Human S-Antigen Determinant Recognition in Uveitis

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PURPOSE. Soluble antigen (S-Ag) is a member of the arrestin family of protein with which it shares a high level of homology. It is an immunologically privileged retinal antigen that can elicit experimental autoimmune uveitis (EAU) and is thought to be a target for ocular inflammatory diseases. This study was conducted to identify in humans, the immunogenic determinants of human S-Ag and to establish whether a specific response profile occurs in particular ocular inflammatory conditions.

METHODS. Peripheral blood lymphocyte responses were measured against a panel of 40 overlapping synthetic peptides of human S-Ag in patients with chronic uveitis and compared with control subjects. Patients with Behçet disease, sarcoidosis, Vogt-Koyanagi-Harada, and sympathetic ophthalmia were tested.

RESULTS. A limited number of immunodominant determinants were identified for Behçet disease and sarcoidosis. These were all located at sites of limited homology with other known arrestins. In addition, several individual patients had prominent proliferative responses to multiple determinants well above that of control subjects. This determinant spread was observed in all disease entities except sympathetic ophthalmia, which did not show any immunoreactivity to S-Ag. Significant response shifts were also noted over time in two patients.

CONCLUSIONS. The results indicate that there are specific immunodominant determinants to human S-Ag in patients with certain forms of uveitis. However, in individual patients, response is not limited to these determinants. In the chronic stage of disease, response is spread over many determinants. (Invest Ophthalmol Vis Sci. 2001;42:3233–3238)

Soluble retinal antigen (S-Ag) also known as rod arrestin, or b-arrestin, is a major component of rod outer segments1 the normal physiological role of which is to quench the visual transduction cascade induced by light activation of rhodopsin.2,3 Although closely related to b-arrestins, which are ubiquitously distributed throughout the body,1–6 rod photoreceptor arrestins are confined to the retina in an immune-privileged site.7 In addition to its normal physiologic function, S-Ag is one of several ocular antigens capable of inducing experimental autoimmune uveitis (EAU) in susceptible hosts.8 EAU is mediated by activated, antigen-specific CD4+ T cells,9 and can be induced by immunization with several S-Ag-derived peptides.10–12 Pathologically, the process resembles a number of human conditions thought to be of autoimmune origin.8 S-Ag is thought to be immunopathogenic in humans, either causing or prolonging certain forms of uveitis. In accordance with this notion, a large number of patients with uveitis were found to respond to bovine S-Ag in lymphocyte proliferation assays.13–15 In addition, lymphocyte responses have been noted to a few peptide determinants derived from both the bovine and human S-Ag sequences.13,16–17

The immune response against a whole protein is usually targeted toward a small number of peptide determinants. To initiate the immune response requires binding to major histocompatibility complex (MHC) molecules on the surface of antigen-presenting cells (APCs) and presentation to receptive T cells.16,19 Because of the polymorphism of MHC molecules, the majority of immunogenic peptide determinants bind strongly to only a single or a limited set of MHC molecules.20 However, some peptide determinants bind to several heterogeneous MHC molecules and may even cross species.21–22 In the case of myelin basic protein, for example, certain peptides, encephalitogenic in experimental animals, have been found to be recognized by lymphocytes from patients with multiple sclerosis.23,24 In this study, we decided to use a panel of overlapping synthetic peptides of human S-Ag to test their immunoreactivity in patients with various forms of uveitis as well as in control subjects. It is then possible to define a spectrum of immune reactivity for each disease entity and to identify the limited set of determinants with particularly high immunoproliferative responses. Response to a few of these determinants was also observed over time.

MATERIALS AND METHODS

Antigen Synthesis and Preparation

Forty overlapping oligomeric peptide determinants of human S-Ag, spanning the entire length of the human S-Ag sequence,25 were synthesized by Applied Biosystems (Foster City, CA). Each peptide determinant measured 20 amino acids in length, except for the last one, which measured 15 amino acids. Each peptide determinant overlapped the previous sequence by 10 amino acids. The exact sequence and the nomenclature used throughout this article are shown in Figure 1. Peptides were synthesized by solid-phase chemistry using tert-butyloxy-carbonyl derivatives of the amino acids on an automated peptide synthesizer and were purified by HPLC to at least 95% purity. The amino acid composition of each peptide was verified using amino acid analysis and automated gas-phase sequencer. In proliferation assays, a final concentration in each well of 20 and 100 µg/ml was used.

Patient Selection

Patients participating in this study were selected from the pool of patients under observation in the uveitis clinic of the National Eye Institute, Bethesda, Maryland. Control subjects were taken from among the NEI medical and paramedical staff, and were age matched as closely as possible with the study population. Before participating in the study, all patients gave informed consent for a protocol approved by the Medical Review Board of the National Eye Institute and in compliance with the tenets of the Declaration of Helsinki. In all patients with active uveitis involving the posterior segment, the disease was controlled with cyclosporine (1.0–5.0 mg/kg · d) and prednisone (2.5–30 mg/d). Any patient with a reactivation of uveitis in the 3 months before this study was considered to have clinically active disease. A decrease in visual acuity by two lines or more on an Early
Treatment Diabetic Retinopathy Study (ETDRS) reading card was associated with evidence of vitreal or retinochoroidal inflammation characterized as active uveitis. Patients had one of the following disorders: Behçet disease, ocular sarcoidosis, Vogt-Koyanagi-Harada (VKH) syndrome, or sympathetic ophthalmia. The diagnosis for each condition was established as described elsewhere.13 Patients with Behçet disease met the diagnostic criteria from the International Behçet’s Disease Study Group.26 Those with ocular sarcoidosis had typical retinochoroidal granulomatous uveitis. Patients with VKH met the criteria of the American Uveitis Society27 or had typical fundus and fluorescein findings. Patients with sympathetic ophthalmia had a clinical picture compatible with the diagnosis and a history of multiple surgeries or ocular trauma in one eye. Characteristics of the patients are given in Table 1.

### Lymphocyte Proliferation Assays

Mononuclear lymphocytes were separated on isolymph gradient (Gallard-Schlesinger, Carle Place, NY) from heparinized blood shortly after the sample was obtained. Cells were resuspended in RPMI 1640 with HEPES (Gibco, Grand Island, NY), supplemented with glutamine (2 mM), penicillin (100 U/ml), streptomycin (100 μg/ml), and 10% commercial heat-inactivated human AB serum (Biocell Laboratories, Carson, CA). These cells were immediately placed in culture at a density of 2 × 10^5 cells/well in the presence of antigen, in flat-bottomed, 96-well plates (Costar, Cambridge, MA). All assays were plated in triplicate. Antigen concentrations were either 20 or 100 μg/ml. Peptides were tested simultaneously. For control of immune reactivity, purified phytohemagglutinin (PHA; Murex Diagnostics, Dartford, UK) and purified phytohemagglutinin (PHA; Murex Diagnostics, Dartford, UK) were also tested. For the last 12 hours before harvesting at day 5, each well was pulsed with [3H]thymidine (2 Ci/mmol, 0.5 μCi per 10 μl/well; New England Nuclear, Boston, MA). Results are expressed as a stimulation index (SI = mean counts per minute in stimulated cultures/mean counts per minute in unstimulated control cultures).

### Data Analysis

SIs for each peptide determinant were analyzed in two ways. First, the results obtained from control subjects were compared with those from patients per disease entity for each peptide determinant. Comparisons were made using a nonparametric test for analysis of variance (Kruskal-Wallis) with a postanalysis correction for comparison of data pairs (Dunn test). Statistical analysis was performed by computer (Prism software, ver. 2.0; GraphPad Inc., San Diego, CA). The second analysis method involved the determination of the frequency with which the SI in patients was above the tolerance limit of control samples. This tolerance limit for a given peptide determinant was set as the mean SI of control subjects ± 2 SD.

### RESULTS

**Patient Characteristics and Assessment of Lymphocyte Response**

Twenty-nine patients and 15 volunteers were tested for their lymphocyte proliferative responses to a panel of overlapping peptide determinants. The nomenclature is indicated under each determinant.

### Table 1. Characteristics of Patients with Uveitis and Control Subjects

<table>
<thead>
<tr>
<th>Diagnosis</th>
<th>Number</th>
<th>Sex (M/F)</th>
<th>Age (y)</th>
<th>Disease Duration (mo)</th>
<th>Disease Activity (Active/Inactive)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control subjects</td>
<td>15</td>
<td>9/6</td>
<td>35 (23–55)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Behçet disease</td>
<td>12</td>
<td>6/6</td>
<td>29 (22–41)</td>
<td>47 (10–96)</td>
<td>6/6</td>
</tr>
<tr>
<td>Sarcoidosis</td>
<td>9</td>
<td>5/4</td>
<td>35 (18–45)</td>
<td>42 (10–82)</td>
<td>4/5</td>
</tr>
<tr>
<td>VKH</td>
<td>4</td>
<td>2/2</td>
<td>27 (21–35)</td>
<td>40 (8–70)</td>
<td>3/1</td>
</tr>
<tr>
<td>Sympathetic ophthalmia</td>
<td>4</td>
<td>2/2</td>
<td>41 (31–47)</td>
<td>44 (11–81)</td>
<td>0/4</td>
</tr>
</tbody>
</table>

Ranges of age and disease duration are in parentheses.
human S-Ag peptide determinants at 20 μg/ml and 100 μg/ml. Because the optimal culture conditions for lymphocyte proliferation with these peptide determinants was not known, both concentrations were used. All patients had shown evidence of retinal inflammatory disease for at least 8 months (8–120 months) before being tested. All patients had a chronic remitting course of disease at the time, with the exception of the patients with sympathetic ophthalmia, in whom the disease was quiescent for more than 1 year before testing. A number of patients had a history of an acute inflammatory episode in the

![Graph](image1)

**Figure 2.** Average SIs for each peptide determinant at a concentration of 20 μg/ml per well. Error bars, SD. Data are shown for the disease categories that showed a significant deviation from the mean of control subjects. The profile of patients with sympathetic ophthalmia was similar to that of control subjects, except for determinant 3 (mean SI, 1.9 ± 1.4) and determinant 6 (mean SI, 2.1 ± 1.6).

![Graph](image2)

**Figure 3.** Average SIs for each determinant at a peptide concentration of 100 μg/ml per well. Error bars, SD. Data are shown for disease categories showing a significant deviation from control subjects. The profile for sympathetic ophthalmia is similar to that in control subjects, except for determinant 3 (mean SI, 4.0 ± 3.5).
6 months before testing (Table 1). Control of lymphocyte responsiveness was determined in each group by testing with PPD and PHA, as indicated in the Methods section. The average SIs were as follows: control subjects: PHA 44, PPD 75; Behçet disease: PHA 78, PPD 24; sarcoidosis: PHA 66, PPD 11; VKH: PHA 42, PPD 27; sympathetic ophthalmia: PHA 47, PPD 7. There was no significant difference between groups in the response to PHA. There was also no significant difference between the various patient groups with regard to the response to PPD. Their responses were lower than in the control group, in which the difference was largely due to a few high responders.

**Determinant Mapping Using Analysis of Variance**

Proliferative responses to individual peptides were highly variable. In general the response to antigen was strongest with the higher antigen concentration (Figs. 3, 4). To test for internal consistency, one volunteer was repeatedly tested at different time points over a period of 1 year. Each proliferative response remained well within the mean + 2 SD of control subjects, except at one of the nine time points, when deviant responses were noted for several determinants. Analysis of variance was used to compare SIs from each patient group to control subjects using a nonparametric analysis of variance. This analysis showed in all disease entities a heterogeneous response to S-Ag. In the case of sympathetic ophthalmia, the following determinants gave significant responses: 2, 6, 11, 21, 25, 27, and 38. In each case, only one patient, but not always the same one, produced an SI above the tolerance limit. For the remaining disease entities, the number of significant responders varied considerably among disease entities and among determinants (Fig. 4).

**DISCUSSION**

Patients with uveitis showed a high degree of heterogeneity in their lymphoproliferative response to determinants of human S-Ag. Such heterogeneity is not surprising, given that patients carried a variety of diagnoses and were at different stages of disease activity and duration. However, two patterns of response were observed, one of which appeared to be disease specific and the other specific to individual patients. The first relates to a generalized increase in lymphocyte proliferation accompanying selected determinants in patients with Behçet disease or sarcoidosis, whereas the second relates to the observation that a number of patients had heightened immune responses to specific determinants, significantly above the mean of control subjects. This heightened response in the presence of whole S-Ag, had previously been shown to be a sign of disease activity within weeks of the assay. The immunogenic determinants associated with Behçet disease and sarcoidosis (Table 2) are adjacent to highly immunogenic or pathogenic determinants in the experimental model.


Determinant Recognition

Table 3. Maximum Number of Immunoproliferative Determinants Recognized by Individual Patients per Disease Category

<table>
<thead>
<tr>
<th>Disease Entity</th>
<th>Antigen Concentration in Culture (μg/ml)</th>
<th>Number of Patients Responding to the Number of Determinants*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>1</td>
</tr>
<tr>
<td>Behçet disease</td>
<td>20</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>0</td>
</tr>
<tr>
<td>Sarcoidosis</td>
<td>20</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>0</td>
</tr>
<tr>
<td>VKH</td>
<td>20</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>0</td>
</tr>
<tr>
<td>Sympathetic ophthalmia</td>
<td>20</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>1</td>
</tr>
</tbody>
</table>

An immunoproliferative determinant is defined as an SI ≥ mean of control subjects +2 SD.
* Number of human S-Ag determinants to which a given patient responds. Max corresponds to the highest number of response sites registered in a patient within the specified disease category and at the specified antigen concentration in culture.

Table 4. Response to Selected Determinants of S-Ag over Time

<table>
<thead>
<tr>
<th>Diagnosis</th>
<th>Time Point (mo)</th>
<th>Response to S-Ag Determinant (SI)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>Control</td>
<td>0</td>
<td>1.4</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>1.1</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>1.2</td>
</tr>
<tr>
<td></td>
<td>12</td>
<td>0.8</td>
</tr>
<tr>
<td>Behçet disease</td>
<td>0</td>
<td>1.0</td>
</tr>
<tr>
<td>Patient 1</td>
<td>6</td>
<td>2.9</td>
</tr>
<tr>
<td>Patient 2</td>
<td>0</td>
<td>0.9</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>1.1</td>
</tr>
</tbody>
</table>

Certain patients showed an immune response, which over time either significantly increased or decreased baseline level (Table 4). However, variations were present among disease entities, because the response in sympathetic ophthalmia was limited to a few determinants. The relatively limited determinant spread in this latter disease may indicate that S-Ag does not play a role in the disease process, because the overall response profile was very similar to that of control subjects. Pathologic changes in this disease are located within the choroid, and experimentally, a similar disease pattern is induced by using choroidal antigens.36 Determinant spreading is likely to be an integral part of the immune response over time to antigens that contribute to the disease process. Inherent in determinant spreading is a shift in response over time. We observed in two patients, but not in a volunteer, such a shift in antigenic responses over time (Table 4). This shift in autoreactivity is unlikely to be an artifact, because repeat testing of a volunteer over a 12-month period showed only limited variations in SIs that remained within 2 SD of the mean of all control subjects. These shifts in responses correspond well to the observations made in multiple sclerosis and experimental autoimmune encephalomyelitis (EAE) and suggest that antigen spreading also occurs in patients with uveitis.36,37 This deserves to be studied in more detail with a number of patients observed after surgery.

In summary, the present study demonstrates the existence of a number of disease-specific antigenic determinants in two disease entities: namely Behçet disease and sarcoidosis. These were associated with unique sequences within the human S-Ag sequence and were related to pathogenic sites in the experimental model. It also demonstrates the existence of a number of determinants associated with immune responses in individ-

EAU. Determinants 19, 20, and 36 induce uveitis in the Lewis rat.29 Determinant 18 is immunoproliferative in the cynomolgus monkey.30 Because the determinants we studied are 20 amino acids long, the minimal immunologic trigger for each determinant is nested somewhere within the peptide sequence. Use of smaller synthetic peptides should make it possible to identify the minimal immunogenic sequence and in particular reveal whether the same sequence is immunogenic in all species. The identification of appropriate targets may be facilitated by selecting sequences that do not have homology in all species. The identification of appropriate targets may be facilitated by selecting sequences that do not have homology in all species. The identification of appropriate targets may be facilitated by selecting sequences that do not have homology in all species. The identification of appropriate targets may be facilitated by selecting sequences that do not have homology in all species.
nal patients. These responses were present in patients with a retinal disease pattern. The observations give credence to the notion that retinal autoantigens play a role in certain forms of uveitis. Further study of determinant responses in patients with uveitis may help to identify the exact site of the immune activation and may provide the rationale for an attempt at peptide-based therapy.

References