Immune Responses to Retinal Autoantigens and Peptides in Equine Recurrent Uveitis

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PURPOSE. To test the hypothesis that autoimmune mechanisms are involved in horses in which equine recurrent uveitis (ERU) develops spontaneously.

METHODS. Material obtained from horses treated for spontaneous disease by therapeutic routine vitrectomy was analyzed for total IgG content and IgG specific for S-Antigen (S-Ag) and interphotoreceptor retinoid-binding protein (IRBP). The cellular infiltrate of the vitreous was analyzed by differential counts of cytopsin preparations and flow cytometry using equine lymphocyte-specific antibodies. Antigen-specific proliferation assays were performed comparing peripheral blood lymphocytes (PBLs) with vitreal lymphocytes by stimulation with S-Ag and several S-Ag- and IRBP-derived peptides.

RESULTS. The total IgG content of specimens from horses with ERU was very high with great variability among the investigated samples (11.5 ± 8.0 mg). Autoantibodies to S-Ag or IRBP or both were found in 72% of vitreous specimens from horses with uveitis. The leukocyte infiltrates (up to 2 × 108 cells per sample) were dominated by lymphocytes (>90%) in most cases (22/32). Flow cytometry showed that more than 50% of these cells were CD4+ T cells. In vitro stimulation of vitreal lymphocytes, but not of PBL, showed a strong proliferative response to peptides derived from S-Ag or IRBP in 9 of 12 patients.

CONCLUSIONS. In the eyes of horses with ERU, IgG antibodies and autoreactive T cells specific for retinal antigens were detected. These results strongly support the hypothesis that ERU is an autoimmune-mediated disease and is highly similar to recurrent uveitis in humans in both clinical and immunologic parameters. (Invest Ophthalmol Vis Sci. 2001;42:393–398)

Equine recurrent uveitis (ERU) is a spontaneous disease affecting up to 15% of horses.1 The disease is characterized by repeated episodes of intraocular inflammation, and many corresponding clinical and pathologic features are similar to recurrent uveitis in humans.2 Clinical findings include biephaphorospasm, photophobia, miosis, and pain in the acute phase.3 The recurrences vary in phase and severity, with a tendency to increase in severity. Vision loss or blindness is the result of the inflammatory process that leads to synechiae, cataract formation, retinal detachment, or phthisis bulb.4 Blind horses have to be euthanatized for ethical reasons and cause large economic losses in the equine industry. Although ERU has been known for centuries and occurs worldwide, the cause remains unclear.5 Research has focused on the identification of infectious agents that may induce uveitis, such as bacteria, viruses, or parasites, especially on a possible role for Leptospira interrogans as an initiating agent in this process.6–8 However, the concept of an infectious factor that exclusively induces and maintains the disease is not sufficient to explain certain aspects of the clinical course and therapeutic approaches. Because of the recurrence of inflammation,3 the positive effect of corticosteroids,2 and the insufficient therapeutic success of antibiotics, the concept has emerged that the disease is immune mediated. Therefore, ERU is of high value for studying uveitis, because horses represent the only species besides humans in which recurrent uveitis develops spontaneously.9 Only a limited number of experiments have been designed to examine the immunologic reactions of diseased horses. Immunohistochemical studies have demonstrated a predominance of T cells in the inflammatory cellular infiltrates and an increase of major histocompatibility complex (MHC) class II expression on retinal pigment epithelial cells.10 Recently, we have established pars plana vitrectomy as a therapeutic approach in ERU,4 and this has enabled us to gain inflammatory cells from eyes with uveitis. The goal of our study was to further investigate the underlying immunopathogenic mechanisms in ERU, providing evidence that the disease is autoimmune mediated with useful and unique properties in comparison with all other uveitis models.

METHODS

Patients

Sixty-four horses with clinical signs of ERU underwent therapeutic pars plana vitrectomy according to the method described by Werry and Gerhards.5 All patients had at least two uveitic episodes, and ERU was diagnosed according to clinical criteria, as described.11 Clinical signs of intraocular inflammation including afebrile flare, synechiae, vitreitis, pigment deposition on the anterior lens surface, retinal detachment, and cataract. The horses were treated with topical atropine a few days before surgery. Neither immunosuppressive therapy nor corticosteroids were applied, because horses undergo surgery only in the quiescent stages of the disease.3 Physical examination included intraocular pressure measurement, slit lamp examination, and direct ophthalmoscopy with dilated pupil. Because the donors of specimens are not experimental but companion animals and were brought to the Department of Equine Surgery in the need of medical help, these studies could not include histologic analysis of the eyes. Enucleation of uveitic eyes is performed rarely, because vitrectomy has been successfully established for horses as a routine treatment for progressive stages of ERU.

Sample Collection

All animals were managed in accordance with the ARVO Statement for Use of Animals in Ophthalmic and Vision Research. Depending on sample size and numbers of collected cells, specimens were used for one or several of the analytical procedures, to determine the type of inflammation and the humoral and cellular immune responses to retinal antigens. Heparinized blood for in vitro proliferation assays and

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serum were collected before ocular surgery. Vitreous samples were obtained during pars plana vitrectomy and assayed immediately. Vitreous was diluted with 200 ml sterile balanced salt solution (BSS) during surgery. BSS is routinely used to replace removed vitreous. Control vitreous was immediately collected and processed after death from eyes of euthanized horses without ocular disease. Control blood samples were obtained from horses without ocular disease.

**Preparation of Cells from Vitreous and Flow Cytometry**

Vitreous humor was filtered through a stainless steel sieve before analysis. Cells were pelleted by centrifugation at 4°C for 20 minutes at 750g. The supernatant was stored at −20°C until use, and the cell pellet was resuspended in RPMI and subsequently analyzed. Differential counts were obtained from Wright-stained cytospin preparations, and the remaining cells were phenotyped by flow cytometry. In 100 μl fluorescence buffer (phosphate-buffered saline [PBS] with 1% bovine serum albumin [BSA], 0.01% sodium azide; Sigma, Deisenhofen, Germany) 1 × 10⁶ cells were incubated for 50 minutes at 4°C with 10 μl of monoclonal mouse anti-equine antibodies directed against the following markers: CD4 (clone CVS4), CD8 (CVS 21), and clone CVSI to detect B cells (all from Biozol/Serotec, Eching, Germany). Cells were subsequently washed three times with fluorescence buffer and stained with fluorescein-isothiocyanate (FITC)-conjugated goat anti-mouse IgG (Sigma). Cells (5 × 10⁶) were each analyzed in a flow cytometer (FACScan; Becton Dickinson, Heidelberg, Germany) using Win MDI 2.5 software (The Scripps Research Institute, La Jolla, CA).

**Quantitative Measurement of Total Vitreal IgG**

IgG concentration was quantified by single radial immunodiffusion, as described,¹² using a polyclonal antiserum specific for the equine γ-heavy chain (Dianova, Hamburg, Germany). Purified equine IgG (Sigma) was used as a standard.

**Detection of Autoreactive Antibodies**

Vitreal and serum samples were tested for the presence of autoantibodies against the retinal antigens S-Ag (S-Ag) and interphotoreceptor retinoid-binding protein (IRBP) by enzyme-linked immunosorbent assay (ELISA). These antigens were purified from bovine eyes, as described previously.¹³ Microtiter plates (Maxisorb; Nunc, Wiesbaden, Germany) were coated with 10 μg of the respective antigen/ml PBS overnight at 4°C. Blocking was performed by 1 hour incubation with 1% BSA in PBS. Samples were diluted (1:1000) in PBS. The cells were added to the wells in duplicates, and incubated for 1 hour at 37°C. As a secondary antibody, 50 μl of a rabbit anti-horse IgG serum with high antibody titer was added. IgG concentration was determined by 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).

**Statistical Methods**

The statistical significance of differences between groups was evaluated using Student’s t-test in the study of vitreous infiltrating cells. The results of the autoantibody assays were compared using the χ² and Fisher’s exact tests. P < 0.005 was considered significant.

**RESULTS**

**Phenotypic Characterization of Vitreous-Infiltrating Cells**

The numbers of infiltrating cells from vitreous samples of 64 horses with ERU were determined. Because of large variations in cell counts, it was not possible to perform all assays (differential analysis of cell type, characterization of cell surface antigens, and antigen specific proliferation) with every intraocular sample. The average cell number obtained was 6.4 × 10⁶ cells ± 2.8 × 10⁵ cells per vitreous sample.

Vitreous samples from unaffected control animals were devoid of infiltrating cells. Cytologic classification of cells in cytospin preparations from eyes of 32 horses with ERU revealed three different types of inflammation, divided into three groups (Fig. 1). Twenty-two patients (group 1) had a predominant infiltrate of lymphocytes in their vitreous (93% ± 5% lymphocytes, significantly higher than in the other groups), a second group (6/32) had 57% ± 15% vitreal lymphocytes and 34% ± 21% vitreal granulocytes (group 2), which is significantly fewer lymphocytes than in group 1. In contrast, horses of group 3 (4/32) had 89% ± 9% intraocular granulocytes and only a few lymphocytes (10% ± 9%) and had hyalitis rather than iridocyclitis. The mean cell number of lymphocytes and granulocytes showed significant differences between every group, using Student’s t-test (P < 0.005).

Vitreous lymphocytes from group 1 horses were further analyzed by flow cytometry. Cell surface markers were determined from vitreal lymphocytes of 17 horses. We found predominantly CD8⁺ T cells (average of 57% ± 17%) and fewer CD8⁻ T cells (29% ± 14%) and B cells (28% ± 16%; by FACScan, data not shown).

**Vitreal Antibodies Specific for Retinal S-Ag or IRBP**

Analysis of serum and vitreal antibodies was performed with specimens of all three groups. A high variation was detected within the amount of total IgG in vitreous samples from 30 tested horses with uveitis. IgG concentrations in vitreous were,
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on average, 11.5 ± 8 mg (data not shown). However, no IgG was detectable in any vitreous specimen of 15 healthy control eyes. Using an ELISA to detect antigen-specific IgG-antibodies in vitreal specimens of ERU-affected eyes revealed a mean optical density (OD) of 0.086 ± 0.094 (SD) for IRBP, 0.080 ± 0.095 for S-Ag, and 0.108 ± 0.139 for Tt-specific antibodies (healthy control animals: IRBP: 0.006 ± 0.005; S-Ag: 0.006 ± 0.005; Tt 0.018 ± 0.012). Patients versus healthy control animals (IRBP: 0.008 ± 0.005; S-Ag: 0.006 ± 0.005; Tt 0.018 ± 0.012) showed intraocular autoantibodies against both retinal antigens (P < 0.001, Fig. 2, top). To separate positive and negative samples, a cutoff was set at a fivefold increased OD compared with healthy control animals, thus minimizing false-positive results. Under these conditions, retina-specific antibodies were detected in the vitreous of 73% of horses with uveitis (24/33). Four specimens had only antibodies to IRBP, whereas six horses had only S-Ag-specific antibodies (healthy control animals: IRBP: 0.060 ± 0.054; S-Ag: 0.054 ± 0.054; Tt: 0.312 ± 0.081). Anti-IRBP and anti-S-Ag-antibodies were significantly higher in ERU-affected versus healthy control animals (P < 0.001, anti-Tt-antibodies in ERU-affected versus healthy control animals, no statistically significant difference).

Of control horse sera, 22% (8/35) were positive for one or both retinal antigens compared with 81% (27/33) of horses with ERU. All horses except for one, which was not vaccinated, were positive for anti-Tt antibodies.

Whereas only 3 of 10 horses with ERU of the inflammatory group 2 (mixed infiltration, Fig. 1) and group 3 (granulocytic infiltration, Fig. 1) showed intraocular autoantibodies, 20 of 22 group 1 horses' lymphocytic infiltrations where positive for retinal autoantibodies in the eye.

**Autoreactive T Cells Preferentially Respond to IRBP Peptides**

Because autoimmune uveitis is a T-cell-mediated disease, we investigated in vitro responses of T cells to retinal antigens. In preliminary experiments we found that PBL of horses with ERU proliferated only weakly in response to retinal antigens (2/10). Lymphocytes of healthy control horses did not respond to stimulation (data not shown).

To further investigate the antigen specificity of lymphocytes from horses with ERU, we took advantage of the availability to gain sufficient numbers of T cells from the target organ eye. Vitreal lymphocyte preparations of group 1 horses (more than 90% lymphocytes as assessed by cytospin preparations) were used. In 14 of 22 horses from this group we collected sufficient numbers of cells to compare the responses of lymphocytes from PBL and vitreous. Whereas in PBL autoantigen-specific cells were more common, in vitreous lymphocytes from horses with ERU, we observed a greater frequency of autoreactive T cells to retinal antigens.

**Figure 1.** Differentiation of vitreal lymphocytes. The mean percentage of lymphocytes (lymph.), granulocytes (gran.) and monocytes (mono.) of vitrectomy samples is shown, which were grouped according to the following criteria: of 32 horses, 22 had a mainly lymphocytic infiltrate (group 1, lymphocytes 95% ± 5%), 5 showed a mixed infiltration (group 2, lymphocytes 57% ± 15%; granulocytes 34% ± 21%), and 5 had a predominant infiltration of granulocytes (group 3, 89% ± 9%). Statistical difference was determined using Student's t-test.

**Figure 2.** Determination of anti-S-Ag and anti-IRBP autoantibodies in vitreous (top) and serum (bottom) of horses with ERU and healthy horses by ELISA. The value (OD$_{450}$) indicates the mean OD corrected for background binding of rabbit anti-horse IgG. Filled symbols: results in horses with ERU; open symbols: healthy control horses. Black horizontal lines: cutoff. Autoantibody titers were significantly higher in horses with ERU than in control horses (eye and serum). Statistical difference was determined using $\chi^2 = P < 0.001$. There was also a significant difference between intraocular anti-Tt antibodies in ERU versus control horses ($P = 0.005$).

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specific responses were either weak in incidence and intensity (3/14 horses) or completely absent, vitreal lymphocytes strongly reacted to IRBP-derived peptides and to the HLA-peptide B27PD (10/14 horses; Fig. 3). In two horses no proliferative responses to any autoantigen were observed with peripheral or with vitreal lymphocytes. Both humoral (Fig. 4) and cellular (Fig. 3) responses of the same horses were examined but due to technical difficulties, the same antigens could not be used for both procedures, thereby hindering any direct comparison.

**DISCUSSION**

ERU is a disease with high prevalence. The disorder shows clinical and pathologic similarities to recurrent uveitis in humans, indicating its potency as a valuable model for this ocular inflammatory disease in humans. In contrast to experimentally induced animal models, ERU develops spontaneously similar to human uveitis. Although some immunohistochemical studies have been performed with eyes and pineal glands of horses with ERU, demonstrating findings that resemble human intraocular inflammatory disease, the function of immune cells using vitreal specimens from ERU-affected eyes has not been analyzed. Our research focused on the immunologic mechanisms involved in ERU. This study provides strong evidence that ERU is an autoimmune-mediated disease comparable to human uveitis, in that we found autoantibodies and vitreal lymphocytes reacting with retinal autoantigens.

Moreover, besides the spontaneously developing disease, the horse offers the possibility to experimentally induce uveitis. This would enable us to compare histopathology and immunopathology of experimentally induced autoimmune disease with naturally occurring disease in the same species.

First attempts to show an autoimmune reaction associated with ERU were made by Maxwell et al.,18 who investigated the humoral immune response to S-antigen and rhodopsin in affected horses and healthy control animals and found no significant difference between both groups. Furthermore, S-Ag, which is the best characterized autoantigen in humans and rats, was not a target for the response of PBL in horses with ERU as shown by Hines and Halliwell.19 To extend these early studies we compared the humoral immune response in the periphery to that of the vitreous from horses with ERU. In addition to S-Ag, we also tested bovine IRBP as autoantigen. We used bovine retinal antigens, because highly immunogenic and uveitogenic epitopes are usually highly conserved among species.20 This, however, remains speculative with respect to the still unknown sequences of equine retinal antigens. The latter fact also prevented us from using peptides with the horsespecific sequences.

There was a marked difference between ERU-affected and healthy horses regarding autoantibodies in the periphery. Whereas 81% of tested ERU sera were positive for one or both retinal antigens, only 22% of sera from healthy horses reacted positively. These findings are in contrast to studies in human uveitis, in which no differences in antibodies were seen between control animals and horses with uveitis.21 Human patients are usually vigorously treated with immunosuppressive agents, probably resulting in impaired B-cell reactivity. ERU-affected horses, however, are usually not treated with immu-

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**FIGURE 3.** Proliferative responses of PBL and vitreal lymphocytes (VL) from the same horses to peptides PDSAg (S-Ag-derived), S-Ag; PDIRBP, R14, P1536 (IRBP-derived), and B27PD (human HLA-class I peptide). Results are given as stimulation index (SI). The cutoff was set at an SI of 2. PBL from 3 of 14 horses responded weakly, whereas VLs from 10 of 14 horses proliferated in response to the antigens.

**FIGURE 4.** Anti-S-Ag and anti-IRBP autoantibodies in vitreous and serum of horses with ERU shown in Figure 3. The value (OD450) indicates the mean OD corrected for background binding of rabbit anti-horse IgG. The cellular response in vitro was not correlated with antibody reaction in antigen specificity and antibody titers.
nose suppression, which may explain the enhanced antibody production to retinal autoantigens.22

This new observation was further confirmed by the demonstration of retinal antigen-specific antibodies in vitreous samples of horses with ERU and the complete absence of autoantibodies in vitreous samples obtained from healthy control animals. In contrast to previous reports,18,19 our findings indicate a specific generation of autoantibodies in affected horses, either in the peripheral immune system or in the eye. A breakdown of the blood-retinal barrier or infiltration of unspecified B cells could be monitored by the presence of T-specific antibodies in the vitreous. Because T is a common vaccine for horses in Germany, most animals have specific serum antibodies.

Penetration of anti-tetanus antibodies from blood into the vitreous is probably due to leakiness of choroidal and iris vessels. As in humans, fluorescein angiograms in nonhuman primates with experimental autoimmune uveitis (EAU) have shown that severe uveitis leads to extravasation of dye21 from retinal vessels. B cells were recently demonstrated in the eyes of horses with ERU by immunohistology, indicating a local antibody production. However, autoantigen specificity of these B cells remains to be determined.10

Because anti-retinal antibodies seem to be nonessential for the induction of uveitis, as shown in several EAU models,23 and the predominating infiltrates in eyes of horses with ERU are T-helper cells, we were highly interested in the cell-mediated mechanisms involved in ERU. The therapeutic routine procedure of vitrectomy allowed us to obtain sufficient numbers of vitreous-infiltrating cells from diseased horses for immunologic studies and to compare the reactions of peripheral and intraocular lymphocytes.

A hallmark of organ-specific autoimmune diseases is the response of lymphocytes to certain autoantigens. In human autoimmune uveitis and EAU animal models, S-Ag, IRBP, and peptides of these autoantigens have been shown to activate CD4+ T cells in vivo and in vitro.25–27 Furthermore, uveitis was induced successfully by adoptive transfer of antigen-specific CD4+ T cells and T cell lines.30 Consequently, we set out to study the cellular response to a selected panel of retinal antigens and their peptides. Our initial attempts to activate PBL with these antigens failed in most horses with ERU. Only two of 14 patients responded to S-Ag- or IRBP-derived peptides; however, no response to the whole antigens could be observed. This is in accordance with the findings of Hines and Halliwell,19 who also could not observe an activation of PBL from horses with ERU to whole S-Ag.

In several autoimmune diseases, responses to autoantigens are rarely seen with PBL. Only in some patients27 or at certain time points29 do PBL respond to autoantigens. The low frequency of antigen-specific PBL, even in advanced cases of uveitis, has been discussed as one reason for poor results in proliferation assays. Because equine patients usually have severe advanced uveitis, proliferative responses cannot be studied during the early phase of disease or even before a relapse, when higher numbers of antigen-specific T cells should be present in the circulation. To circumvent this problem, we investigated the proliferative capacity of target organ infiltrating lymphocytes in vitro. In contrast to PBL, cells from the eye strongly responded to several of the tested peptides. In most cases, a clear response to one or several IRBP-derived peptides was observed. The S-Ag-derived peptide PDSAg activated vitreal lymphocytes in 5 of 14 cases. PDSAg was chosen, because it is a major pathogenic epitope of S-Ag in the rat model and has also been shown to be highly immunogenic in humans.15

In general, the response to whole S-Ag was weak, suggesting S-Ag may not be a major autoantigen for T-lymphocytes in ERU or that processing of the protein in cell culture was not adequate. IRBP was not tested as a whole antigen because of the content of concanavalin A within the preparations as a result of the purification procedure. However, the results obtained with IRBP peptides clearly point to IRBP as the dominant autoantigen in ERU. In 9 of 14 horses, the vitreal lymphocytes reacted to at least one IRBP-derived peptide. R14 (aa 1169-1191) is the immunodominant determinant in the Lewis rat and can induce EAU in these animals.29 In accordance with observations in human uveitis,30 there was no positive or negative correlation between detectable autoantibodies and the frequency of cellular response. There was also no clear pattern of reactivity to certain antigens in individual horses. In conclusion, we observed a predominant location of retinal antigen-specific T cells within the eye (10/14 vitreous cell samples proliferating to one or several antigens and peptides), whereas only 4 of 14 horses showed intraocular but no peripheral autoantibodies as indicators for B-cell reactivity. Although not supportive of a Th1 immune response, these findings demonstrate strong T-cell-mediated autoreactivity and are consistent with the previous demonstration of a Th1 cytokine pattern in eyes of affected horses.51,52

In humans, a possible mechanism for the induction of uveitis has been described by antigenic mimicry between retinal autoantigen peptide and a peptide derived from disease-associated HLA-antigens (B27PD).14 This work inspired us to test the reactivity of horse lymphocytes to these peptides, although the equine MHC antigens known so far do not contain a sequence corresponding to peptide B27PD. It was interesting that 6 of 14 horse vitreal lymphocyte preparations tested with this peptide strongly proliferated in response to B27PD. HLA-peptide B27PD was shown to mimic S-Ag peptide PDSAg in the rat model of EAU and also to play a role in human uveitis. It is also used as an oral tolerogen in rat EAU15 and in human patients.53 To explain the tolerogenic properties of B27PD in IRBP-induced EAU in the Lewis rat,55 peptide P1556 and PDIRBP were selected as possible cross-reactive epitopes according to certain amino acid sequence homologies.16

In conclusion, our results point toward an autoimmune pathogenesis in ERU. Wittebost postulated regarding the autoimmune origin of human diseases that the autoimmune response must be mediated by an autoantibody or an autoreactive T cell, the corresponding antigen must be identified, and the analogous autoimmune response must be induced in an experimental animal, which should develop a similar disease.53 These criteria are now fulfilled with the demonstration of the presence of autoantibodies and autoreactive lymphocytes specific for S-Ag and IRBP in horses with ERU. Moreover, we found significant proliferation of retinal autoantigen-specific T lymphocytes within the target organ eye compared with peripheral blood, indicating that autoimmunity plays an important role in this disease. However, the initiating events of the immunopathology remain obscure. In many autoimmune diseases, infections have been discussed as triggering events, either by antigenic mimicry with a pathogen’s antigen or as a bystander effect due to the general systemic or local immune stimulation by the pathogen.15 The induction of an autoimmune disease through molecular mimicry between a linear epitope of an infectious agent and host protein was demonstrated only sporadically.54

The role of infection with L. interrogans in ERU is currently under investigation. Recently, immunohistopathologic examinations of equine ocular tissues with leptospiral antisera showed cross-reactivity with iris pigment epithelium and retina from horses with ERU. The investigators underscored the relevance of these studies as a possible model of postinfectious immunopathogenesis.35 It is unclear at present, whether infection with L. interrogans leads to an autoimmune reaction.
through molecular mimicry between the pathogen and retinal tissue. The immune reactions to ocular autoantigens demonstrated in the present study may be the clue for the maintenance of ocular inflammatory disease. Because lymphocytes in horses with ERU proliferate strongly in response to peptide B27PD, which is derived from HLA-B antigens that are associated with uveitis in humans, a similar mechanism of molecular mimicry between a self-MHC peptide and a peptide from a retinal autoantigen could also be speculated for horses; however, no horse MHC antigen with the respective sequence has been identified so far.

Therefore, further investigations of the intraocular inflammatory process of horses with ERU could aid in understanding the initiating and maintaining processes in recurrent uveitis both in horses and humans.

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