Repertoire Analysis and New Pathogenic Epitopes of IRBP in C57BL/6 (H-2\textsuperscript{b}) and B10.RIII (H-2\textsuperscript{r}) Mice

Lizette M. Cortes\textsuperscript{1}, Mary J. Mattapallil\textsuperscript{1}, Phyllis B. Silver\textsuperscript{1}, Larry A. Donoso\textsuperscript{2}, Gregory I. Liou\textsuperscript{3}, Wei Zhu\textsuperscript{1}, Chi-Chao Chan\textsuperscript{1}, and Rachel R. Caspi\textsuperscript{1}

PURPOSE. Interphotoreceptor retinoid binding protein (IRBP) is the major uveitogenic retinal antigen eliciting experimental autoimmune uveoretinitis (EAU) in mice. The most frequently used mouse strains are B10.RIII and C57BL/6, but to date only one uveitogenic epitope for each has been identified. The purpose of this study was to identify and characterize additional uveitogenic epitopes in B10.RIII and C57BL/6 mice and to compare epitope recognition in wild-type versus IRBP-deficient mice on both backgrounds.

METHODS. Mice were immunized with IRBP. Spleen cells were stimulated in culture with overlapping peptides representing the entire IRBP molecule, and lymphocyte proliferative responses were measured. Peptides determined to be immunodominant were used to immunize mice for EAU. Cytokine profile and proliferation of the CD4 versus CD8 subsets were analyzed for the most pathogenic peptides.

RESULTS. Two new major pathogenic epitopes were identified in WT C57BL/6 mice, residues 461-480 and 651-670. These epitopes induced EAU of severity similar to that induced by the previously known peptide, 1-20. Several other peptides elicited mild disease with lower incidence. Some peptides elicited EAU only in WT recipients of IRBP KO splenocytes. In the B10.RIII strain, two new uveitogenic epitopes were identified, 171-190 and 541-560, and several others elicited moderate disease. Unlike in C57BL/6 mice, adoptive transfer of WT B10.RIII with IRBP KO splenocytes did not reveal additional uveitogenic epitopes. Both CD4 and CD8 lymphocyte subsets proliferated to pathogenic peptides.

CONCLUSIONS. Several new pathogenic peptides of IRBP were identified in C57BL/6 and B10.RIII mice. Differences in epitope recognition between WT and IRBP KO mice were observed in C57BL/6 mice, but not in B10.RIII mice, suggesting more extensive culling of the repertoire in C57BL/6 mice by endogenously expressed IRBP. (Invest Ophthal Vis Sci. 2008;49: 1946–1956) DOI:10.1167/iovs.07-0868

From the \textsuperscript{1}Laboratory of Immunology, National Eye Institute, National Institutes of Health, Bethesda, Maryland; the \textsuperscript{2}Philadelphia Retina Endowment Fund, Philadelphia, Pennsylvania; and the \textsuperscript{3}Department of Ophthalmology, Medical College of Georgia, Augusta, Georgia.

Supported by National Eye Institute Intramural funding and by the Elizabeth C. King Fund. Submitted for publication July 10, 2007; revised December 6, 2007, and January 20, 2008; accepted March 20, 2008.

Disclosure: L.M. Cortes, None; M.J. Mattapallil, None; P.B. Silver, None; L.A. Donoso, None; G.I. Liou, None; W. Zhu, None; C.-C. Chan, None; R.R. Caspi, None.

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be marked "advertisement" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Corresponding author: Rachel R. Caspi, Laboratory of Immunology, National Eye Institute, National Institutes of Health, 10 Center Drive, 10/10N222, Bethesda, MD 20892-1857; rcaspi@helix.nih.gov.

Experimental autoimmune uveoretinitis (EAU) is a T-cell-mediated autoimmune disease induced by immunization with uveitogenic retinal antigens, or by the adoptive transfer of uveitogenic T cells.\textsuperscript{1–4} EAU can be induced in mice by a major uveitogenic antigen, the interphotoreceptor retinoid-binding protein (IRBP).\textsuperscript{6} EAU resembles some types of human uveitic diseases of a presumed autoimmune nature and serves as their model.\textsuperscript{7} Uveitic patients have shown serum levels of autoantibodies to retinal antigens such as arrestin (S-Ag), recoverin, recoverin, IRBP, and others,\textsuperscript{8} suggesting their involvement, primary or secondary, in the disease process.

IRBP is a retinoid and fatty acid–binding glycolipoprotein (molecular mass, 140 kDa) found in the retinal interphotoreceptor matrix, in pinealocytes, and in some retinoblastoma-derived cell lines. It plays an important, though apparently a partly dispensable, role in the visual cycle by binding free fatty acids and retinoids and transporting them between the photoreceptor layer and the retinal pigment epithelium during the bleaching and regeneration of visual pigments\textsuperscript{9} and is important in retinal development and maintenance.\textsuperscript{10} The IRBP gene is evolutionarily conserved among species. It is a 145-kDa retinal protein encoded by a 11.6-kb genomic region containing four exons and three introns. It is transcribed into a 6.4-kb long mRNA, comprising four repeat units due to gene duplication. The repeats themselves have 30% to 40% sequence identity and many conservative substitutions between any two of the four protein repeats. The third and fourth repeats are the most similar to each other. All three of the introns in the IRBP gene fall in the fourth protein repeat.\textsuperscript{11}

Mice of the H-2\textsuperscript{b} haplotype (B10.RIII) are highly susceptible to whole IRBP or to its dominant pathogenic murine epitope (residues 161-180).\textsuperscript{12} C57BL/6 (H-2\textsuperscript{b}) strain is only moderately susceptible to IRBP or its component peptide IRBP(1-20).\textsuperscript{13} but C57BL/6 is the background on which most knockout and transgenic mice are available, and so it is an important strain for basic studies. Although identifying even a single uveitogenic peptide for each of the strains is extremely useful, knowledge of more than one epitope is needed to permit studies such as linked suppression and epitope spreading. In the present study, we have sought to define additional pathogenic sites of IRBP for the C57BL/6 and B10.RIII strains. As a second goal, we used IRBP-deficient (knockout; KO) mice,\textsuperscript{14} to examine the impact of endogenous IRBP expression on the development of the IRBP-specific T-cell repertoire, as defined by IRBP epitope recognition in the adults. Toward that end, IRBP peptides that were recognized in the context of whole IRBP (immunodominant)—that is, those that elicited high proliferation in WT and/or IRBP KO mice primed with IRBP, were tested for their ability to induce disease (pathogenic). Using this approach, we were able to identify new pathogenic epitopes for both haplotypes that could be used for experimental protocols and to define specificities that are eliminated in WT mice as a result of endogenous expression of IRBP.

Copyright © Association for Research in Vision and Ophthalmology

1946
MATERIALS AND METHODS

Mice

Mice with a targeted disruption of the IRBP gene were generated using embryonic stem (ES) cells of strain 129/Ola and C57BL/6 oocytes, as previously described. In brief, the targeting vector lacked the promoter region (from \(1246\) to \(106\)) and 81% of the translated sequence of the IRBP gene (from \(107\) to \(3151\)). These chimeric founder mice were backcrossed either to the C57BL/6J strain (H-2b) or to the B10.RIII (H-2r) strain (Jackson Laboratory, Bar Harbor, ME). After 12 backcrosses, the heterozygous mice were allowed to mate among themselves, to generate homozygous IRBP KO mice from both strains. Wild-type mice from the C57BL/6J and B10.RIII strains were used as the control (Jackson Laboratory). Treatment of the animals conformed to institutional guidelines and to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

Antigens and Reagents

IRBP was isolated from bovine retinas by affinity chromatography on concanavalin-A (Con A)-Sepharose followed by fast-performance liquid chromatography (FPLC), as previously described. Twenty amino acid overlapping peptides were synthesized according to the human IRBP sequence, including all four repeats. The sequence and nomenclature of these peptides has been reported. The human IRBP peptide 1-20 (GPTHLFQPSLVLDMAKVLLD) was synthesized in-house (432A Peptide Synthesizer; Applied Biosystems, Inc. [ABI]) using Fmoc chemistry, as previously described. Murine peptide 161-180 (SGIPYVISYLHPGNTVMHVD) was synthesized by Anaspec, Inc. (San Jose, CA). Methyl-\(\text{mannopyranoside (MMP)}\), Bordetella pertussis toxin (PT), complete Freund’s adjuvant (CFA), and Mycobacterium tuberculosis strain H37RA were purchased from Sigma-Aldrich (St Louis, MO). HyQ DMEM/high-glucose medium was purchased from HyClone (Logan, UT). Medium supplements other than normal mouse serum (which was prepared in-house) were from BioWhittaker (Gaithersburg, MD).

Determination of Immunodominance by Lymphocyte Proliferation Assay

WT and KO mice from C57BL/6J and B10.RIII strains were immunized with 100 \(\mu\)g of bovine IRBP in PBS emulsified 1:1 vol/vol in CFA that had been supplemented with Mycobacterium tuberculosis to 2.5 mg/mL (1:1). A total of 200 \(\mu\)g of emulsion was injected SC, divided among three sites: base of the tail and both thighs. Concurrent with immunization, the mice received 0.4 \(\mu\)g of PT (in DMEM containing 1% normal mouse serum) given by IP injection. Fourteen days after immunization, splenocytes from five mice were collected, pooled, and distributed into triplicate 0.2 mL cultures in 96-well round-bottomed plates (5 \(\times\) 10^5 cells per well). Proliferative responses were recalled with 25 \(\mu\)g/mL (10 \(\mu\)M) of each of the overlapping 124 synthetic peptides representing the entire sequence of human IRBP. DMEM/high-glucose culture medium (HyQ; HyClone) was supplemented as necessary.

![Image](http://tvst.arvojournals.org/)
described plus 20 mg/mL of α-MMP to neutralize any traces of ConA, which is used during the initial stages of IRBP purification. Cultures were incubated for 48 hours and were pulsed with 3H-thymidine (1 μCi/well) for an additional period of 18 hours. To ensure further the specificity of the responses, antigen preparations were tested and confirmed free of lipopolysaccharide (LPS) contamination by using an assay for endotoxin (Limulus Amoebocyte Lysate LAL QCL-1000; Cambrex Bio Science, Inc., Walkersville, MD), according to the manufacturer’s protocol.

**Determination of Pathogenicity by EAU Induction**

IRBP peptides that elicited meaningful proliferative responses (i.e., double the background of 1296 cpm for C57BL/6 and 2541 cpm for unstimulated B10.RIII) in WT and/or KO mice were tested for pathogenicity in WT mice or in WT mice infused with IRBP KO spleen cells of the appropriate strain to detect clonotypes that may have been deleted in the WT. Groups of 5 to 15 mice were immunized SC in both thighs and the base of the tail with 300 μg of each selected peptide, as a 0.2 mL emulsion in mycobacterium-enriched CFA (1:1, vol/vol) and 0.4 μg of PT, intraperitoneally. Positive control groups were immunized in parallel with known uveitogenic stimuli—that is, whole IRBP and human peptide 1-20 (for C57BL/6 mice) or whole IRBP and murine peptide 161-180 (for B10.RII mice).

Adoptive transfer of KO splenocytes into WT was performed from naïve donors to naïve recipients intravenously, and 1 to 2 days later the recipients were immunized with the selected peptides. Cells from five donors were pooled, washed, and counted. The cell suspension was adjusted to 80 × 10⁶ cells/mouse. The cells were infused intravenously at a ratio of one donor to one recipient. The recipient mice were slightly irradiated (100 rads), to facilitate engraftment. This radiation
dose is not immunosuppressive, as it does not reduce subsequent EAU scores in WT mice that do not receive adoptive transfer. Clinical disease in the immunized mice was evaluated by funduscopy, and the eyes were collected for histopathology on day 28 after immunization.

**Histopathology and Scoring of EAU**

In all experiments, the clinical appearance of disease was followed by fundus examination, to confirm that the disease was progressing and developing as expected. The final readout was by histopathology, and these data are presented in Figures 3 and 6. To analyze histopathology results, eyes collected immediately after euthanatization were prefixed for 1 hour in 4% phosphate-buffered glutaraldehyde and transferred into 10% phosphate-buffered formaldehyde until processing. Tissue sections (3–6 μm) were stained with hematoxylin and eosin. Incidence and severity of disease were examined in a masked fashion by one of the authors (C-CC), an ophthalmic pathologist. The severity of EAU was scored on a scale of 0 (no disease) to 4 (maximum disease) in half-point increments, according to a semiquantitative system described previously that takes into account the severity of inflammation and retinal damage, as follows17,18: 0, normal; 0.5 (trace), mild inflammatory cell infiltration with no tissue damage; 1, infiltration, with retinal folds and focal retinal detachments, a few small granulomas in the choroid and retina, and perivasculitis; 2, moderate infiltration, with retinal folds, detachments, and focal photoreceptor cell damage, small-to-medium sized granulomas, perivasculitis, and vasculitis; 3, medium to heavy infiltration, extensive retinal folding with detachments, moderate photoreceptor cell damage, medium-sized granulomatous lesions, and subretinal neovascularization; and 4, heavy infiltration, diffuse retinal detachment with serous exudate and subretinal bleeding, extensive photoreceptor cell damage, large granulomatous lesions, and subretinal neovascularization. Figure 1 shows a representative example of EAU scoring.

**Immunological Responses**

WT and KO mice were immunized with IRBP or with selected pathogenic peptides in emulsion with CFA and were given PT as for EAU induction. For detection of Ag-induced cytokines in cell culture supernatants, draining lymph nodes (inguinal and iliac) from five mice per peptide were collected and pooled after 21 days and cultured in triplicate 0.2-ml cultures containing 1 × 10^6 cells/well. They were seeded in flat-bottomed, 96-well microtiter plates, either alone or stimulated with 25 μg/ml of each one of the peptides, respectively. The supernatants were collected after 48 hours and were kept frozen in small aliquots at −70°C. The cytokine concentrations in the supernatants of IFN-γ, IL-5, IL-17, IL-23, IL-10, IL-6, IL-13, TNF-α, and TGF-β were measured by multiplex ELISA (SearchLight Technology; Pierce Boston Technology, Woburn, MA).
Proliferation of CD4 and CD8 Subsets by CFSE Dilution

Freshly isolated cells (~2 x 10^6) from draining lymph nodes and spleen of immunized mice were stained with a 5-μM solution of CFSE (carboxy fluorescein diacetate succinimidyl; Invitrogen Corp., Carlsbad, CA) at room temperature for 8 minutes. After staining, the cells were incubated with warm fetal bovine serum at 37°C for 10 minutes. The cells were washed twice in phosphate-buffered saline (PBS) and were cultured in HL-1 medium (Lonza Ltd., Allendale, NJ) for 4 days at 37°C in 7.5% CO₂ in the presence or absence of the immunizing antigen (IRBP 10 μg/mL; peptides 25 μg/mL each). The cells were harvested after 4 days, washed, and stained with anti-mouse CD4 or CD8 antibody conjugated to APC (allophycocyanin; BD Biosciences, Franklin Lakes, NJ) according to standard protocol. After the cells were washed, they were resuspended in PBS for flow cytometry (FACSCalibur; BD Biosciences). Propidium iodide (PI; Roche Pharmaceuticals, Branchburg, NJ) was added to each sample 2 minutes before flow cytometry, to exclude dead cells from the analysis. Approximately 75,000 to 100,000 cells were collected for each subpopulation. The percentage of proliferated cells was calculated for each peptide by dividing the percentage of CD4 by that of CD8. The ratio of CD4 to CD8 proliferation was calculated for each peptide, as evaluated by multiplex ELISA.

Data Presentation and Analysis

Groups typically were composed of five or more mice. Experiments were repeated at least three times with similar results. Graphs represent average of all mice tested from pooled experiments.
Table 3. Immunologic and Immunopathologic Properties of IRBP Peptides in B10.RIII Mice

<table>
<thead>
<tr>
<th>IRBP Peptides</th>
<th>Proliferation</th>
<th>Pathogenicity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>KO</td>
<td>WT</td>
</tr>
<tr>
<td>21-40</td>
<td>✓</td>
<td>✓</td>
</tr>
<tr>
<td>51-70</td>
<td>✓</td>
<td>✓</td>
</tr>
<tr>
<td>121-140</td>
<td>✓</td>
<td>✓</td>
</tr>
<tr>
<td>141-160</td>
<td>✓</td>
<td>✓</td>
</tr>
<tr>
<td>161-180</td>
<td>✓</td>
<td>✓</td>
</tr>
<tr>
<td>171-190</td>
<td>✓</td>
<td>✓</td>
</tr>
<tr>
<td>181-200</td>
<td>✓</td>
<td>✓</td>
</tr>
<tr>
<td>301-320</td>
<td>✓</td>
<td>✓</td>
</tr>
<tr>
<td>331-350</td>
<td>✓</td>
<td>✓</td>
</tr>
<tr>
<td>421-440</td>
<td>✓</td>
<td>✓</td>
</tr>
<tr>
<td>471-490</td>
<td>✓</td>
<td>✓</td>
</tr>
<tr>
<td>521-540</td>
<td>✓</td>
<td>✓</td>
</tr>
<tr>
<td>531-550</td>
<td>✓</td>
<td>✓</td>
</tr>
<tr>
<td>541-560</td>
<td>✓</td>
<td>✓</td>
</tr>
<tr>
<td>581-600</td>
<td>✓</td>
<td>✓</td>
</tr>
<tr>
<td>771-790</td>
<td>✓</td>
<td>✓</td>
</tr>
<tr>
<td>901-920</td>
<td>✓</td>
<td>✓</td>
</tr>
<tr>
<td>931-950</td>
<td>✓</td>
<td>✓</td>
</tr>
<tr>
<td>961-980</td>
<td>✓</td>
<td>✓</td>
</tr>
<tr>
<td>1001-1020</td>
<td>✓</td>
<td>✓</td>
</tr>
<tr>
<td>1011-1030</td>
<td>✓</td>
<td>✓</td>
</tr>
<tr>
<td>1081-1100</td>
<td>✓</td>
<td>✓</td>
</tr>
<tr>
<td>1121-1140</td>
<td>✓</td>
<td>✓</td>
</tr>
<tr>
<td>1131-1150</td>
<td>✓</td>
<td>✓</td>
</tr>
</tbody>
</table>

* Bold type indicates high pathogenicity.

RESULTS

The strategy we adopted for identifying new peptide epitopes was to first determine the immunodominant epitopes that are recognized in the context of whole IRBP. The determination was made by immunizing mice with the native IRBP molecule and recalling proliferative responses in vitro with a panel of 124 peptides that contain 20 amino acids each, overlapping by 10 residues, representing the entire IRBP molecule. The immunodominant epitopes were then tested for pathogenicity by immunizing mice with each peptide individually and scoring the eyes for EAU. To orient the reader in the pathology that is observed, Figure 1 shows examples of EAU pathology typical of the different disease scores.

Responses of C57BL/6 Mice

C57BL/6 mice are moderately susceptible to EAU. The only pathogenic peptide characterized for this strain thus far is IRBP 1-20. To identify additional peptides to which this strain responds, C57BL/6 WT and IRBP KO mice on C57BL/6J background were immunized with whole IRBP. Peptide recognition was assessed by in vitro proliferative responses to overlapping 20-amino-acid peptides spanning the entire IRBP molecule (Fig. 2). The proliferation pattern revealed 17 peptides recognized by both IRBP WT and KO mice (Table 1). Eighteen additional peptides elicited proliferation only in KO splenocytes (Table 1), pointing to a difference in the IRBP-specific T-cell repertoire between WT and IRBP KO C57BL/6J mice (Fig. 2). To ascertain that the proliferative responses were indeed antigen specific, all peptides that elicited proliferation were tested for LPS contamination by the LAL assay. LPS was under the limit of detection (<0.1 endotoxin units per milliliter; data not shown).

Immunodominant IRBP peptides that elicited high proliferative responses in splenocytes of WT mice were tested for pathogenicity by immunizing the mice with the peptides. The peptides selected for pathogenicity testing are marked with arrows in Figure 2. In addition, splenocytes obtained from IRBP KO mice were adoptively transferred into WT mice, to identify pathogenic specificities that may have been eliminated in the WT. The recipients were subsequently immunized with the peptides that elicited responses in both WT and KO. Testing pathogenicity directly in the KO mice was not possible, as they do not develop EAU, due to lack of the target antigen in the retina. The pathogenicity results are shown in Figure 3a (unmodified WT) and 3b (WT mice infused with KO cells). The sequences of the pathogenic peptides are shown in Table 2. It should be noted that for logistic reasons (availability of mice) it was not possible to immunize all mice with all peptides at the same time, so that WT mice and WT infused with KO cells had to be immunized in separate experiments. Therefore, because of interexperiment variability, the absolute scores are not comparable between experiments, only within each experiment.

As depicted in Figure 3, in the WT C57BL/6J mice, peptides 461-480 (LRHNPQPSAVPLLSYFQ) and 651-670 (LAQGAYRTAVDLESASQLT) were the most pathogenic and were comparable in scores to the previously described peptide 1-20. Four other peptides elicited disease, albeit with minimal scores. Of interest, six additional peptides were pathogenic in WT recipients of IRBP KO splenocytes, suggesting that WT C57BL/6 mice eliminate potentially pathogenic specificities from the repertoire. Thus, not all peptides that exhibited immunodominance by proliferation, either in WT or KO mice, appeared to encode pathogenic specificities.

Peptide 1-20, which was used along with the others as a control for immunization, was pathogenic, but not highly immunodominant in the WT and slightly immunodominant in the KO. Peptide 271-290 elicited high proliferation in the KO but not in the WT mice; however, it was pathogenic in the WT and highly pathogenic in WT recipients of KO cells. Peptide 651-670 was not immunodominant in the WT but only in the KO mice; however, it was highly pathogenic (Fig. 3). Disease scores were not significantly decreased by immunizing the mice with a lower dose (150 μg) of peptide (data not shown).

Next, we examined the immunologic responses to the peptides themselves, using the most strongly pathogenic peptides. It should be noted, that unlike in Figure 2, which shows mice that had been immunized with IRBP and therefore their proliferative responses to peptides reflected crossreactivity to the whole molecule (immunodominance), now each peptide served as both immunogen and recall (immunogenicity). Antigen-specific T-cell proliferation to each immunizing peptide was assessed by3H-thymidine uptake (Fig. 4a). Typically, KO cells exhibited stronger proliferative responses than did WT cells. The proliferation pattern did not exactly correspond to that shown in Figure 2, indicating that immunodominance and immunogenicity of pathogenic peptides may differ.

Since peptides can bind directly to class I or II molecules, without undergoing processing, we also examined the respective proliferation of the CD4 and CD8 lymphocyte populations to each peptide by flow cytometry of CFSE-labeled cells costained for CD4 or CD8. Shown as an example are responses to peptide 1-20. In the case of all peptides, and to our surprise also in the case of whole IRBP (which should be processed through the class II pathway), proliferation occurred not only in the CD4, but also in the CD8 population, which in some cases was stronger than that of CD4 (Figs. 4b, 4c). Ag-specific cytokine responses for each peptide were evaluated by multiplex ELISA (Fig. 4d) and, for most cytokines, roughly paralleled the pattern of proliferation shown in Figure 4a.

Downloaded From: http://tvst.arvojournals.org/ on 02/24/2018
Responses of B10.RIII Mice

Using a similar approach to the one described for the C57BL/6 mice, we immunized B10.RIII WT and IRBP KO mice on a B10.RIII background with IRBP and, after 2 weeks, responses were recalled in vitro with the IRBP peptide panel. Seven peptides (21-40, 161-180, 171-190, 531-550, 581-600, 901-920, and 1011-1030), were immunodominant, in that they elicited proliferation in splenocytes of IRBP-immunized WT as well as in KO mice. For reasons that are unclear (enhanced positive selection?), peptide 531-550 was recognized mainly by WT mice. Thirteen additional peptides elicited proliferation only in KO mice (Table 3). All peptides that elicited proliferation, whether in WT or in KO mice or both, were tested for pathogenicity in unmodified WT mice and in WT mice infused with IRBP KO splenocytes.

Of interest, B10.RIII mice, which are more susceptible to EAU than C57BL/6 mice, responded to fewer peptides than did the C57BL/6 mice (Fig. 5). The reason for this is not clear, and might be connected to the ability of IAb vs IAa to bind and present processed fragments of IRBP. No LPS contamination was detected in any of the peptides.

Similar to C57BL/6 mice, only some of the peptides that were immunodominant, turned out to also be pathogenic (51-70, 161-180, 171-190, 521-540, 541-560, 1011-1030, and 1131-1150). The sequences of peptides that were pathogenic are shown in Table 2. Peptide 161-180 was reported previously as a pathogenic epitope. A new pathogenic peptide was identified, 541-560 (SLGWATLVGEITAGNLLHTR), that elicited EAU with high incidence. On the other hand, peptides 21-40, 531-550, and 581-600, that were immunodominant in the WT, and
peptides 121-140, 181-200, 1001-1020, recognized only by KO, were not pathogenic (Figs. 5, 6).

Ag-specific responses to direct immunization with each of the pathogenic peptides and participation of CD4 versus CD8 compartments were examined as described for C57BL/6 mice (Fig. 7). Ag-specific proliferative and cytokine responses of KO mice were again more pronounced than those of WT mice (Figs. 7a, 7d), with most cytokines showing a pattern similar to proliferation. Notably, as in C57BL/6 mice, not only CD4 but also CD8 cells proliferated to the peptides and to IRBP, although CD4 versus CD8 “preference” varied for individual peptides (Figs. 7b, 7c).

**DISCUSSION**

In the present study we identified new pathogenic specificities of IRBP and examined repertoire differences due to endogenous expression of IRBP in the C57BL/6 and B10.RIII strains, the two most commonly used strains for the EAU model. Our method relies on crossreactivity between species for epitope identification. For objective and for historical reasons having to do with availability of reagents and with prior work done in the field, the whole IRBP used to immunize mice is a natural molecule of bovine origin, the 124 overlapping 20 amino acid peptides are based on the human IRBP sequence, and the target antigen in the eye is, of course, mouse IRBP. Thus, some of the epitopes showing up as immunodominant by proliferation may be fragments shared between the bovine and human species, but foreign to the mouse. Despite being highly proliferative, they would not be pathogenic, as they are not sufficiently similar to the autologous target sequence. Examples of such epitopes are 421-440 and 931-950 on the C57BL/6 background. On the other hand, the pathogenic epitopes are expected to be shared among all three species, and will probably be the conserved epitopes whose precursors are not well deleted in the mouse by the process of central tolerance. However, as previously reported for bovine S-Ag and IRBP peptides in the rat, we found also in the mouse that there is only a partial correlation between proliferation to particular peptides and their pathogenicity. This may mean that some peptides, which were not well recognized by proliferation in the context of whole IRBP and therefore were not pursued further, may have been missed as pathogenic sites. In addition, because most class II epitopes consist of 12 to 19 amino acids, our 20-amino-acid peptides, having 10 amino acid overlaps, may have failed to detect some pathogenic epitopes. Last, a peptide would not be detected as pathogenic if the immunization dose of 300 μg was insufficient to elicit disease; however, the pathogenicity of such peptides by definition would have been borderline.

With all these caveats in mind, we nevertheless identified several previously unrecognized pathogenic sites for each of the tested strains.

In the C57BL/6 strain, we further defined a series of specificities that are recognized by the IRBP KO repertoire but not by the WT, some of which were pathogenic. This included peptide 271-290 which was previously reported by us to elicit proliferation in the IRBP KO but not in WT C57BL/6 mice and is confirmed in the present study to be pathogenic. These findings support our previous data, obtained using thymus transplantation and direct IRBP-specific precursor frequency
analysis, showing that central tolerance due to endogenously expressed IRBP shapes the IRBP-specific repertoire.19

Unexpectedly, in B10.RIII mice, unlike in C57BL/6 mice, there were no new epitopes unmasked by transferring KO cells, which did not undergo thymic selection to IRBP, into WT mice. We speculate that this may be a consequence of relatively inefficient culling of pathogenic specificities, due to the unusually low expression of IRBP in the thymus of B10.RIII mice,24 resulting in a greater similarity between the KO and the WT repertoires than in C57BL/6. However, Avichezer et al.25 clearly demonstrated that there is repertoire culling and a lower precursor frequency of IRBP-specific cells in B10.RIII mice.

Two non–mutually exclusive explanations can be proposed to reconcile these seemingly disparate observations: (1) It is possible that because of inefficient deletion in B10.RIII mice, the precursor frequencies for all specificities in B10.RIII are reduced to some extent, but none are completely eliminated; and (2) the method of direct transfer of spleen cells from KO to WT to identify uveitogenic specificities that may have been eliminated in WT, while simple and straightforward, is not the most sensitive. The number of spleen cells transferred represents only part of the lymphoid cell population of the donor, and not all may engraft in the recipient. Therefore, this method may underdetect or even entirely miss certain epitopes. Indeed, in a previous study, we were able to detect a uveitogenic specificity, present in peptide 121-140, that is culled in B10.RIII, by making a T-cell line specific to this peptide from KO and transferring the cells to WT.25 Another approach would have been surgical removal and replacement of the WT APCs.
thymus with a KO thymus, followed by complete immunobloration and bone marrow reconstitution, which would have resulted in a KO repertoire in the WT. Unfortunately, neither of these methods is feasible when examining a large number of peptides, as was done in the present study.

The concept of inefficient culling in B10.RII mice is supported by the pattern of proliferative responses to the peptides themselves, as shown in Figures 4 and 7, which reflect immunogenicity (rather than the immunodominance reflected by cross-reactivity to IRBP). On the B10.RII background, responses of IRBP KO mice were double or better than of WT to all but one of the pathogenic peptides examined. In contrast, on the C57BL/6 background, the difference between KO and WT proliferation was low to minimal. In general, cytokine responses were also more strongly upregulated in B10.RII KO than in C57BL/6 KO over the respective WT. We believe that the higher responses of KO mice reflect lack of negative selection rather than lack of Ag-specific “natural” thymic regulatory T cells (Tregs). This conclusion is based on our recent findings that responses to IRBP of IRBP KO mice immunized for EAU are efficiently regulated by “polyclonal” Tregs that are triggered by mycobacterial components of CFA.26

Some interesting peptides deserve individual mention such as, 11-30 and 271-290 in the C57BL/6J mice. Peptide 11-30 was immunodominant and was pathogenic in WT as well as in WT mice that received KO cells. This is an overlapping peptide with 1-20, an epitope previously identified as pathogenic. Splenocytes from WT C57BL/6 mice proliferated more to this peptide than splenocytes of IRBP-KO C57BL/6J. Another example where WT reproducibly mounted higher responses than KO is 531-550 in B10.RII. It is unclear whether this represents a proportional increase in frequency of this clonotype due to reduction of other specificities, or enhanced positive selection. Peptide 271-290 was highly immunodominant in C57BL/6 IRBP KO mice and pathogenic in the C57BL/6 recipients of IRBP KO cells, but elicited only minimal responses in the unmodified WT mice. This specificity seems to represent a dominant pathogenic epitope present in the KO that becomes deleted or anergized in the WT. Finally, some peptides, (e.g., 521-540 and 1131-1150) that induced mild disease in B10.RII mice, have been reported to be pathogenic in Lewis rats,26 and so may represent “promiscuous” fragments. However, the core epitope(s) recognized by the different species remain to be defined.

IRBP protein originated from an evolutionary process that resulted in a symmetrical fourfold molecule, containing extensive sequence similarity between the four homologous domains. By examining the amino acid sequences of those uveitopathogenic peptides, we were able to identify some pathogenic blocks and shared pathogenic regions conserved among the strains. There were pathogenic peptides within all four IRBP repeats in both strains; however, the majority were contained in repeats I and II. In C57BL/6 (H2-b) mice, the peptides 11-30 (repeat I), and 311-330 (repeat II) represent homologous sequences that were pathogenic, as were 331-350 (repeat II) and 651-670 (repeat III). Peptides 471-490 (repeat II) and 771-790 (repeat III) were also homologous. Some peptides that were pathogenic turned out to be overlapping sequences, such as 11-30 (repeat I), which has an overlapping sequence with the known pathogenic epitope 1-20. In this case, it is probably not an epitope within the shared sequence that is being recognized, since amino acids 1-5 were shown to be critical for pathogenicity of peptide 1-20.15 Other pathogenic peptides with overlapping sequences were 461-480 with 471-490 (repeat II). In B10.RII (H2-r) mice, we identified a pathogenic homologous region that contained peptides 521-540 and 1131-1150 (repeats II–IV). The main pathogenic epitope (161-180) had an overlapping sequence with 171-190 (repeat I).

In summary, we identified several new pathogenic peptides for the H-2b and H-2r haplotypes, as well as peptides representing specificities recognized by IRBP KO mice, that are reduced or eliminated in WT mice. All peptides elicited proliferation in CD4 as well as CD8 T-cell compartments, although CD4/CD8 ratio varied. Many of the pathogenic epitopes represented either overlapping peptides or homologous sequences in different repeats. These findings will be useful in immunological studies that require knowledge of multiple epitopes and will help in understanding the pathogenic potential of IRBP as an autoantigen.

Acknowledgments

The authors thank Mary Alice Crawford, Joseph Hackett, Iris Wise, and Julius Boykin from the NEI Histology Core Facility for technical assistance in the preparation of the histologic specimens.

References
