RPE65 Is Highly Uveitogenic in Rats

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PURPOSE. To examine the hypothesis that RPE65, a protein specific to the retinal pigment epithelium, is uveitogenic in rats.

METHODS. Rats of four inbred strains (Lewis, Brown Norway, Fischer, and SHR) were immunized with native or recombinant bovine RPE65, or with S-antigen (S-Ag), emulsified with complete Freund adjuvant, and treated simultaneously with killed Bordetella pertussis bacteria, as indicated. Development of ocular changes was examined and scored both clinically and histologically.

RESULTS. Lewis rats immunized with RPE65 showed development of acute and severe inflammatory eye disease that affected most ocular tissues. The minimum uveitogenic dose of RPE65 was similar to that of S-Ag (1 μg per rat), but the changes induced by RPE65 at higher dose ranges were less severe than those induced by S-Ag. Concurrent treatment of the RPE65-immunized rats with B. pertussis bacteria was not critical for disease induction, but enhanced dramatically the pathogenic reaction. Unlike the results with several other retinal proteins, no pinealitis was detected in rats immunized with RPE65. Fischer (F344) rats resembled Lewis rats in being similarly affected by RPE65 or S-Ag. In contrast, Brown Norway (BN) rats developed severe disease when immunized with RPE65, but showed minimal changes in response to S-Ag. SHR rats responded poorly to disease induced by RPE65, and S-Ag-induced disease failed to develop.

CONCLUSIONS. RPE65 is highly uveitogenic in rats, thus suggesting that this molecule could be involved in pathogenic autoimmunity in the human eye. (Invest Ophthalmol Vis Sci. 2002;43:2258–2263)

The complex process of vision uses a large battery of molecules that are unique to the corresponding eye tissues. A remarkable number of these ocular-specific molecules, most of which are proteins, have been found to be uveitogenic—that is, capable of initiating ocular inflammation when injected to susceptible experimental animals.1–3 So far, at least eight ocular-specific molecules have been reported to be immunopathogenic, a number greater by far than the numbers of immunopathogenic antigens identified in other organs in which autoimmune diseases can be induced, such as the brain, thyroid, or testes. The known uveitogenic proteins include S-antigen (S-Ag, arrestin), interphotoreceptor retinoid-binding protein (IRBP), rhodopsin, recoverin, phosducin, pigment epithelial polypeptide preparation (PEP-65), melanin-associated antigen, and tyrosinase related protein-1.4–11 The inflammatory eye diseases induced by these proteins vary in the tissues mostly affected and have been designated accordingly: experimental autoimmune uveoretinitis (EAU); experimental autoimmune anterior uveitis (EAU); also known as experimental melanin-induced uveitis (EMIU); and experimental autoimmune posterior uveitis (EAU).

In this study we investigated the uveitogenic capacity of RPE65. RPE65 is a 61-kDa protein, specifically and abundantly expressed in the retinal pigment epithelium (RPE) and associated preferentially with the microsomal membrane fraction of the RPE.12–15 RPE65 is highly conserved across vertebrates from human to salamander, and plays an essential role in vitamin A metabolism necessary for the synthesis of the visual pigment chromophore 11-cis retinal.16–19 Mutations of RPE65 were identified among patients with Leber congenital amaurosis, retinitis pigmentosa, and autosomal recessive childhood-onset severe retinal dystrophy.20–22 In view of its eye-specific expression, we hypothesized that RPE65 could be uveitogenic.

Our data showed that, indeed, RPE65 was a potent uveitogenic molecule, inducing EAU in Lewis rats at doses as low as 1 μg per rat. In addition to Lewis rats, RPE65 was found to be uveitogenic in three other inbred rat strains. RPE65-induced EAU was found to exhibit certain distinct clinical and histopathologic features. Unlike in rats immunized with several other retinal proteins, no pinealitis was detected in RPE65-immunized rats.

MATERIALS AND METHODS

Animals

Male Lewis, Brown Norway (BN), Fischer (F344), and SHR rats, approximately 8 weeks old, were supplied by Charles River (Raleigh, NC). All animal procedures were performed in adherence to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

Antigens

Native RPE65 was purified from the microsomal fraction of fresh bovine RPE, prepared as previously described15 and stored at −80°C until used. Thawed microsomes were pelleted at 100,000g for 30 minutes in a ultracentrifuge (TL-100; Beckman Instruments, Carlsbad, CA) at 4°C. They were then dispersed in 1 M acetic acid and incubated on ice for 30 minutes with occasional agitation. The suspension was centrifuged at 100,000g for 30 minutes, and the supernatant, containing RPE65, was dialyzed against phosphate-buffered saline containing 0.3% 3-[3-cholamidopropyl]dimethylammonio-2-hydroxy-1-propane-

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Supported by a grant from Research to Prevent Blindness to the Department of Ophthalmology, University of Florida.

Submitted for publication December 27, 2001; revised March 4, 2002; accepted March 15, 2002.

Commercial relationships: N.

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sulfonate (CHAPS). 10 μM FeSO₄, 1 mM tris(2-carboxyethyl)-phosphine hydrochloride (TCEP), and 1 Complete (EDTA-free) protease inhibitor tablet (Roche Molecular Biochemicals, Indianapolis, IN) per 50 mL, with three changes. The dialyzed protein was then concentrated with ultrafiltration units (Centricon 10; Millipore Corp., Bedford, MA). Protein concentration was determined by the method of Bradford.²³

Recombinant RPE65 was prepared in an in vitro transcription-translation system (RTS 500; Roche Diagnostics GmBH, Mannheim, Germany). The coding sequence of bovine RPE65 was ligated into pIVEX2.4b, according to the manufacturer’s instructions. The RPE65-pIVEX plasmid was amplified in XL-1 Blue cells and isolated with a kit (QiAmp Plasmid Midikit; Qiagen, Valencia, CA) at a final concentration of 1.9 μg/mL. The 5’ and 3’ sequences of the insert were verified with an automatic sequencer (CEQ 2000; Beckman Instruments). Exposure of the plasmid was accomplished with the transcription-translation system. Plasmid DNA (10 μg) was used in a 1 mL volume of kit accompanying the transcription-translation system (RTS 500 Escherichia coli H’Y kit. Roche Diagnostics), reconstituted according to the manufacturer’s instructions, plus 10 μM FeSO₄ in both chambers and 0.1% CHAPS in the reaction chamber. Incubation proceeded at 30°C for 24 hours, with a stirring speed of 120 rpm. The expressed protein (approximately 250 μg) was localized entirely in the 16.000g pellet of the reaction mixture and was evaluated by immunoblot analysis with an antibody against residues 150-164 of bovine RPE65²⁴ (described later). The crude pellet, containing the expressed protein and insoluble components of the reaction volume, was taken up in 0.5 mL Tris-HCl (pH 7.4) and frozen.

Bovine S-Ag was prepared from PBS extracts bovine retinas that had been treated with ConA-Sepharose, as described by Adler et al.²⁵ The resulting unbound supernatant was then dialyzed against 10 volumes of 10 mM HEPES, 15 mM NaCl, 1 mM EDTA, 1 mM benzamidine (pH 7.0), with the buffer changed once. The S-Ag was prepared by the method of Palczewski et al.,²⁶ with modifications described by Puig et al.²⁶ The final elution from the heparin-agarose column was through a gradient from 10 mM HEPES, 15 mM NaCl (pH 7.0) to 10 mM HEPES, and 750 mM NaCl (pH 7.0) and poded based on the optical density profile at 278 nm. The resultant S-Ag was essentially homogeneous by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

**SDS Gel Electrophoresis and Immunoblotting**

The purity of the antigenic preparations was tested using SDS gel electrophoresis and immunoblot analysis. Samples were loaded onto a polyacrylamide gel (8% to 25% gradient, Phastgel; Amersham Pharmacia Biotech, Uppsala, Sweden) for SDS gel electrophoresis, and silver staining was performed according to the manufacturer’s protocol. For immunoblot analysis, electrophoresed samples were transferred to membranes (Immobilon-P; Millipore Corp.) by heat, as described in the gel manufacturer’s manual (Amersham Pharmacia Biotech). The blots were developed by the rapid dry blot method described in the user’s guide for the membrane (Immobilon-P; Millipore). The primary antibody used was a rabbit antibody made to residues 150-164 of murine RPE65. The secondary antibody was a goat anti-rabbit alkaline phosphatase conjugate (Novagen, Madison, WI), detected colorimetrically with 5-bromo-4-chloro-3-indoyl phosphate/nitroblue tetrazolium (BCIP/NBT) phosphatase substrate (KPL, Gaithersburg, MD).

**Immunization of Rats**

Rats were immunized by a single injection of various amounts of antigen, as indicated, emulsified by sonication in complete Freund adjuvant (CFA) prepared by grinding killed Mycobacterium tuberculosis bacteria (Difco, Detroit, MI) in incomplete Freund adjuvant oils (Difco) to a concentration of 2.5 mg/mL. The emulsion, in a volume of 0.2 mL, was injected subcutaneously into the tail base. Killed Bordetella pertussis bacteria (lot 94; Michigan Department of Public Health, Lansing, MI) were injected intravenously, 10⁹ organisms per rat, concurrently with the antigen emulsion.

**RESULTS**

**Purity of Antigenic Preparations**

The native bovine RPE65 preparation was evaluated for homogeneity by silver-stained SDS gel (Fig. 1A) and immunoblot analysis with antibody against peptide 150-164 of murine RPE65²⁴ (Fig. 1B). Similar results were obtained with the acetic acid extract of the RPE microsomes (lane 1) and the neutralized and Fe₃⁺-reconstituted preparation (lane 2). The major band, comprising more than 90% of the stained protein, was immunoreactive for RPE65. Minor smaller bands also showed RPE65 immunoreactivity, indicating some fragmentation of this protein. The identity of the recombinant protein was also evaluated and showed a strong band of RPE65 immunoreactivity (Fig. 1C) with more extensive fragmentation than in the microsomal extracts.

**Uveitogenic Capacity of RPE65**

Groups of Lewis rats were injected with differing doses of RPE65, and the development of EAU was monitored by both clinical and histologic examinations. Additional groups of rats were immunized with S-Ag, at the same doses, to compare the uveitogenicity of RPE65 with that of S-Ag, a well-investigated molecule. Table 1 summarizes data of three repeated experiments and shows that RPE65 was a highly potent uveitogen, matching S-Ag in its capacity to induce EAU at the tested low doses of 1 or 3 μg per rat. A good correlation was observed between the clinical and histologic analyses. Immunization of Lewis rats with RPE65 without B. pertussis treatment induced EAU, but the severity was less than that in the B. pertussis-treated rats (Table 1). A preparation of recombinant RPE65

![Figure 1. Gel electrophoresis and immunoblot evaluation of RPE65 preparations. (A) Silver-stained gel of an RPE65 preparation from bovine RPE (lane 1: acetic acid extract; lane 2: neutralized and Fe₃⁺-reconstituted acid extract). (B) Immunoblot of a duplicate to gel in (A). (C) Immunoblot of a recombinant RPE65 preparation. Molecular weight standards are to the left of (A) and (C).](image-url)


resembled the native preparation in its uveitogenic capacity (Table 1).

**Clinical Features of RPE65-Induced EAU**

Clinical changes were bilateral in all rats with RPE65-induced EAU, with very similar severity levels scored in both eyes. This feature differs from that in rats immunized with S-Ag, in which 9 of 30 diseased rats showed asymmetrical clinical EAU (score difference of >1.0), and unilateral disease observed in four of these animals. The disease onset range was similar in rats immunized with RPE65 and S-Ag (days 8–15 and 8–14 after immunization, respectively). The peak of clinical severity was observed in most rats on the second or third day after onset. The typical clinical changes in eyes with RPE65-induced EAU are shown in Figure 2B. These changes included engorgement of the iridic blood vessels, haziness of anterior chamber, inflammatory cellular deposits on the posterior corneal surface (keratic precipitates), hypHEMA, and decreased fundus light reflex. It is of note that RPE65 differed from S-Ag in usually producing intermediate levels of clinical changes, even at the higher doses of 10 μg or more. Thus, whereas all rats immunized with S-Ag at 30 μg showed clinical changes with the highest score of 4.0, the clinical changes in RPE65-immunized rats did not exceed 3.0, and mean scores were below 2.0. Moreover, intermediate scores of clinical changes were also observed in rats immunized with RPE65 at 100 μg (data not included in Table 1). The clinical changes induced by RPE65 subsided rapidly and disappeared within 3 to 9 days, markedly more rapidly than those in S-Ag-induced EAU.

**Histologic Features of RPE65-Induced Inflammation**

The presence of histologic changes correlated well with the presence of clinical disease. The histopathology in eyes with EAU induced by RPE65 generally resembled that of S-Ag-induced EAU in that it showed most ocular tissues to be affected. In the early phase, inflammatory cells infiltrated the ciliary body, iris, and around blood vessels of the limbus (Fig. 2C). Infiltrated cells accumulated in the anterior chamber and pars plana. They were both polymorphonuclear (PMN) and mononuclear leukocytes, with PMNs being the majority. At the same time, retinal blood vessels showed perivascular infiltration, and the retina became edematous (Fig. 2D). Inflammatory cells were also present in the neural retina and vitreous. In the peak phase, infiltrated cells in the anterior chamber disappeared rapidly, and the anterior segment was relatively intact, but the retina usually showed extensive serous and exudative detachment (Fig. 2F). Aggregates of inflammatory cells were observed in the choroid and subretinal space adjacent to the RPE (Fig. 2E). A unique histologic characteristic of RPE65-induced disease was accumulation of inflammatory cells in the pars plana and anterior peripheral retina (Fig. 2G). As the disease progressed, inflammatory cells gradually disappeared, and loss of the photoreceptor outer segment layer and outer nuclear layer was apparent (Fig. 2H). It is of interest, however, that there was no total loss of inner retinal layers in rats immunized with RPE65, features often seen in EAU induced by S-Ag3,28 (observations not shown here). Eyes of rats immunized with RPE65 often showed focal accumulations of cells beneath the RPE layer, simulating the Dalen-Fuchs nodule (Fig. 2I).

No pineal inflammation was detected in any of the rats immunized with RPE65 (Fig. 3B). In contrast, six of the 30 Lewis rats immunized with S-Ag had pinealitis caused by mononuclear cell infiltration (Fig. 3A and Ref. 29).

**Uveitogenicity of RPE65 in Different Inbred Rat Strains**

Groups of rats of the four inbred strains, with different major histocompatibility complex (MHC) haplotypes, were tested for susceptibility to EAU induced by RPE65 or S-Ag (Table 2). Moderate to severe EAU was induced by RPE65 in all rats of the Lewis, BN, and F344 strains, whereas SHR rats showed only minimal disease. Lewis, BN, and F344 rats also showed development of EAU after immunization with S-Ag, but with remarkable differences in severity. Whereas Lewis and F344 had EAU with severity scores of 4.0, the BN rats showed only mild disease with this antigen, in contrast to the development of severe RPE65-induced EAU in these animals. Typical changes in eyes of BN rats immunized with RPE65 are shown in Figures 2J, 2K, and 2L. It is of interest that the pigmented RPE in BN rats made it easier to identify the disruption of this layer by the inflammatory process (Fig. 2L). SHR rats did not respond to disease induced by S-Ag.

**DISCUSSION**

In the present study, RPE65 was a potent uveitogenic molecule, inducing EAU in Lewis rats at the low dose of 1 μg per rat.

### Table 1. Uveitogenicity of RPE65 in Lewis Rats

<table>
<thead>
<tr>
<th>Antigen</th>
<th>Dose (μg/rat)</th>
<th>Affected Rats/Total</th>
<th>Day of Onset (mean)</th>
<th>Severity (mean ± SE)*</th>
<th>Affected Rats/Total</th>
<th>Severity (mean ± SE)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>S-Ag</td>
<td>30</td>
<td>6/6</td>
<td>8.7</td>
<td>4.0 ± 0</td>
<td>6/6</td>
<td>3.9 ± 0.2</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>6/6</td>
<td>10.5</td>
<td>3.0 ± 1.3</td>
<td>9/9</td>
<td>4.0 ± 0</td>
</tr>
<tr>
<td></td>
<td>3–5</td>
<td>9/9</td>
<td>10.8</td>
<td>2.2 ± 1.3</td>
<td>9/9</td>
<td>3.8 ± 0.5</td>
</tr>
<tr>
<td>RPE65</td>
<td>1</td>
<td>1/9</td>
<td>14</td>
<td>0.5 ± 0</td>
<td>2/9</td>
<td>3.2 ± 0.3</td>
</tr>
<tr>
<td></td>
<td>30–50</td>
<td>9/9</td>
<td>9.2</td>
<td>1.6 ± 0.9</td>
<td>9/9</td>
<td>2.1 ± 1.1</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>9/9</td>
<td>10.8</td>
<td>1.4 ± 0.7</td>
<td>9/9</td>
<td>1.7 ± 0.6</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>4/6</td>
<td>11.5</td>
<td>1.8 ± 0.8</td>
<td>5/6</td>
<td>1.9 ± 1.3</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>2/6</td>
<td>10.5</td>
<td>0.9 ± 0.3</td>
<td>2/6</td>
<td>1.9 ± 0.3</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>1/9</td>
<td>8.0</td>
<td>0.6 ± 0.5</td>
<td>3/3</td>
<td>1.7 ± 0.6</td>
</tr>
<tr>
<td>RPE65†</td>
<td>30</td>
<td>2/3</td>
<td>10.5</td>
<td>1.0 ± 0.5</td>
<td>3/3</td>
<td>2.9 ± 0.2</td>
</tr>
<tr>
<td>rRPE65‡</td>
<td>35</td>
<td>3/3</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Only rats with disease were used for calculating the mean severity.
† No *B. pertussis* treatment was given to these rats.
‡ Recombinant RPE65.
This uveitogenic capacity resembled that of S-Ag (Table 1) and was only slightly lower than that of IRBP, a protein with the minimal uveitogenic dose of 0.3 μg per rat.5,12 The uveitogenicity of RPE65 exceeded that of all the other known retin-specific uveitogenic molecules, including rhodopsin, recoverin, phosducin, melanin-associated antigen, and tyrosinase-related proteins.6–11 The purity of the RPE65 preparation was demonstrated by SDS-PAGE and immunoblot assay, with an antibody specific against an RPE65 peptide, and the activity of a recombinant preparation further verified the uveitogenicity of this protein.

Our data suggest that RPE65 and the uveitogenic protein designated PEP-65 by Broekhuyse et al.9,30 are the same molecule. The molecular identity of PEP-65 was not determined by those investigators, however. In addition to their similarity in size, both proteins are extracted from the microsomal fraction of RPE. Another similarity between RPE65 and PEP-65 is that no pineal inflammation was detected in rats immunized with these two proteins. This observation is significant in view of the finding that pinealitis develops in rats immunized with at least three uveitogenic retinal antigens, S-Ag, IRBP, and recoverin.5,8,29 It is consistent with the absence of RPE65 in pineal gland in immunoblot assay.15

The ocular diseases described in the present study and that of Broekhuyse et al.9 differed in three aspects: (1) Pertussis toxin was essential for disease induction by PEP-65, even at the...
uveitis, minimally affects the anterior segment or the retina. In addition, typical features of RPE65-induced disease included retinitis and retinal detachment with exudate in the subretinal space (Fig. 2, SRS), features that are not reported to be common in PEP-65-induced uveitis. Unlike the acute ocular inflammation in the present study in rats immunized with RPE65, with rapid regression of inflammation, the ocular inflammation induced by PEP-65 is characterized by its chronicity.

It is conceivable that the differences between the ocular diseases induced by RPE65 and PEP-65 are mainly due to differences between the uveitogenic antigen-CFA emulsions used in the two studies. Thus, the CFA we used contained tubercle bacilli at a concentration of 2.5 mg/mL, which is higher than that in any known commercial preparations. In contrast, Broekhuysen et al. used a commercial preparation of an unspecified bacterial concentration. In addition, we prepared the immunizing emulsion by sonication, a procedure that was reported to enhance the immunopathogenicity of tissue-specific molecules substantially.

Of particular interest are the observations concerning the uveitogenicity of RPE65 and S-Ag in different inbred strains of rats. Whereas Lewis and F344 rats were similarly susceptible to the two antigens, BN rats were highly susceptible to RPE65 but poorly affected by S-Ag (Table 2). This observation with BN rats resembles our previous finding, that these rats are susceptible to EAU induced by IRBP, in contrast to their poor response to S-Ag. It is also noteworthy that BN rats are completely resistant to EAE induced by myelin basic protein (MBP). Our findings that BN rats were susceptible to EAU induced by RPE65 or IRBP suggest that the resistance to diseases induced by S-Ag or MBP is due to deficiencies in the T-cell repertoire, rather than to dominance of T helper 2 (Th2) cells in these rats. Unlike the other tested strains, SHR rats responded poorly to EAU induction by RPE65 and did not develop any disease when immunized with S-Ag.

The high uveitogenic capacity of RPE65 suggests that this protein could be involved in certain uveitic processes in which autoimmunity has been proposed to play a major pathogenic role. In addition, immune processes have been suggested to participate in the pathogenesis of conditions such as age-related macular degeneration (AMD), in which the RPE cell layer is a major target for the disease process. We are continuing to examine the possible participation of autoimmunity against RPE65 in the etiology of human eye diseases.

**Acknowledgments**

The authors thank Paul Hargrave for support and critical reading of the manuscript, Barbara P. Vistica for excellent technical assistance, the National Eye Institute’s Histolab for preparation of tissue sections, and Shauna Everett and Ricardo Dreyfuss for digital microphotography.

**Table 2. Susceptibility of Rats of Different Inbred Strains to EAU Induced by RPE65 or S-Ag**

<table>
<thead>
<tr>
<th>Strain</th>
<th>MHC Haplotype</th>
<th>RPE65 Affected Rats/Total</th>
<th>Severity (mean ± SE)*</th>
<th>S-Ag Affected Rats/Total</th>
<th>Severity (mean ± SE)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lewis</td>
<td>1</td>
<td>6/6</td>
<td>2.1 ± 0.9</td>
<td>3/3</td>
<td>4.0 ± 0</td>
</tr>
<tr>
<td>BN</td>
<td>n</td>
<td>6/6</td>
<td>3.2 ± 0.7</td>
<td>3/3</td>
<td>0.8 ± 0.3</td>
</tr>
<tr>
<td>F344</td>
<td>lvl</td>
<td>6/6</td>
<td>1.6 ± 0.5</td>
<td>3/3</td>
<td>4.0 ± 0</td>
</tr>
<tr>
<td>SHR</td>
<td>k</td>
<td>6/6</td>
<td>0.9 ± 0.6</td>
<td>0/3</td>
<td>—</td>
</tr>
</tbody>
</table>

All rats were immunized with RPE65 or S-Ag at 30 μg per rat emulsified with CFA and were treated concurrently with *B. pertussis* bacteria. Recorded severity levels are of histopathologic changes.

* Only rats with disease were used for calculating the mean severity.
References


