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Chapter 6

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Cellular Immune Responses of Patients With Uveitis to Retinal Antigens and Their Fragments

Marc D. de Smet, M.D., Joyce H. Yamamoto, M.D., Manabu Mochizuki, M.D., Igal Gery, Ph.D., Vijay K. Singh, M.D., Tochimichi Shinohara, Ph.D., Barbara Wiggert, Ph.D., Gerald J. Chader, Ph.D., and Robert B. Nussenblatt, M.D.

Of two patient populations totaling 82 patients, one in the United States and the other in Japan, we studied the cellular immune responses against S-antigen and interphotoreceptor retinoid binding protein as well as to fragments of each antigen. Behçet's disease, birdshot retinochoroidopathy, pars planitis, ocular sarcoid, sympathetic ophthalmia, and the Vogt-Koyanagi-Harada syndrome were diagnosed in these patients. The response profile of both antigens paralleled each other. This profile was more commonly seen in patients suffering from diseases affecting the retina. Responders reacting to both antigens or to several fragments of an antigen were present. This pattern of response was seen in 26 of the patients tested. Patients with uveitis appeared able to recognize several autoantigens. This might be a consequence of the breakdown of the blood-retinal barrier and may help perpetuate the inflammatory process. Several patients were capable of responding to more than one epitope of the same antigen, which indicates that there are major differences between the experimental model and human autoimmune diseases in the response to autoantigens. Both of these findings may help develop new immunotherapeutic strategies in the treatment of uveitis.

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S-AG AND IRBP IMMUNE RESPONSES IN PATIENTS

Intraocular Inflammatory Disease (uveitis) is the cause of about 10% of severe visual loss in

Patients and Methods

Patients participating in this study were seen in the uveitis clinic of the National Eye Institute, Bethesda, Maryland, and at the Tokyo University Branch Hospital, Tokyo, Japan. All patients gave informed consent before participating in the study. They were part of an ongoing protocol approved by each institution's committee on human investigation. All patients had active uveitis involving the posterior segment or had a history of active disease involving the retina or choroid. The patients
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tested had one of the following disorders: Behçet's disease, birdshot retinochoroidopathy, pars planitis, ocular sarcoid, sympathetic ophthalmia, or the Vogt-Koyanagi-Harada syndrome. Patients with Behçet's disease met at least the minimal criteria for incomplete Behçet's set by the Behçet's Disease Research Committee of Japan with all patients having ocular disease. Patients with birdshot retinochoroidopathy had cream-colored lesions in the posterior segment, macular edema, and retinal vascular changes. These patients were HLA A-29 positive as well. Patients with sympathetic ophthalmia had a history of penetrating trauma or multiple operations followed by a bilateral granulomatous uveitis. Patients with Vogt-Koyanagi-Harada syndrome were of either Japanese or American Indian heritage and had ocular and systemic changes compatible with the disorder. The patients with ocular sarcoid had bilateral granulomatous uveitis usually accompanied by either a positive gallium scan or a noncaseating granuloma on a biopsy specimen. Patients were tested irrespective of their current medical therapy (usually consisting of cyclosporine, prednisone, or both) or of their level of activity. Since the antigens tested were of retinal origin, anterior segment inflammation was not considered as part of the definition of active disease. The presence of retinal infiltrates, perivasculitis, snowbanking, or vitreous haze were accepted as evidence of activity. Additionally, cystoid macular edema confirmed by fluorescein angiography was considered a sign of active disease. All the diagnostic categories were based on clinical criteria except ocular sarcoid and birdshot retinochoroidopathy, in which confirmation by another test was required. Control subjects were selected from either nonresearch staff or from clinic patients not being seen for a uveitic condition, and in whom a retinal or choroidal disorder had been ruled out.

Antigens used in this assay included bovine interphotoreceptor retinoid binding protein purified to homogeneity, as described by Redmond and associates, and bovine S-antigen purified by the method described by Dorey, Cozette, and Faure. Peptides derived from interphotoreceptor retinoid binding protein were synthesized and purified by Applied Biosystems Inc., Foster City, California, using the t-BOC chemistry, on a peptide synthesizer 430A. The peptide sequences were derived from the sequence of bovine interphotoreceptor retinoid binding protein as determined and reported by Borst and associates. This consisted of sequence 1158-1180 (HVDTDDYLTIP-TARSVGAADGS) for R-4 and of sequence 1169-1191 (PTARSVGAADGSSWEGVGVP-DV) for R-14. Peptides derived from S-antigen were based on the bovine S-antigen sequence reported by Shinohara and associates. The peptides were synthesized in accordance with the method of Donoso and associates, on a benzhydrolyamine resin using an automated peptide synthesizer (SAM II, Biosearch, Inc., San Rafael, California). The sequence for peptide M corresponded to positions 303 to 320 (DTNLASSTIKEGTGV), and peptide N corresponded to positions 281 to 302 (VPLLANNERRGIALDKIKIKHE) of the S-antigen.

Proliferation assays were performed in the same way in Japan and the United States, except where indicated. Mononuclear leucocytes from heparinized blood samples were separated on Isolymph gradients (Gallard-Schlesinger, Carle Place, New York) and cultured in Roswell Park Memorial Institute (RPMI) 1640 medium with HEPES (GIBCO, Grand Island, New York), supplemented with glutamine (2 mmol/l), penicillin (100 units/ml), streptomycin (100 μg/ml), and heat inactivated human AB serum. The National Eye Institute used 20% serum from a single donor in the cultures, whereas Tokyo University Branch Hospital used 10% commercial serum (lot No. 14510, Pel Freez, Brown Deer, Wisconsin).

The cells were cultured by two methods. In the first method, 2 × 10^5 cells/well were cultured in flat-bottom, 96-well plates for five days. In the second method, which under certain circumstances is believed to increase responses by increasing cell to cell interactions, 5 × 10^5 cells/well were incubated in round-bottom, 96-well plates for seven days. All cultures were in a total volume of 200 μl and were set up in triplicate with or without stimulants. The antigen concentration was either 4, 20, 50, or 100 μg/ml. The cultures were incubated for the specified time at 37 °C with 100% humidity and 5% carbon dioxide in air, pulsed for 16 hours with 3H-thymidine (3H-TdR, New England Nuclear, Boston, Massachusetts; 2 Ci/mmol, 0.5 μCi per 10 μl/well) and harvested on glass fiber filters using a MASH II harvester. After drying, the filter pads were placed in vials with 3 ml of toluene-based fluor and counted in a Beckman L3801 liquid scintillation counter. Several peptides were tested simultaneously; however, not all peptides could be tested on each patient. Cells from a control subject were
usually tested simultaneously with cells from one or more patients.

The mean of the triplicate cultures in counts per minute was calculated for each set of replicate cultures. A stimulation index was derived by dividing the mean for each of the antigen stimulated cultures by the mean for the control cultures in which no antigen was added. For each testing center and for each antigen, a mean stimulation index ± standard deviation was calculated for the control subjects. A significant response in a patient was considered to be present when the patient's stimulation index for a given peptide or determinant was above the mean for the controls by two S.D.

The stimulation indices for each antigen tested were also compared by disease category to the control subjects to determine if any statistically significant difference was present. Significance was assessed by a standard nonpaired Student's t-test. Patients were also assessed by their clinical activity. Testing for statistical significance was done using chi-square. Results are given as the mean stimulation index ± standard error.

**Results**

A total of 30 control subjects and 82 patients were tested; 47 patients were from the United States and 35 patients were from Japan. The average age of the patients in both groups was comparable; 41 years of age (range, 10 to 70) for the American patients and 43 years of age (range, 20 to 70) for the Japanese. The duration of follow-up was also similar in the two groups: 44 months in the United States (range, six to 108) and 57 months in Japan (range, two to 247). On average, uveitis had been diagnosed in the patients for 63 months (range, two to 247). All of the patients examined in Japan were of Japanese descent, whereas in the American group, 41 patients were white, five were black, and one patient was Oriental. The number of patients with clinically active disease varied among the various categories (Table 1) and between countries. Overall, half of the patients tested had active ocular disease. The largest discrepancy was found among patients with sarcoid, in which a greater number of the Japanese patients had active disease. Of all the groups, the patients with birdshot retinochoroidopathy had the lowest incidence of activity, and none were tested in Japan, where the disease is extremely rare. The proportion of patients with active disease was highest among those suffering from Behcet's disease.

The various disease entities responded differently to the uveitogenic antigens (Table 2). A similar response profile, however, was found for S-antigen and interphotoreceptor retinoid binding protein. Patients with diseases involv-

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**TABLE 1**

CHARACTERISTICS OF AMERICAN AND JAPANESE PATIENTS

<table>
<thead>
<tr>
<th>CLINICAL ENTITY</th>
<th>TESTING CENTER</th>
<th>MEAN AGE (YRS)*</th>
<th>DURATION OF DISEASE (MOS)*</th>
<th>NO. OF PATIENTS</th>
<th>MALE</th>
<th>FEMALE</th>
<th>CLINICAL ACTIVITY</th>
<th>CYCLOSPORINE</th>
<th>PREDNISONE</th>
<th>CYTOTOXICS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Behcet's disease</td>
<td>U.S. 34 (28-42)</td>
<td>50 (10-96)</td>
<td>0.5/3.3/53</td>
<td>04.7</td>
<td>04.7</td>
<td>0.4/1.8/010</td>
<td>04.7</td>
<td>04.7</td>
<td>—</td>
<td></td>
</tr>
<tr>
<td>Vogt-Koyanagi- Harada syndrome</td>
<td>Japan 38 (24-60)</td>
<td>54 (12-126)</td>
<td>016.0/16.0/115</td>
<td>08.0</td>
<td>04.7</td>
<td>04.7</td>
<td>04.7</td>
<td>04.7</td>
<td>—</td>
<td></td>
</tr>
<tr>
<td>Ocular sarcoid</td>
<td>U.S. 47 (29-65)</td>
<td>93 (4-247)</td>
<td>010.5/5/50</td>
<td>01.0</td>
<td>04.7</td>
<td>04.7</td>
<td>04.7</td>
<td>04.7</td>
<td>—</td>
<td></td>
</tr>
<tr>
<td>Pars planitis</td>
<td>Japan 45 (20-70)</td>
<td>43 (2-180)</td>
<td>09.3/6/63</td>
<td>00.0</td>
<td>04.7</td>
<td>04.7</td>
<td>04.7</td>
<td>04.7</td>
<td>—</td>
<td></td>
</tr>
<tr>
<td>Birdshot retino- chorioidopathy</td>
<td>U.S. 31 (16-49)</td>
<td>45 (24-60)</td>
<td>06.2/4/51</td>
<td>02.0</td>
<td>04.7</td>
<td>04.7</td>
<td>04.7</td>
<td>04.7</td>
<td>—</td>
<td></td>
</tr>
<tr>
<td>Sympathetic ophthalmia</td>
<td>U.S. 56 (46-66)</td>
<td>67 (24-108)</td>
<td>09.5/4/18</td>
<td>02.0</td>
<td>04.7</td>
<td>04.7</td>
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<tr>
<td>Normal</td>
<td>U.S. 35 (26-50)</td>
<td>—</td>
<td>010.10</td>
<td>01.0</td>
<td>04.7</td>
<td>04.7</td>
<td>04.7</td>
<td>04.7</td>
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<td></td>
</tr>
<tr>
<td>Japan 37 (14-67)</td>
<td>—</td>
<td>06/4</td>
<td>01.0</td>
<td>04.7</td>
<td>04.7</td>
<td>04.7</td>
<td>04.7</td>
<td>04.7</td>
<td>—</td>
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</tr>
</tbody>
</table>

* Average. The range is given in parentheses.

* Nine patients with Behcet's disease also received colchicine.
ing the retina were more likely to have a significant proliferative response. In American patients, the mean proliferative responses to the S-antigen (20 µg/ml) were significant for Behcet’s disease, 2.8 ± 1.0 (P = .05) and birdshot retinochoroidopathy, 2.7 ± 0.7 (P = .02) whereas the control was 1.3 ± 0.2. For the interphotoreceptor retinoid binding protein (20 µg/ml), the proliferative responses were lower, with a significant difference in Behcet’s disease, 1.5 ± 0.4 (P = .04), birdshot retinochoroidopathy, 1.4 ± 0.3 (P = .04), and pars planitis, 1.4 ± 0.02 (P = .04) as compared to the control, 0.9 ± 0.1. A similar pattern of response was found in the Japanese patients, with the highest number of responders found in Behcet’s disease. The patients with ocular sarcoid gave few positive responders. Their responses to phytohemagglutinin and purified protein derivative, however, were strong, which indicates that the lack of response is not caused by a generalized state of unresponsiveness, but probably reflects an inability to recognize bovine S-antigen or bovine interphotoreceptor retinoid binding protein. Culturing the cells for seven days in round-bottom wells increased the number of significant responses in some groups, but not in all. There was an increase in sensitivity mainly for patients with the Vogt-Koyanagi-Harada syndrome where there was an increase in the number of significant responders to the S-antigen and the interphotoreceptor retinoid binding protein. The mean response to the S-antigen for lymphocytes from patients with Vogt-Koyanagi-Harada syndrome in the United States was 2.8 ± 1.4, as compared to control subjects (1.8 ± 0.1), which was a statistically significant difference (P = .03).

In addition to testing in vitro responses to the interphotoreceptor retinoid binding protein and the S-antigen, the responses to peptide fragments of each of these antigens were determined (Table 2). There was a correlation between the intensity of the proliferative response to the interphotoreceptor retinoid binding protein or the S-antigen and the existence of a significant proliferative response to one or more of the peptide fragments of that antigen. In five-day cultures, if considering only statistically significant responders to S-antigen, seven of nine American patients had a significant response to one or both peptide fragments tested. In seven-day cultures, three of five Japanese patients responsive to S-antigen had a response to either M peptide or N peptide, or to both. In six of the nine American patients who responded to the interphotoreceptor retinoid binding protein, there was a response to either R-4 or R-14, or to both. No such correlation was seen in the Japanese responders to interphotoreceptor retinoid binding protein.

### Table 2

<table>
<thead>
<tr>
<th></th>
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<tbody>
<tr>
<td>Interphotoreceptor retinoid binding protein</td>
<td>3/4</td>
<td>6/16</td>
<td>1/9</td>
<td>1/10</td>
<td>0/9</td>
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<tr>
<td>R-4</td>
<td>4/8</td>
<td>—</td>
<td>2/9</td>
<td>—</td>
<td>1/9</td>
<td>—</td>
<td>5/10</td>
<td>—</td>
<td>1/6</td>
<td>2/6</td>
</tr>
<tr>
<td>R-14</td>
<td>2/8</td>
<td>2/16</td>
<td>2/9</td>
<td>3/10</td>
<td>2/9</td>
<td>2/9</td>
<td>3/9</td>
<td>1/5</td>
<td>2/5</td>
<td></td>
</tr>
<tr>
<td>S-antigen</td>
<td>3/8</td>
<td>3/16</td>
<td>4/9</td>
<td>2/10</td>
<td>1/9</td>
<td>0/9</td>
<td>3/9</td>
<td>1/6</td>
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<tr>
<td>M peptide</td>
<td>6/8</td>
<td>2/16</td>
<td>4/9</td>
<td>1/10</td>
<td>3/9</td>
<td>3/9</td>
<td>3/9</td>
<td>1/6</td>
<td>0/6</td>
<td></td>
</tr>
<tr>
<td>N peptide</td>
<td>3/4</td>
<td>6/16</td>
<td>1/4</td>
<td>5/10</td>
<td>0/4</td>
<td>7/9</td>
<td>4/9</td>
<td>0/6</td>
<td>1/5</td>
<td></td>
</tr>
</tbody>
</table>

* The numerator refers to the number of positive responders. The denominator refers to the total number of patients tested. In most cases, five-day cultures with 20 µg/ml of antigen in each well were found to be optimal except where indicated.

1 Cells were cultured with 4 µg/ml for seven days.

2 Antigen concentration was 100 µg/ml.

3 Cells were cultured for seven days with 20 µg/ml of antigen.
Several patients' lymphocytes had a proliferative response to a peptide fragment, but did not recognize the parent antigen. In 12 of 22 American patients (54%) responding to one or the other S-antigen fragment at 20 or 100 µg/ml in five-day cultures, there was no cross reaction with the whole molecule. Under similar conditions, the responses to interphotoreceptor retinoid binding protein fragments gave no cross reaction in 16 of 20 (84%) American patients. The Japanese responders to S-antigen fragments recognized S-antigen in only six of 19 cases (cells cultured for seven days).

Patients with Behçet's disease showed a response to fragments of both antigens but the responses were strongest for fragments of S-antigen. At 20 µg/ml, M peptide gave the highest response with a mean stimulation index of 5.3 ± 1.0 for patients and 1.3 ± 0.2 for control subjects (P = .0001). The response to N peptide was also significant with patients having a mean stimulation index three times higher than control subjects. Patients with birdshot retinochoroidopathy had similar responses to both sets of fragments. The mean stimulation indices in patients were twice those of control subjects for both sets of fragments.

Several patients demonstrated an ability to give simultaneously a significant proliferative response to at least one determinant of each antigen, but not necessarily to the whole antigen. A total of 32 patients out of the 82 patients tested (39%) were found to give such responses. 18 among the American patients and 14 among the Japanese patients. They were found in all disease categories but were more frequently found among the patients with Behçet's disease or birdshot retinochoroidopathy. A similar distribution of patients was found in the two countries. Of the American patients 11 had active disease at the time they gave a response to both antigens as compared to six of 18 nonresponders (P = .02). There is little difference, however, with the number of patients with active disease (seven of 11 patients) responding to only one antigen (P = .05). A correlation between active disease and a significant lymphoproliferative response was not found among the Japanese patients.

In comparing S-antigen to interphotoreceptor retinoid binding protein, it appears that S-antigen is more frequently correlated with active disease (P = .003). As is shown in Table 3, however, the profile in each disease entity is similar for the two antigens. An attempt was made to correlate proliferative responses with therapy, but no correlation was possible. Patients with active disease were more likely to be treated with cyclosporine or prednisone.

### Discussion

Our aim in this study was twofold. First, we wanted to determine the response profile of interphotoreceptor retinoid binding protein in patients with uveitis and to compare it to that of S-antigen. Secondly, we wanted to determine whether patients were able to respond to fragments of these antigens, which have been shown to be uveitopathogenic in animals. Since the cellular immune response centers around different epitopes in different animal species, we did not know if patients would be able to react to any of these fragments in cellular proliferation assays.

Several previous studies demonstrated that cellular proliferative responses to S-antigen

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### Table 3

<table>
<thead>
<tr>
<th>CLINICAL ENTITY</th>
<th>TESTING CENTER</th>
<th>INTERPHOTORECEPTOR RETINOID BINDING PROTEIN</th>
<th>S-ANTIGEN</th>
</tr>
</thead>
<tbody>
<tr>
<td>Behçet's disease</td>
<td>U.S.</td>
<td>2/7</td>
<td>3/7</td>
</tr>
<tr>
<td>Vogt-Koyanagi-Harada syndrome</td>
<td>Japan</td>
<td>6/9</td>
<td>3/4</td>
</tr>
<tr>
<td>Ocular sarcoid</td>
<td>U.S.</td>
<td>0/2</td>
<td>1/2</td>
</tr>
<tr>
<td>U.S.</td>
<td>0/5</td>
<td>0/2</td>
<td></td>
</tr>
<tr>
<td>Pars planitas</td>
<td>Japan</td>
<td>1/5</td>
<td>1/5</td>
</tr>
<tr>
<td>Birdshot retinochoroidopathy</td>
<td>Japan</td>
<td>2/3</td>
<td>0/0</td>
</tr>
<tr>
<td>Sympathetic ophthalmia</td>
<td>U.S.</td>
<td>2/5</td>
<td>2/5</td>
</tr>
</tbody>
</table>

* Numerator refers to those patients with active disease. The denominator refers to all patients with a significant proliferative response to the antigen at 20 µg/ml or 100 µg/ml. The data include all responders with a stimulation index above 2.0 in both five- and seven-day cultures.
were present in inflammatory conditions affecting the posterior pole, and in particular the retina of patients with uveitis. Table 2 suggests that the response profile for interphotoreceptor retinoid binding protein is nearly identical to that of S-antigen. Patients with Behcet's disease gave the highest number of positive responders. This was true whether the data were analyzed in terms of the number of patients with a stimulation index substantially above that of control subjects, as in Table 2, or using the Student's t-test, which compares each group as a whole. Using the Student's t-test, a significant response to interphotoreceptor retinoid binding protein is also found in patients with birdshot retinochoroidopathy, even though Table 2 does not show this result. The discrepancy develops from a few control subjects who were able to give a strong proliferative response to the antigens tested, which caused a substantial increase in the value of the standard deviation.

Interphotoreceptor retinoid binding protein does not appear to be as predictive of active disease as is S-antigen (Table 3). There are, however, more patients responding to interphotoreceptor retinoid binding protein than to S-antigen. In the Japanese population, the number of patients with active disease who respond to interphotoreceptor retinoid binding protein is greater. A much larger number of patients would be required to determine the exact relationship to disease activity. Patients who show an ability to proliferate concurrently to both antigens have a slight increase in their probability of having active disease, but the difference did not reach statistical significance. In relation to the mechanism of disease induction and its propagation, it is the existence of a response in culture and its greater prevalence in patients with active disease that is significant. In a given patient, an in vitro proliferative response indicates that lymphocytes sensitized to the antigen tested are circulating in the peripheral blood. In the animal models, sensitized lymphocytes capable of an in vitro proliferative response were one of the necessary conditions for the induction of autoimmune disease. It is likely, however, that the induction of uveitis is caused by multiple factors. Therefore, it is not surprising to find some control subjects whose lymphocytes demonstrate an in vitro response to these antigens. Sensitization to these antigens might occur through a variety of means such as minor trauma or mimicry with other peptides. We believe that the difference between a patient and a control subject lies in the inability of control subjects to initiate an inflammatory response or to maintain it once initiated. This could occur because all of the mechanistic criteria have not been met or because suppressor mechanisms are strong enough to shut down the response. The ability of some patients to respond to both retinal antigens in culture is a unique finding, which may help to elucidate certain aspects of the mechanisms involved in chronic uveitis. We believe that a patient is initially sensitized to probably only one antigen; however, after the breakdown of the blood-retinal barrier, the immune system becomes exposed to several new sequestered antigens. These autoantigens are likely processed by circulating or resident antigen presenting cells. Partial digestion of the antigen will generate fragments that are then able to associate with appropriate class II antigens. In the presence of these antigens a complex is formed, which when expressed on the cell surface is able to interact and activate T lymphocytes. These activated, proliferating T lymphocytes can potentiate an acute inflammatory episode and possibly help perpetuate the inflammatory episode. Patients who responded to S-antigen or interphotoreceptor retinoid binding protein were able to recognize one or both fragments tested, but none of the responses to the fragments were of a similar magnitude to that of the parent antigen. This lesser response suggests that these fragments do not appear to be the primary mediators of the cell-mediated immune response in humans. Hence, we conclude that they are not immunodominant sites. Some patients demonstrated an ability to generate an in vitro response to both fragments of a given antigen. This capacity to recognize more than one epitope of S-antigen and interphotoreceptor retinoid binding protein was not found in the animal model. In the Lewis rat, immunization with interphotoreceptor retinoid binding protein generates a strong proliferative in vitro response to peptide R-14, whereas R-4, which we also tested, is a nondominant fragment and is not recognized by animals immunized with the whole protein. In the case of S-antigen, only one immunodominant site has been reported. It is likely that this disparity reflects a fundamental difference between the experimental model and naturally occurring autoimmune diseases. Future studies are needed to elucidate the importance of the various epitopes of S-antigen and interphotoreceptor
retinoid binding protein. Although most responders to S-antigen and interphotoreceptor retinoid binding protein were able to respond to one or both fragments of these antigens, most of the responders to the fragments were not able to recognize the parent antigen.

This study shows that interphotoreceptor retinoid binding protein has a response profile that is virtually identical to that of S-antigen. Several patients were able to mount a response to both antigens in culture. Several patients also showed the ability to respond to more than one epitope of a given antigen. Both phenomena indicate that human disease is much more complex than suggested by the current autoimmune models of disease. Several autoantigens appear to be implicated in chronic uveitis in humans, and the interactions between each of these antigens is unknown. The extent of the plurispecificity of response to autoantigens also remains to be determined. It may be a generalized characteristic of human class II antigens, and it is also possible that patients possess an enhanced ability to interact with several different fragments as compared to normal individuals. Further studies on cell lines, and by using multiple fragments of the antigens, may help answer these questions.

References


