Inter- and Intramolecular Epitope Spreading in Equine Recurrent Uveitis

Cornelia A. Deeg, Barbara Amann, Albert J. Raith, and Bernd Kaspers

PurPOSE. To test the hypothesis that inter- and intramolecular spreading to S-antigen (S-Ag) and interphotoreceptor retinoid binding protein (IRBP)-derived epitopes occurs in a spontaneous model of recurrent uveitis in the horse.

METHODS. The immune response of eight horses with equine recurrent uveitis (ERU) was compared with that of five control horses with healthy eyes. Lymphocytes derived from peripheral blood (PBLs) were tested every 8 weeks for their reactivity against S-Ag and various S-Ag and IRBP-derived peptides for 12 to 39 months (median, 22 months). During uveitic episodes, additional blood samples were analyzed.

RESULTS. Intermolecular epitope spreading was detectable in all ERU cases during the study. Intramolecular spreading occurred in seven (of eight) horses with ERU. Fourteen relapses were analyzed during the observation period. Ten uveitic episodes were accompanied by neoreactivity to S-Ag or IRBP-derived peptides during the relapse. Shifts in the immune response profile were also detectable without any clinical signs of inflammation. Eye-healthy control horses were negative at all time points in the in vitro proliferation assays.

CONCLUSIONS. Inter- and intramolecular spreading was detectable in a spontaneous model of recurrent uveitis. The shifts in immunoreactivity could account for the remitting-relapsing character of the disease. (Invest Ophthalmol Vis Sci. 2006;47: 652–656) DOI:10.1167/iovs.05-0789

Current concepts to explain the origin and perpetuation of autoimmune diseases include molecular mimicry, bystander activation, and epitope spreading. These mechanisms do not exclude each other but could appear together and even interact.

Epitope spreading occurs in the context of inflammation and destruction of the target tissue, promoting spreading of the immune response from one autoantigenic determinant to other epitopes not previously recognized by the immune system. Autoimmune uveitis is assigned to the class of organ-specific, T-cell-mediated autoimmune diseases. Two major autoantigens have been identified so far: the retinal proteins S-antigen (S-Ag) and interphotoreceptor retinoid binding protein (IRBP). Peripheral blood-derived, autoaggressive T cells specific for the S-Ag and IRBP epitopes were detected in patients with autoimmune uveitis, and there is experimental evidence of molecular mimicry in the rat model of experimental autoimmune uveitis (EAU). Therapeutic studies with cytokine blockage also point to a possible role of bystander activation. S-Ag determinant recognition in humans with uveitis has been examined by testing peripheral blood-derived leukocyte (PBL) proliferation induced by S-Ag-derived peptides. In that study, the immune response of two patients with Behçet disease was retested after 6 and 2 months, and a shift in the S-Ag-specific response pattern toward new determinants was observed in both patients, an event called intramolecular epitope spreading. To our knowledge, these are the only data that support the occurrence of epitope spreading in spontaneous autoimmune uveitis. We have shown before in experimentally induced uveitis that intramolecular spreading occurs to S-Ag in horses immunized with IRBP. To investigate the occurrence of epitope spreading in spontaneous disease, we used a model of spontaneous human autoimmune uveitis in the horse called equine recurrent uveitis (ERU). This is a remitting-relapsing disease that is clinically similar to human autoimmune uveitis.

We decided to examine the long-term immune response of uveitic horses and repeatedly tested the pattern of immunologic responses to various epitopes of S-Ag and IRBP in eight ERU horses and five eye-healthy control horses over a mean observation period of 22 months. We found intramolecular epitope spreading in all tested ERU horses and intramolecular spreading in seven horses. In addition, we could correlate the clinical relapse of disease to neoreactivity toward new peptide epitopes in 10 uveitic episodes.

METHODS

Animals and Blood Samples

Eight horses with at least three clinical signs of spontaneous ERU and at least three uveitic episodes were included in this study (Table 1). Clinical signs of intraocular inflammation included aqueous flare, synechiae, vitritis, pigmentation on the anterior lens surface, retinal detachment, and cataract. The history was given by the owners and the veterinarians that treated the horses. Only animals with an observation period of at least 3 years before starting the study were included. Five sex-matched, visually healthy horses from two breeders served as control subjects (Table 2). The genetic background was standard outbred (German warmblood). Blood samples were collected from all animals regularly every 8 weeks during the study (12–39 months). During overt acute uveitic episodes, additional blood samples were obtained. The horses did not receive any systemic medication during the study period. Relapses were treated with topical anti-inflammatory drugs until the cessation of the episode and then were withdrawn. This is the standard therapy for ERU-diseased horses, due to severe side effects of systemic immunosuppressive drugs and due to European Union and FEI (International Federation of Equestrian Sports) legislation. Blood samples were obtained at onset of uveitic episodes before topical medication was given. All animals were treated according to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

IRBP and S-Ag Derived Autoantigens

Bovine S-Ag was isolated from retinas, as described previously. S-Ag- and IRBP-derived peptides were purchased from Biotrend (Co-
The following peptides were used for stimulation in in vitro proliferation assays: S-Ag-derived peptides: S-Ag 281 (bovine S-Ag; amino acids [aa] 281-290),13 peptide M (bovine S-Ag; aa 303-320),13 S-Ag 286 (bovine S-Ag; aa 286-297),20 and PDSAg (bovine S-Ag; aa 342-355);12 IRBP-derived peptides: PI 536 (bovine IRBP; aa 536-549),10 PI731 (human IRBP; aa 731-745),13 PI1137 (human IRBP; aa 1137-1153),13 R4T (bovine IRBP; aa 1163-1176),15 R14 (bovine IRBP; aa 1169-1191),22 and PDIRBP (bovine IRBP; aa 1174-1187).10

### Endotoxin Measurement in Peptides and S-Antigen

To rule out contamination with endotoxins, especially lipopolysaccharides, we regularly tested both antigen preparations and purchased peptides using the semiquantitative limulus amebocyte lysate method22 (E-Toxate; Sigma-Aldrich, Deisenhofen, Germany). The endotoxin concentration of antigen preparations was below the detection limit (0.125 EU/mL) in the peptides.

### T-Cell Proliferation Assays

PBLs were obtained from fresh heparinized horse blood samples by density gradient centrifugation (Ficoll; GE Healthcare, Braunschweig, Germany) every 8 weeks. Cells were cultured in triplicate (5 × 10^5 per well) in flat-bottomed microtiter plates (Nunc, Wiesbaden, Germany) for 5 days in the presence of antigens (5 μg/mL whole S-Antigen or S-Ag- or IRBP-derived peptides). Cells were then labeled with [3H] thymidine (GE Healthcare) per well for an additional 18 hours and harvested on day 6. [3H] thymidine incorporation was measured by β-scintillation counting. The results are presented as stimulation index (SI; mean counts per minute [cpm] of triplicates with antigen/mean cpm of triplicates without antigen). An SI ≥ 2 was considered a positive reaction.

### Results

#### Variable T-Cell Recognition Patterns in ERU-Diseased Horses

We monitored the peripheral T-cell reaction every 2 months for a total observation period of 22 months in eight horses with spontaneous ERU and compared the results with those of similar assays of healthy control horses. We detected a positive peripheral T-cell reaction to S-Ag or IRBP-derived peptides in all tested ERU horses (Fig. 1; the immune reaction of ERU horses at various time points during the observation period; all reactive epitopes are shown; PI 536 and R4T were negative throughout the study). The visually healthy control horses never reacted to the tested peptides (data not shown, n = 5). In addition, intermolecular epitope spreading was detectable in all ERU horses (Fig. 1) but the response pattern varied between individual horses. Five of eight horses’ lymph cells first recognized S-Ag-derived peptides before intermolecular spreading to IRBP-derived peptides occurred (Fig. 1, ERU cases 2, 3, 5, 7, and 8). The remaining three cases showed a shift in the reaction from IRBP to S-Ag derived peptides (Fig. 1, ERU cases 1, 4, and 6). In addition to intermolecular spreading, intramolecular spreading patterns were detectable in the animals in six ERU cases, which reacted to different S-Ag-derived epitopes during the observation period (Fig. 1; ERU cases 1, 2, 3, 4, 5, and 8). Five ERU horses showed a shift in reaction to different IRBP-derived epitopes during the observation period (Fig. 1; ERU cases 1, 4, 5, 7, and 8).

#### Shifts in Antigen Recognition in Uveitic Episodes

During a uveitic episode, we analyzed additional blood samples of each affected horse and identified a neoreactivity in five of seven horses that had a uveitic episode during the observation period (Fig. 1, ERU cases 1, 2, 4, 5, 6, and 8; episodes are marked as shaded bars). These horses had not reacted to the respective epitope in the proliferation assays before the episode. A shift of the immune reaction could be correlated to new uveitic episodes in 10 of 14 relapses that occurred during the observation period. In three relapses, the specificity of peripheral T cells could not be determined (Fig. 1, ERU cases 2, 3, and 6). ERU case 7 had a blind and phthisical eye at the commencement of the study, and so uveitic episodes could not be clinically observed despite a T cell reaction at three time points. In addition to periods of acute uveitic episodes, autoimmune responses were also measurable in quiescent stages of uveitis (Fig. 1, ERU cases 1, 3, 4, 5, 6, 7, and 8). The autoantibody titers remained stable within each horse, despite the changes in T-cell reaction (data not shown).

### Discussion

Epitope spreading was previously discovered in experimental allergic encephalitis (EAE)23 and was verifiable in patients with multiple sclerosis (MS).24 To our knowledge, there are no data on epitope spreading in experimental autoimmune uveitis (EAU). The progression of both EAE and MS was accompanied by the decline of primary T-cell autoreactivity associated with disease onset and by the concurrent emergence of an epitope spreading cascade.24 Recognition of further self-epitopes as a perpetuating mechanism of autoimmune diseases is a well-established concept in many animal models of autoimmune diseases and is also observed in patients (e.g., with type 1 diabetes mellitus).25 The spreading to new determinants ex-

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**Table 1. Characteristics of ERU Diseased Horses**

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<thead>
<tr>
<th>Subject</th>
<th>Birth Year</th>
<th>Clinical Uveitis</th>
<th>Duration of Uveitis at Beginning of Study</th>
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<td>12 Months</td>
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**Table 2. Characteristics of the Control Group**

<table>
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<td>5</td>
<td>1989</td>
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1. [Reference](#)
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4. [Reference](#)
5. [Reference](#)
plains the character of remitting–relapsing diseases as the autoimmune reaction to one epitope goes down after the peak of inflammation. The progression of disease depends on the generation of autoaggressive T cells with a new repertoire. Several different autoantigens are involved in the reaction pattern of patients in various autoimmune diseases, leading to intermolecular determinant spreading. In experimental mouse models, a hierarchic spreading cascade was detected, indicating that chronic progression of EAE involves a shift of autoreactivity from primary initiating self-determinants to defined cascades of secondary encephalitogenic determinants. However, in a study of patients with MS during disease progression, a spreading pattern was detected in the immune reaction, but the target of the next autoaggressive reaction was not predictable compared with the SWXJ mice. This finding is not surprising, as the heterogeneous background of the patients with MS and the difference in the clinical picture of MS should lead to an individually different pattern of inflammation and autoimmune response. This observation was confirmed in other studies examining the autoaggressive T-cell repertoire of MS or patients with type I diabetes.

Our study is the first examination of epitope spreading in an animal model of spontaneous uveitis, ERU. A key feature of recurrent uveitis is the remission and relapse of uveitic episodes. This phenomenon could be explained by determinant spreading. Reaction to a new epitope could lead to a new uveitic episode that ceases when regulatory cells get the upper hand in inflammation. The next uveitic episode would then be generated by a shift of response to another epitope of the same autoantigen (intramolecular spreading) or another autoantigen...
Epitope-Spreading in Uveitis

Disease progression. A first hint of this mechanism came from our ERU horse model induced with IRBP. In five of seven horses immunized with bovine IRBP, intra- and intermolecular spreading to S-Ag was observed, although the animals received only IRBP by injection. We therefore sought to verify these findings in the spontaneous disease monitored in this study. Accordingly, we detected a response in the T-cell proliferation assay in all eight ERU horses during the observation period (Fig. 1). In contrast, eye-healthy control subjects never reacted in these assays. The SI is low in the responders, but comparable to the indices obtained after immunization with IRBP or in other autoimmune diseases involving PBLS. We tried to prevent false-positive reactions through endotoxin contaminated preparations of peptides through choosing preparations with an endotoxin concentration of <0.125 EU/mL for the in vitro test. For that reason, we think the proliferative response clearly indicates a specific autoimmune reaction to the respective peptides in the ERU responders. Of note, all horses showed autoreactive lymphocytes to both tested autoantigens, S-Ag and IRBP, during the course of the disease (Fig. 1). This is a clear indication of intermolecular spreading in ERU. Intramolecular spreading patterns were also observed to S-Ag in six horses and to IRBP determinants in five. A small study in two patients with uveitis also indicated S-Ag intramolecular spreading in these individuals. In our study, the clinically observed uveitic episodes were accompanied by a neoactivity in the autoimmune response in five of seven horses. The animals in ERU cases 3 and 6 did not react to any of the tested peptides during the episodes, revealing the lack of the appropriate determinants for these horses in the study. Although we tested a large panel of peptides from S-Ag and IRBP including the immunodominant ones, this study can only describe a part of the whole reaction pattern accompanying the progression of uveitis in ERU. In three horses, we were able to directly compare the immune reaction before a uveitic episode with the reaction during a relapse (ERU cases 1, 2, and 4). We were able to link them to a shift in the immune response to a newly recognized determinant at the time of the new episode. The association between a relapse and a changed immunologic response pattern points to a critical role for antigen spreading in the recurrent disease onset. Nevertheless, we observed major shifts of the immune response in quiescent stages. Therefore, the meaning of the observed neoactivities remains uncertain. Determinant spreading was also detectable during clinically quiescent stages in three cases (ERU cases 3, 5, and 8). There are several possible reasons for this phenomenon. First of all, we could have missed signs of posterior uveitis in the horses, as this is not accompanied by pain and can therefore only be detected by repeated examinations of the fundus after application of mydriatic drugs. Therefore, the observed shifts in the reaction pattern could indicate ongoing disease activity in the horses concerned. If the autoimmune response is directed toward minor uveitogenic epitopes in these cases, no overt uveitic episode can be detected. It has been shown that epitope spreading in the EAE model is only relevant to the disease if it targets an encephalitogenic determinant. A shift in the immune response to a nonencephalitogenic epitope could be measured in the EAE mice but this was not accompanied by disease progression.

A further explanation of the observed spreading reaction not leading to a uveitic episode could be an epitope presentation by antigen-presenting cells in the context of an anti-inflammatory cytokine milieu. A possible interpretation of the observed spreading reaction in quiescent stages could be an unknown regulatory function or protective function of these T-cell clones as speculated in other diseases as multiple sclerosis. The uveitogenicity of peptides could also vary in the tested horses due to the heterogeneous major histocompatibility complex (MHC) backgrounds leading to a different potential in peptide processing and presentation. This could therefore account for a variance in the uveitis-inducing potential between determinants in vivo.

Third, the observed reaction patterns could be an epiphenomenon of uveitis. The self-reactivity could be caused by the tissue destruction and therefore may just reflect the retinal damage without direct implications for pathogenesis. Further experiments are needed to determine the significance of determinant spreading for autoimmune uveitis.

In contrast to mouse models of epitope spreading, but in agreement with early data from patients with autoimmune disease, we saw no hierarchical pattern of targeted determinants that allows us to predict the next targeted peptide. This results most likely from the heterogeneous backgrounds of human and equine cases in comparison to the inbred mouse strains. Thus, the concept of determinant spreading holds true in spontaneous disease. However, a therapeutic intervention targeting the next peptide in the hierarchy does not seem a successful approach in ERU. The finding of epitope spreading in a spontaneous model for human autoimmune uveitis underlines the importance of examining the full autoantigenic response pattern in patients with uveitis.

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References


