELISA-type assay, where sera raised against Ov39 expressed from pEX3b, pGEXI, and the carrier expressed from pEX34b (14) were used in a 1:5,000 dilution. Serum raised to the electroeluted ocular antigens was used in 1:100 dilution. The bleeds taken from the rabbits before immunization were also tested. The assays were carried out in accordance with the supplier's instructions.

T Cell Response Assays. Lewis rats (Harlan, Bicester, UK) were immunized once by plantar injection with 50 μ g of antigen in 100 μ g FCA supplemented with 5 mg \cdot ml⁻¹ of mycobacteria H37Ra, whole organism (Difco Laboratories, Detroit, MI). The antigens used were Ov39, hr44, and Ov3.11 as control. 10 d after immunization, the cells of the inguinal lymph nodes were isolated and cultured to establish antigen-specific T cell lines following a protocol by Ben-Nun et al. (23). The cells were restimulated at intervals of 10–14 d using irradiated (6,125 rads) rat thymus-derived APCs. Specific T cell lines for Ov39 and hr44 were stimulated five times and the specific T cell line for Ov3.11 four times with the respective homologous recombinant antigen at a concentration of 2.5 μ g \cdot ml⁻¹ before stimulation experiments were carried out with heterologous antigens at various concentrations. Stimulation was measured after incorporation of [³H]thymidine, 25 Ci \cdot mmol⁻¹ (Amersham International, Amersham, UK), which was added at 2×10^{-4} μ Ci per well for 18–20 h.

Immunocytochemistry. Rat eyes were fixed in 4% glutaraldehyde in PBS. The sections were mounted on slides coated with APES (3-aminopropyltriethoxy-silane) (Sigma) (24). The tissue sections were treated with 'STUF' (Serotec Unmasking Fluid) following the supplier's recommendations. Indirect immunoperoxidase staining was performed as described elsewhere (15) using rabbit serum (dilution range 1:20–1:160) and mAbs (dilution range 1:5–1:160 of the 50 \times concentrate). Species-specific biotin-

ylated secondary Abs and ExtrAvidin peroxidase (Sigma) were used for detection of Ab binding.

Controls for specificity of immunostaining with rabbit antiserum were (a) omission of the primary Ab; (b) use of serum taken before immunization; and (c) use of an irrelevant antiserum raised following the same immunization protocol using FCA. The controls for specificity of immunostaining with the mAbs were (a) omission of the primary Ab; and (b) use of irrelevant mAb 39/21A1.

Results

hr44, a cDNA Clone Derived from Human Retinal Tissue. To characterize the ocular antigen and the molecular basis of the immunological cross-reactivity, 5×10^5 phages of a cDNA library prepared from human retinal tissue were screened using rabbit serum raised against Ov39 expressed in pEX34b. Of the five clones obtained, one expressed an $\sim 155,000$ - M_r antigen with β -galactosidase, which was detected by Western blot (data not shown). This clone was designated hr44. The cDNA insert consists of 1,183 bp containing one open reading frame encoding 338 amino acid residues with a calculated molecular mass of 37281.3 D (Fig. 1). The 3' untranslated region downstream of the TAG stop codon at position 1016 consists of 167 bp. This region is highly AT rich, and it has several stop codons in all three reading frames. It does not contain a poly (A)⁺ tail nor a polyadenylation sequence, presuming the 3' EcoRI site is a gene-specific sequence rather than a restriction site introduced by added linker sequences in the process of cDNA cloning.

Serum to the native 44,000- M_r antigen was used to establish the identity of the clone hr44. As demonstrated by Western blot, this serum, like the serum to Ov39, reacts with a 44,000- M_r component of optic nerve (Fig. 2). It also recognizes the recombinant antigen hr44, which was expressed in an inducible fashion from pTrcHisB (Fig. 3). This reactivity is not based on recognition of the carrier peptide, since the serum does not react with the control antigen Ov3.11 (data not shown). Hr44-specific, affinity-purified Abs of this serum were used in Western blot and shown to react with a 44,000- M_r antigen of bovine retina (Fig. 4). This provides unequivocal evidence for clone hr44 to represent the native ocular antigen originally identified by serum to the parasite antigen Ov39.

mAbs to hr44 and Ov39. Additional evidence in support of the identity of hr44 is provided by data obtained using mAbs. Ab 39/24C5, one out of five mAbs to Ov39, was demonstrated to cross-react with hr44 by Western blot (Fig. 5). The cross-reactivity of 39/24C5 was not based on recognition of the carrier peptide, since ELISA results have shown that this mAb does not react with the control nor with the synthetic carrier peptide (Fig. 6). Binding of the antigens to ELISA plates was monitored using an mAb 39/21A1, which is specific for the carrier peptide of pTrcHisB (Fig. 6, inset). None of the 11 other mAbs to recombinant hr44 that have been characterized so far recognize Ov39. mAb 44/72C2 recognizes hr44, hr44-Sal, and hr44-3 by Western blot and ELISA (data not shown).

gaatccggAAGACACTTGAACCTGATACAGTTACAGGTAAGCTCTGGTGAGAAGATTGAC	60
(E) (F) (R) K T L E T D T V T G K S G E K I D	20
TACTCTACAACACCTCGAATTGATGAATGGCGTGAC AAGGGTTATCGCTTAGTTGAAGAT	120
Y S T T P R I D E W R D K G Y R L V E D	40
GGTTTCGCTGAAGCAGGCACACCGGTTTATGATGATAATGATGATGTCGAACAAATCTTT	180
G F A E A G T P V Y D D N D D V E Q I F	60
ATCGTTAAATTGGATCATGATACTGCACCAAGTTGGGCCAAATGATCCACATGAACAGAA	240
I V K L D H D T A P V G P N D P H E P E	80
ACACCAATCAATCCAAACGATCCAAATGGACCAAGTGGCCAGCTAAGGATAGCTACACA	300
T P I N P N D P N G P K W P A K D S Y T	100
AAACAATATACGTC AACGGTTCATTTGTAGATGAAAACGGTAATAAATACACGATTAT	360
K Q Y T S T V H F V D E N G N K L H D Y	120
AACGTTCAACATCAAATGGACACGAACATGATGTCGATAAGGCTACTGGTGAATC	420
N V Q T S N W T R T L I V D K A T G E I	140
TTGAATCCAGATGAAGCATGGCATTCTGATATTGATAAGTATTCTGATGTCAAAGTTGAT	480
L N P D E A W H S D I D K Y S D V K V D	160
CCTATTAAAGGGCTACTATACTGCCCAATAGTGTACCAGGTGAAGTACACACAACAA	540
P I K G Y Y T D R N S V P G E V A Q Q Q	180
GATATTGTGAATACGGTTGTTTATAAGCAAATGGTAAAGTGTACCGGTTGATCCATCA	600
D I V N T V V Y K Q I G K I V P V D P S	200
GGAAACCAATCCAGATGCACCAACACCACAATACAGAAACAATCCATACAGTCCAACT	660
G N P I P D A P T P Q Y R N N P N D P T	220
AAGGTTATCGTAAACGAATACGCCAGAAATCCAGGTATGACACCAGAATACCAAGTGT	720
K V I V T N T P E I P G M T P E V P S V	240
ACACCAGAAAATCCAGGTGAAGATACACCAATTTGTACAAACAGGTTGAAACACCAACA	780
T P E N P G E D T P I V Y N K V E T P T	260
CCAAGACAGTAGATGTCACCCAGAAACAGTCCAGGTTACGTCAGAAACA	840
P K T V D V T P E T P K T V D V T S E T	280
CCAAAGGCAACACCAGTTAAAACCTGCGCCTCAAGTTGAAAAGTTAAGCGCCAGAAATTA	900
P K A T P V K T A P Q V E K V K A P E L	300
CCACAACTGGTGAAGCAAAAATAGCGGTTAACTGCCGTCGGTGCATCTATTATGACT	960
P Q T G E K Q N K A L T A V G A S I M T	320
GGTCTATTAGGAATCCTTGCCTTTGCTAAGAAAAGAGGATGACAAAGAAATCTAGTCA	1020
G L L G I L A F A K K K K D D K E I *	338
TTTGGTTTGAAGAAAGAGTCCGAAAGACTCTTTTATTTATGGTGTGCGTGATAATAGGT	1080
TAATTTATTTTGGTAATATCTCATGAAAACATATGCATAAATTTAGGTGCAAAATTAGCC	1140
AACGATATATCAATATTTTATATAATAGTTCGCCGGAATC	1183

Figure 1. cDNA sequence and deduced protein sequence of hr44. The single-letter amino acid code is given below the first nucleotide of each codon. The amino-terminal three residues are encoded by the linker sequence. Repetitive amino acid sequences are underlined. Arrows 1 and 2 indicate the 3' end of the truncated molecules hr44-Sal and hr44-3, respectively. These sequence data are available from EMBL under accession number X91103. Composition: A, 16; G, 19; L, 11; Q, 11; V, 33; D, 32; H, 5; M, 2; R, 7; W, 4; E, 24; I, 19; N, 20; S, 12; Y, 12; F, 5; K, 30; P, 38; T, 38. Total: 339.

The Primary Structure of hr44. The primary structure of hr44 was deduced from the nucleotide sequence and is presented in Fig. 1. Proline and threonine, each comprising 11.2% of the total amino acid content, are the most abundant. Unlike threonine, the proline residues are not equally distributed throughout the molecule. A cluster of proline residues is found between positions 70 and 94. 55% of the proline residues, however, are positioned between residues 203 and 298. A number of repetitive sequences are located in this area. The fragment of the protein up to position 310 is predominantly hydrophilic. This is consistent with the finding that the purified recombinant antigen is soluble in aqueous solution. The amino acid sequence between positions 310 and 329, however, has an exclusively apolar char-

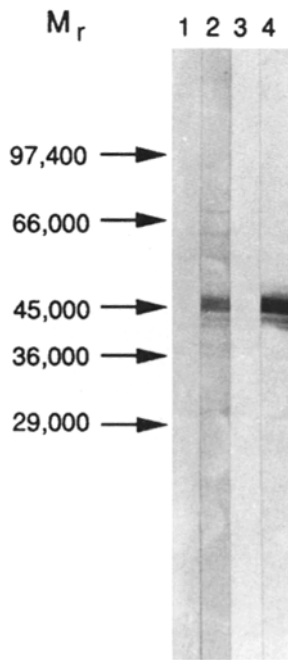


Figure 2. Recognition of the 44,000- M_r antigen in a total extract of bovine optic nerve. Proteins were electrophoretically separated on a 10% acrylamide/SDS gel under reducing conditions, and the antigen was detected by Western blot using rabbit sera. (Lane 1) serum taken before immunization with Ov39. (Lane 2) Serum raised against recombinant Ov39 expressed from pEX34b. (Lane 3) Serum taken before immunization with ocular antigens. (Lane 4) Serum raised against the 44,000- M_r antigen of optic nerve.

acter with typical features of a transmembrane domain, not interrupted by nonpermissive residues such as K, H, R, D, E, N, or Q. This sequence is predicted to form the helical structure of a single transmembrane-spanning region. A cluster of consecutive basic residues, KKKKDDK, is found

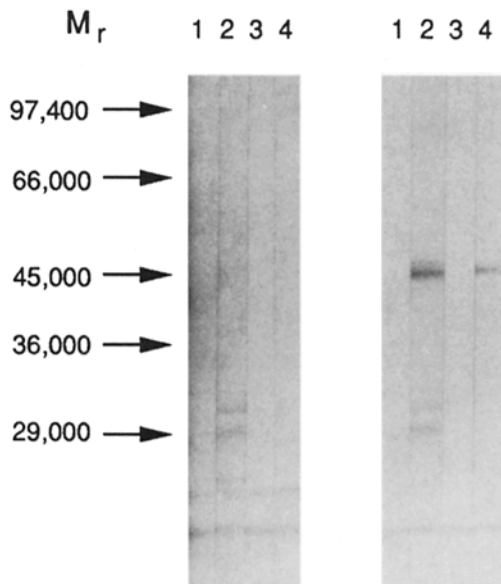


Figure 3. Induced expression of the hr44 from pTrcHisB in *E. coli* NM522. Proteins of an uninduced (right) and an induced (left) bacterial lysate were electrophoretically separated on a 10% acrylamide/SDS gel under reducing condition, and the antigen was detected by Western blot using rabbit sera. (Lane 1) Serum taken before immunization with Ov39. (Lane 2) Serum taken after immunization with Ov39 expressed from pEX34b. (Lane 3) Serum taken before immunization with ocular antigens. (Lane 4) serum taken after immunization with serum raised the 44,000 M_r antigen of the optic nerve.

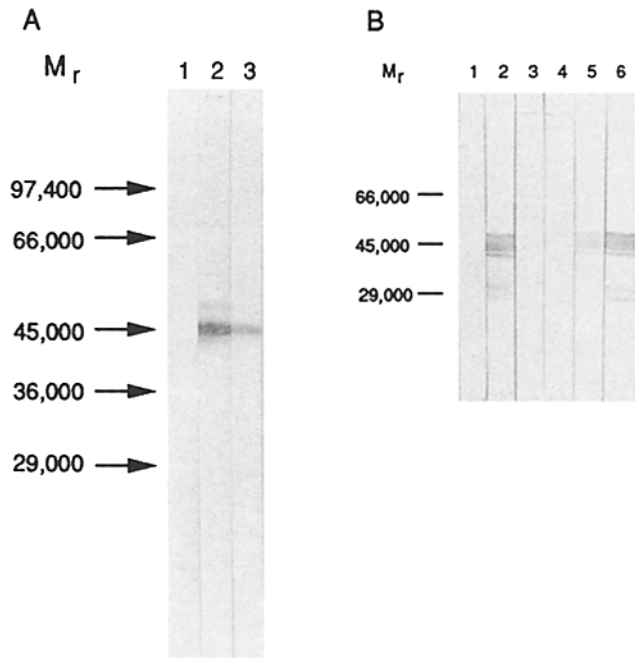


Figure 4. Recognition of the 44,000- M_r native ocular antigen by affinity-purified Abs. The antigens of an extract of bovine retina (A) and a lysate of *E. coli* NM522 induced to express hr44 from pTrcHisB (B) were electrophoretically separated on a 10% acrylamide/SDS gel, and the recombinant and native antigens were detected by Western blot. (A, lane 1) Serum taken before immunization with the ocular antigens, dilution 1:800. (A, lane 2) serum taken after immunization with the native 44,000- M_r optic nerve antigen, dilution 1:800. (A, lane 3) Abs that were affinity-purified from serum to native ocular antigen using the recombinant antigen hr44, dilution 1:50. (B, lane 1) Serum taken before immunization with the native 44,000- M_r ocular antigen, dilution 1:800. (B, lane 2) rabbit serum to the native 44,000- M_r ocular antigen, dilution 1:800. (B, lanes 3 and 4) serum to the native antigen depleted of Abs specific for recombinant hr44, dilutions 1:800 and 1:200, respectively. (B, lanes 5 and 6) affinity-purified Abs specific for hr44, dilutions 1:200 and 1:50, respectively. These Abs were used in A, lane 3.

at the very carboxy terminus at position 330. This is consistent with the requirement of positively charged residues in the cytoplasmic segments of transmembrane proteins (25, 26).

The sequence from residue 300–329 has also some homology with the proposed sites of cleavage and glycosylphosphatidylinositol attachment sequences found in glycosylphosphatidylinositol (GPI)¹-anchored proteins of eucaryotes. At position 300–305, the sequence contains a hexapeptide, LPQTGE, identical to the consensus sequence that has been described as the membrane anchor region for surface proteins, particularly of Gram-positive bacteria (27). This sequence of hr44 has homology with GPI-anchored molecules associated with cell adhesion, including the neural cell adhesion molecule N-CAM; the lymphocyte function-associated protein LFA-3, and the *Dictyostelium discoideum* contact site A molecule CsA, compiled by Ferguson and Williams (28). The sequence downstream of the hexapeptide has homology with GPI-anchored cell surface molecules

¹Abbreviation used in this paper: GPI, glycosyl-phosphatidylinositol.

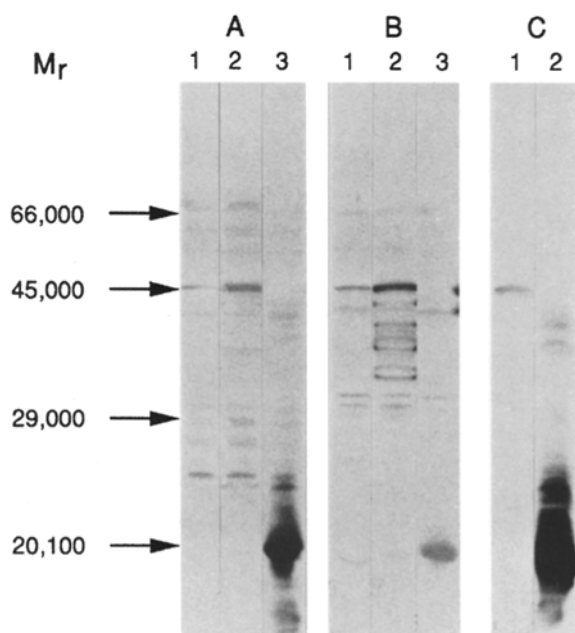


Figure 5. Recognition of Ov39 and hr44 by the cross-reactive mAb 39/24C5. Bacteria extracts were electrophoretically separated on a 10% polyacrylamide/SDS gel, and the antigens were detected by Western blot. (A) Serum to Ov39. (B) Serum to native 44,000- M_r optic nerve antigen. (C) mAb 39/24C5. (Lanes 1 in A and B) Lysate of uninduced *E. coli*. (Lanes 2 in A and B) Lysate of *E. coli* induced to express hr44 from pTrcHisB. (Lane 3) Lysate of *E. coli* induced to express Ov39 from pTrcHisB. (Lane 1 in C) Lysate of *E. coli* induced to express hr44. (Lane 2 in C) lysate of *E. coli* induced to express Ov39.

such as the acetylcholinesterase of *Drosophila*, liver alkaline phosphatase, and gp190 of *Plasmodium falciparum*. The hr44 clone does not contain the amino terminus of the antigen, and a signal sequence could not be predicted.

Primary Structure Comparisons. Comparative analysis of the primary structures of hr44 and Ov39 showed that the two antigens are not homologues, and the local identities found between these antigens are not extensive (Fig. 7). With reference to the respective amino acid residues, these are located at positions 129-140 (hr44) and 88-99 (Ov39) with 41.7% identity in the 11-amino acid overlap (Fig. 7 A); at positions 133-145 (hr44) and 99-111 (Ov39) with 30.8% identity in the 12-amino acid overlap (Fig. 7 B); at positions 143-147 (hr44) and 55-59 (Ov39) with 60.0% identity in the 5-amino acid overlap (Fig. 7 C); and at positions 248-255 (hr44) and 18-25 (Ov39) with 62.5% identity in the 8-amino acid overlap (Fig. 7 D). This localizes identities in three areas of Ov39. In hr44, the identities are confined to two areas comprising residues 129-147 and 248-255.

Both hr44 and Ov39 were compared with entries of DNA and protein data bases. Neither hr44 nor Ov39 is homologous to any of the registered protein sequences to an extent that might give any information as to the potential functions of these antigens. For Ov39, however, sequence identity was found with bovine and human visual rhodopsin over a stretch of eight residues. The octapeptide CAV-

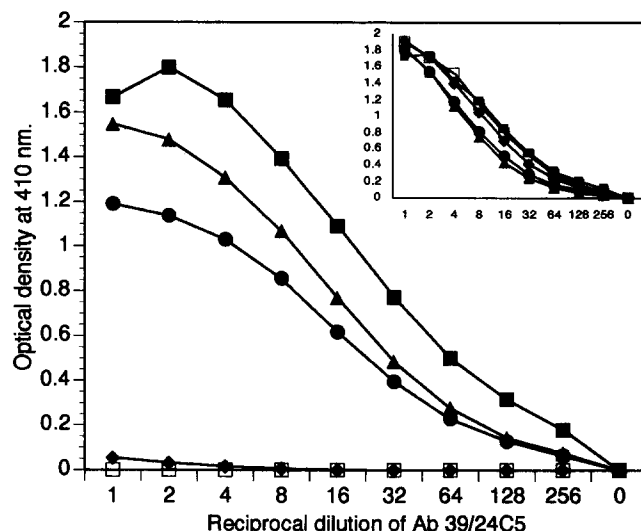


Figure 6. ELISA demonstrating the cross-reactivity between Ov39 and hr44 using the mAb 39/24C5. ELISA plates were coated with optimum dilutions of the following antigens, Ov39, hr44, hr44-Sal, Ov3.11, and the synthetic peptide representing the carrier of pTrcHisB. Plates were probed with doubling dilutions of 39/24C5 and 39/21A1 (inset), which are specific for the carrier peptide. The reactivity of 39/24C5 with each antigen was determined at a time point when the control Ab 39/21A1 gave an OD of ~ 1.8 . —■—, Ov39; —●—, hr44; —▲—, hr44-Sal; —◆—, Ov3.11; —□—, carrier.

PPFVG residue 10-17 of Ov39 is 75% identical to human rhodopsin residue 167-174 comprising CAAPPFAG.

Linear Epitopes of Ov39. Immunological cross-reactivity between Ov39 and hr44 may either be based on linear sequences and/or on recognition of discontinuously positioned residues. Whether or not linear sequences of Ov39 are recognized by sera was tested by ELISA using a set of 36 peptides (Fig. 8). Rabbit sera to two constructs of Ov39 were used in these experiments. Both sera recognize peptides 4, 5, 17, and 18. In addition, sera to Ov39 expressed from pGEXI also recognizes peptides 6, 7, and 16 (Fig. 8, B and C). Serum raised against the electroeluted 44,000- M_r optic nerve antigen recognizes peptides 16 and 17 (Fig. 8 E). The cross-reactive mAb 39/24C5 did not react with any linear sequence of Ov39.

T Cell Response Assays. The T cell lines that were established are all specific for recombinant antigens that share

A 41.7% identity	B 30.8% identity
129 ATLIUDKATGEI 140	133 VDKATGEILNPDE 145
: :	:: :: : :
88 ATLNYDLKKQEL 99	99 LSKLREEILSKKQ 111
C 60% identity	D 62.5% identity
143 PDEAH 147	248 DTPIVVVK 255
: :	
55 PDEUR 59	18 DLPIAENK 25

Figure 7. Identities between the deduced protein sequence of hr44 and Ov39 (SwissProt, accession No. P31730). The top sequence in each alignment represents hr44 compared with the sequence of Ov39 that is printed underneath. Numbers indicate the amino acid residue position within the respective sequence.

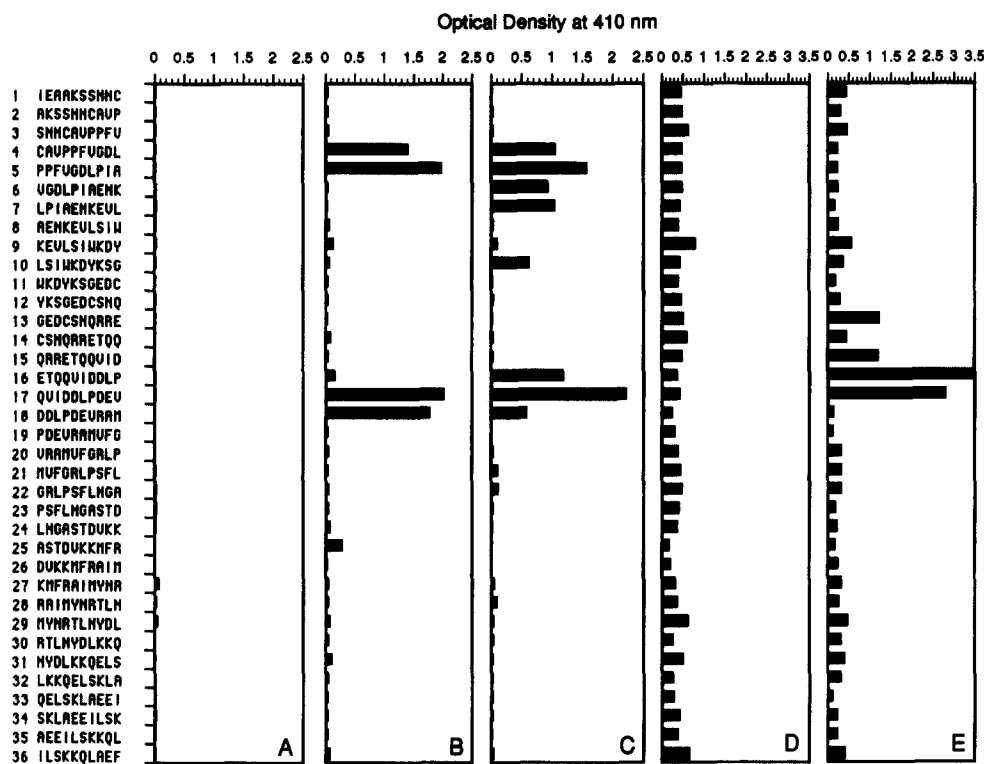


Figure 8. Mapping of linear epitopes of Ov39 by ELISA. Peptide 1 represents the amino-terminal 10 amino acids of Ov39. Each peptide consists of 10 residues that overlap the preceding peptide by 7 residues. (A) Serum to the expressed carrier of pEX34b. (B) Serum to Ov39 expressed from pEX34b. (C) Serum to Ov39 expressed from pGEXI. (D) Rabbit serum taken before immunization with the native 44,000- M_r ocular antigen. (E) Serum to the native 44,000- M_r antigen.

the same carrier peptide. These are Ov39, hr44, and Ov3.11 (Fig. 9). The cell line specific for Ov3.11 was used as control and was shown to respond only to antigen Ov3.11 (Fig. 9 A). The synthetic carrier peptide derived from pTrcHisB was used as a control and does not stimulate the Ov3.11-specific T cell line nor the cell lines specific for Ov39 or hr44 (Fig. 9, B and C). hr44-specific cells respond to the homologous challenge with hr44 and with both the truncated molecules, hr44-Sal and hr44-3. Background incorporation obtained without stimulation is highest for hr44-specific cells and similar counts to the background are obtained after stimulation with Ov39 and Ov3.11. In contrast, T cells specific for Ov39 show proliferative responses to both the derivative molecules of hr44. These cells, however, do not respond to the entire molecule of hr44.

Immunocytochemical Localization. The immunocytochemical localization of the ocular antigen was conducted using rabbit antiserum to the native 44,000- M_r antigen and two mAbs, 44/72C2, which is specific for the recombinant hr44 antigen, and 39/24C5, which has been shown to react with hr44 and the parasite antigen Ov39 (Fig. 10). The 44,000- M_r antigen is detectable in all neural-derived tissues of the eye, including the optic nerve (Fig. 10, A and B), the retina, the retinal pigment epithelium (Fig. 10, C-F), the epithelial (neural-derived) layers of the iris, ciliary body, and pars plana (Fig. 10, G-f). The inner and outer segments of the photoreceptor cells were not stained except for the myoid region. This region of the photoreceptors was stained by 44/72C2 but not by the other reagents. This mAb also showed stronger reactivity with both the retinal

pigment epithelium and ciliary and iris epithelium than the other two reagents.

Discussion

The role of molecular mimicry in autoimmunity (29-31) finds increasing support as mechanisms are identified that show how a sustained autoimmune response can develop from serologically cross-reactive antigens that involve not only B cells but also allow priming of autoreactive T cells (32). Ov39 and hr44 are antigens that may drive such a response. These antigens are not homologous and the serological crossreactivity seems to be based on coincidentally shared conformational structures similar enough to engage Ab cross-reactivity. This is supported by the reactivity of the mAb 39/24C5 that has affinity for both molecules but does not recognize any decamer peptides of Ov39. In contrast, polyclonal sera to Ov39 and the native ocular antigen react with a peptide of Ov39 (QVIDDLPDEV), for which some sequence identity with hr44 was found (PDEA, residue 143-147). Various degrees of identity was also found for other peptides of Ov39 and hr44. The contribution of the corresponding peptides to the antigenicity of Ov39 and hr44 will be entirely dependent on the conformation of the native proteins. These peptides, therefore, may or may not contribute to the formation of a nonlinear cross-reactive epitope.

Ov39 and hr44 may share at least one T cell epitope, since rat T cells specific for Ov39 can be stimulated with both truncated versions of hr44, hr44-Sal, and hr44-3. This

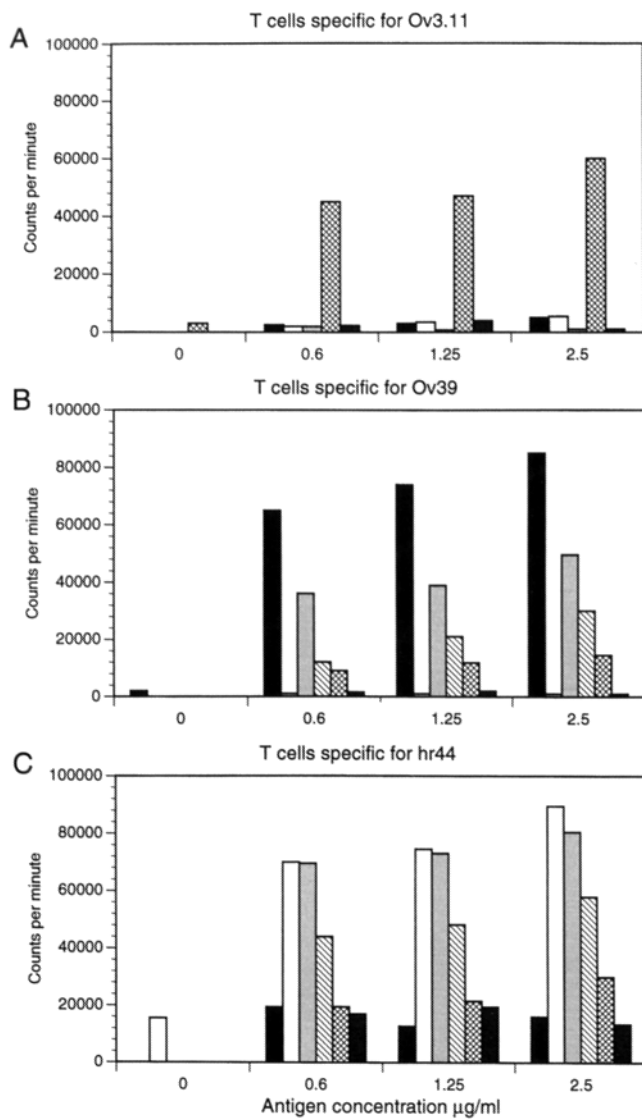


Figure 9. T cell response assays. (A) T cell line specific for Ov3.11. (B) T cell line specific for Ov39. (C) T cell line specific for hr44. The first column in each graph demonstrates the background counts without addition of antigen. The antigens used for stimulation were Ov39, Ov3.11, hr44, hr44-Sal, hr44-3, and the synthetic peptide representing the carrier of pTrcHisB. ■, Ov39; □, hr44; ▨, hr44-Sal; ▩, hr44-3; ■, Ov3.11; ■, carrier.

suggests that the cross-reactive epitope(s) is located within the amino-terminal 219 amino acid residues. The full-length molecule of hr44, however, does not stimulate Ov39-specific T cells. The hydrophobic carboxy terminus of hr44 does not appear to influence the uptake by APCs, since hr44-specific T cells responded to stimulation with the full-length molecule. The various derivative molecules of hr44 may be processed in different ways, generating the cross-reactive epitope from the truncated molecules of hr44 but probably not at all, or not sufficiently enough, from the full-length antigen. Contamination of the antigen preparations with bacterial components can be excluded since Ov3.11-specific cells are not stimulated by any other antigen prepared from the same bacterial strain. The carrier

peptide common to all antigens may also be excluded as a potential source of cross-reactivity since none of the T cell lines responded to stimulation with the synthetic carrier molecule.

Several mechanisms of antigen processing have been suggested to be responsible for the generation of either immunodominant or cryptic epitopes (33). In healthy humans, there exists an extensive T cell repertoire that seems to have escaped tolerance and is directed against determinants that are considered nondominant (34). In the development of pathology recruitment of these cells may play a vital role. It has been speculated that the initial priming event could be by exposure to a dominant determinant on a protein from an infective agent that may induce a response that is cross-reactive with a cryptic self determinant (35). In Ov39, the cross-reactive epitope seems dominant, since Ov39-specific T cells still respond to truncated versions of hr44 after repeated stimulation with Ov39. The T cell repertoire specific for hr44 does not contain or maintain a population of T cells that respond to stimulation with Ov39. Consequently, it is possible that the cross-reactive T cell epitope of hr44 is subdominant or cryptic and may only be generated from the truncated derivatives of hr44.

The cross-reactive T cell epitope(s) may be within the peptides for which primary sequence identities have been found. A peptide derived from Ov39 (residue 88-111) and a peptide derived from hr44 (residue 129-145) can be matched in two different alignments and may therefore make a good candidate for a cross-reactive T cell epitope. The peptide of hr44 consisting of residue 123-139, also has identity with the conserved consensus of a defined motif, the "HLA-DR α " motif that has been described for seven human proteins, four of which are implicated in development of autoimmune disease. These include HLA-DR α , glutamic acid decarboxylase, heat shock protein 65, and insulin receptor (36). For HLA-DR α , this sequence has been identified as a self T cell epitope (37).

Although the amino terminus with a potential signal sequence has not been cloned, analysis of the primary structure suggests that hr44 is a type I membrane protein with an amino-terminal extracytosolic domain, a transmembrane domain, and a very short carboxy-terminal cytosolic fragment consisting of basic residues. Such residues are vital in determining the topology of transmembrane proteins (26, 38), and they tend to be localized in those parts of the protein that have not been translocated across the bilayer (39). The residues that constitute the cytoplasmic domain can be comprised of as few as three amino acid residues (40). In addition, the protein also has homologies with the proposed cleavage and GPI attachment sequences of some adhesion molecules (28). Hr44 may be the primary translation product and, similar to some other GPI anchored proteins, the hydrophobic carboxy terminal sequence may serve as an intermediate transmembrane anchor for the protein in the endoplasmic reticulum before the formation of the glyphospholipid anchor. Isoforms of surface molecules ascribed to the generation of alternative splice products, as shown for N-CAM, have also been identified. By Western

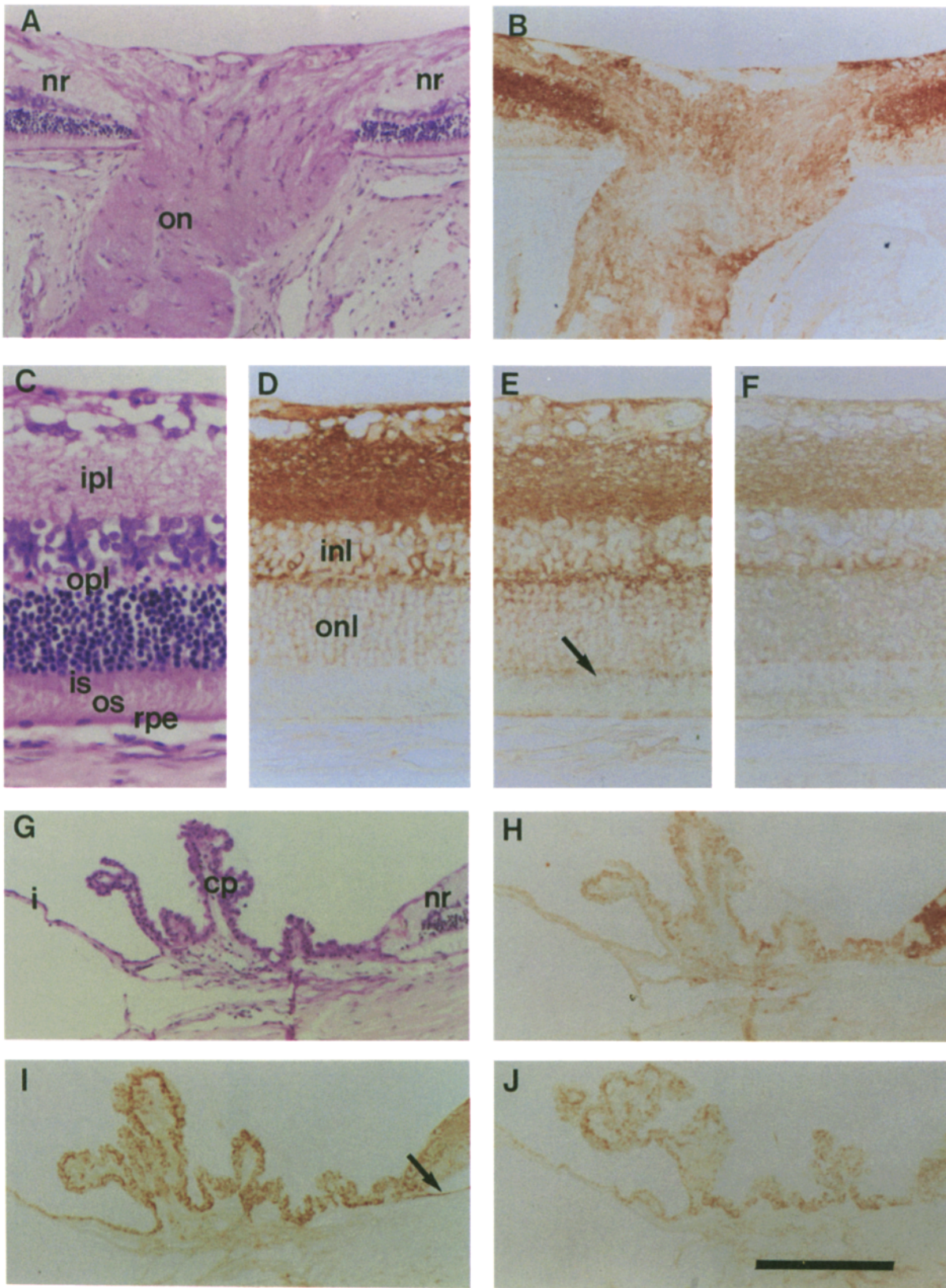


Figure 10.

blot analysis, it has been observed that the native ocular antigen often appears as a doublet (15).

Hr44 has no cysteine residues that would allow for disulfide bridges and formation of Ig-like C2 domains, as shown for many eucaryotic surface molecules involved in cell adhesion. Since antiserum to the native antigen recognizes the recombinant hr44 molecule, extensive posttranslational modification may not occur. hr44, however, has 20 potential N-glycosylation sites and 2 serine-threonine clusters that could serve as acceptors for O-linked carbohydrates (41). It does not show glycosaminoglycan attachment sites that contain features of the described consensus acceptors (42, 43). The calculated molecular mass of the fusion protein expressed from pTrcHisB is 41.957,33 D, of which the amino terminal carrier peptide contributes 4.676 D. This is not consistent with the electrophoretic mobility of hr44. The aberrant slow migration of hr44 may be due to its high content of prolines and basic residues (44). The proline-rich repeat region may serve as a hinge, conferring some flexibility to the extracytosolic part of the molecule.

The mAbs to Ov39 and hr44 and the rabbit serum raised to the native ocular antigen localize hr44 in the plexiform layers of the retina that are particularly rich in membrane structures. The myoid region of the photoreceptor cells, which was stained by the mAb 44/72C2, is known to be the location of Golgi bodies and endoplasmic reticulum in these cells, and staining may result from reactivity with newly synthesized protein.

The membrane-associated antigen localized in the neurotissue and the blood-ocular barrier may become the target of antiparasite-directed, Ab-dependent cellular cytotoxicity, as well as complement-mediated cell lysis by formation

of a cytocidal membrane attack complex (MAC). Most human cells and tissues are protected against complement-mediated lysis by expression of an inhibitory molecule CD59 (45). However, the absence of CD59 from some cell types, such as oligodendrocytes (46) in the central nervous system, which includes the optic nerve, may make these cells susceptible to MAC attack in conditions where the blood-tissue barrier is damaged.

Direct sequence comparisons between host molecules and molecules from potentially infective agents by searching protein data bases have frequently been used to suggest potential immunological cross-reactions. Using this approach it was found that an Ov39-derived peptide is identical to an octapeptide of human rhodopsin, CAAPPFAG, except for two conserved exchanges. The biological turnover of photoreceptor outer segment involves ingestion and degradation of rod outer segment material by the retinal pigment epithelium (47). Rhodopsin may be presented by those cells in the context of HLA molecules or released from damaged retinal pigment epithelial cells. This sequence of rhodopsin is not found in any of the three human cone pigments. This may be of relevance when considering the typical sparing of the macula seen in eyes affected with otherwise widespread chorioretinal lesions.

The immune responses to a diversity of retinal antigens in human infection sera has been reported before (48-50) and may be explained by determinant spreading (51) caused by an underlying disease process. We suggest that cross-reactive cellular and humoral responses to hr44 might play a significant role in the initiation and perpetuation of ocular pathology seen in onchocerciasis.

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Figure 10. Immunocytochemical localization of the 44,000- M_r antigen in rat ocular tissues. (A) Hematoxylin and eosin staining of a section of optic nerve and retina (*nr*, neural retina; *on*, optic nerve). (B) Similar section immunostained with serum to the native 44,000- M_r antigen. The antigen is present in the inner layers of the neural retina and the entire optic nerve. (C) Neural retina stained with hematoxylin and eosin: *ipl*, inner plexiform layer; *opl*, outer plexiform layer; *is*, photoreceptor inner segments; *os*, photoreceptor outer segments; *rpe*, retinal pigment epithelium. (D) Serum to the native 44,000- M_r antigen. Reactivity is detectable throughout the neural retina, particularly in the plexiform layers with weaker staining of both the inner nuclear layer and the outer nuclear layer (*inl* and *onl*, respectively). The photoreceptor inner and outer segments do not stain. Faint patchy staining of the retinal pigment epithelium can be seen. (E) mAb 44/72C2. A similar staining pattern apart from additional staining of the myoid region of the photoreceptor inner segment (*arrow*) can be seen. (F) mAb 39/24C5 produces similar though less intense staining of the neural retina. (G) Hematoxylin and eosin staining of: *i*, iris; *cp*, ciliary process; *nr*, peripheral neural retina. (H) Serum to the native 44,000- M_r antigen. Staining of the epithelial layers of the iris and ciliary processes with stronger staining of the peripheral neural retina can be seen. (I) mAb 44/72C2. Staining of the epithelial layers of the iris and ciliary body and also the retinal pigment epithelium of the peripheral retina is detectable. (J) mAb 39/24C. The staining is similar to but weaker than that obtained with the mAb to hr44. Bar, 200 μ m in A, B, G, and H-J, and 80 μ m in C-F.

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