

Recurrent Uveitis in Horses: Vitreal Examinations with Ultrastructural Detection of Leptospires

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Summary

This study documents the examination of 17 horses (both sexes, 3–18 years old) suffering from spontaneous equine recurrent uveitis (ERU). Vitreal samples obtained by pars plana vitrectomy were examined macroscopically and ultrastructurally, and in most cases also by cultural examination, by microscopic agglutination test (MAT) and by polymerase chain reaction. In 24% (4/17) of the animals, ultrastructural examination by electron microscopy revealed intact leptospiral bacteria in the vitreous. The leptospires were detected freely in the vitreous and also incorporated by a phagocyte. They were surrounded by a rim of proteinaceous material which was reduced around a phagocytosed leptospira. Ninety-four per cent (16/17) of the vitreal samples presented significant antibody levels in the MAT, mostly against leptospiral serovar Grippotyphosa. Seventy-five per cent (9/12) of bacterial culture examinations were positive for leptospira. Polymerase chain reaction was positive in all (16/16) examinations performed. Our findings support previous reports suggesting that leptospires play an important role in the pathogenesis of ERU. Interestingly, this study found leptospires after secondary and later acute episodes. A persistent leptospiral infection is therefore suggested as the cause of ERU.

Introduction

Equine recurrent uveitis (ERU) is one of the most frequent ocular diseases in horses. It is characterized by repeated acute episodes of ocular pain and intraocular inflammatory precipitates in one or both eyes which alternate with quiescent intervals. Intraocular damage including liquefaction of the normally homogenous gelatinous vitreous structure and loss of transparency increases with each acute episode, leading to impaired vision and potentially blindness.

Scientists have been debating the cause of ERU for over 100 years. Following initial reports hypothesizing that the disease may be caused by certain environmental, weather-related or potentially genetic conditions (Haase, 1879), infective agents were proposed as the cause of equine non-purulent panophthalmitis (Bayer, 1900). In the 1940s, high antibody titres against leptospires were found in aqueous fluid (Gesell *et al.*, 1947), after which leptospira was first taken in culture from diseased animals in the 1950s (Hartwig and Stoebbe,

1952). More recently, the involvement of immune-related or autoimmune reactions has also been discussed (Parma *et al.*, 1992; Romeike *et al.*, 1998; Gilger *et al.*, 1999; Wildner and Diedrichs-Mohring, 2003).

Vitreous examination of ERU-affected eyes has revealed high antibody titres against various leptospiral serovars, most frequently serogroup Grippotyphosa followed by serogroup Australis (Wollanke *et al.*, 1998). The existence of viable leptospiral bacteria in serologically positive vitreal samples has also been demonstrated bacteriologically, thus confirming leptospiral infection as a possible cause of ERU (Brem *et al.*, 1998, 1999; Wollanke *et al.*, 2001; Hartskeerl *et al.*, 2004).

Ocular leptospirosis has also been reported in humans. It typically develops from systemic leptospirosis, but commonly exhibits a prolonged symptom-free period making causal diagnosis difficult (Rathinam and Namperumalsamy, 1999). As in humans, a systemic leptospiral infection exhibiting mostly subclinical or mild clinical symptoms also precedes ocular involvement in horses, both with experimentally induced and natural infections (Roberts, 1958; Williams *et al.*, 1971). But the pathogenesis of the ocular leptospiral infection remains unclear, with outstanding questions such as (i) when, after bacteremia, do the leptospires enter the eye? (ii) where are they localized until occurrence of ERU? (iii) where are they located during the quiescent intervals? Therapeutically, vitreous replacement has proved to be a good method of conserving vision, or at least the globe, in advanced cases. Pars plana vitrectomy to remove the diseased vitreous is performed, followed by a lavage and vitreous replacement with a balanced saline solution containing gentamicin. Eyes with anterior uveitis are treated just as successfully as eyes with predominantly posterior uveitis. Relapses are rare following such treatment, and have been reported in only 2% of cases (Gerhards and Wollanke, 2005).

The aim of this study was to ultrastructurally analyse equine vitreous for leptospiral bacteria and inflammatory cells in horses with spontaneously occurring ERU. The affected horses experienced an acute episode recently before vitreal examination with one exception of a still painful eye by the time of vitrectomy. Furthermore, we correlated macroscopic and ultrastructural findings with the results from bacteriological culture examinations, antibody titres, PCR and clinical history of the horses.

Materials and Methods

Vitreous samples were taken from horses kept in southern Germany and Austria and brought to the Equine Clinic, University of Munich. In the period from May to December 2005, 17 horses presenting with diffuse yellow vitreous clouding were selected for examination. The group consisted of nine females, seven geldings and one male animal, aged 3–18 years.

All horses had experienced an acute phase, characterized by clinical symptoms like blepharospasm, vitreal discoloration, intraocular inflammatory precipitates, between 10 days and several months before vitrectomy. Vitreous samples were taken after disappearance of acute clinical symptoms which was obtained by a 3-day systematic treatment with phenylbutazone and a local ocular application of β -dexamethasone. The eye of horse no. 1 did not respond to clinical treatment and was still painful. At least the last acute episode was diagnosed before vitrectomy in the Equine Clinic, the preceding episodes were reported by private veterinarians or by the owners.

Vitrectomies were performed as described by Gerhards et al. (1999). Approximately 3–4 ml of undiluted vitreous was obtained during the first phase of vitrectomy, which comprised a large liquid fraction and a slippery, slightly more compact fraction of variable size. Alterations in composition, structure and colour were also evaluated. This material was divided up for subsequent examination as follows.

For culture examination, 0.4 and 0.7 ml undiluted vitreous respectively, were placed in bovine-albumin-tween transport medium with 0.15% agar (BBL-Agar, Becton, Dickinson and Co., Cockeysville, MD, USA) and fluorouracil (100 mg/ml). On the day of arrival in the laboratory the samples were subcultured. Aliquots (0.5 ml) of the transport medium samples were placed in bovine-albumin-tween medium containing 10 mg of vancomycin per litre or 100 mg of fluorouracil per litre and 10 mg of vancomycin per litre respectively. Cultures were maintained at 29°C and examined weekly for leptospire, using dark-field microscopy at 250 \times magnification (Brem et al., 1998).

For vitreal antibody titre evaluation, a sample of 0.5 ml was tested for specific antibodies against *Leptospira interrogans* serovars via the microscopic agglutination test (MAT) [Office International Des Epizooties (OIE), 2004]. Routinely used leptospiral serovars included serovar Bratislava, Canicola, Copenhageni, Grippotyphosa, Hardjo, Javanica, Pomona and Pyrogenes.

A further 0.5-ml sample was stored at room temperature and analysed the day after vitrectomy by PCR in a commercial laboratory (VetMedLabor, Ludwigsburg, Germany). This method is based on detection of a 16S-rDNA gene sequence of *L. interrogans* (Merien et al., 1992, 1995). A conserved leptospiral gene sequence cloned into a bacterial plasmid and multiplied in bacteria served as positive control. Several negative controls regarding the check-up of reagents, DNA isolation and contamination were performed with every approach. DNA extraction was performed by using a commercial purification kit (Qiagen GmbH, Hilden, Germany). All samples, including vitreous samples and controls of one exclusive experimental approach, were analysed at the same time point. Differentiation of leptospiral serovars was not possible using this method.

Transmission electron microscopical examination was performed on the remaining vitreal material (0.9–1.9 ml). For this

purpose, the liquid and the more compact vitreous fractions were fixed separately in 6.5% glutaraldehyde (Serva GmbH, Heidelberg, Germany) in Sørensen's phosphate buffer 0.15 M for 24 h at room temperature. The more compact vitreous fraction was post-fixed in osmium tetroxide (Plano W. Plannet GmbH, Wetzlar, Germany) after washing in 0.1 M phosphate buffer, dehydrated in a graded series of ethanol and embedded in epoxy resin (Epon[®]; Serva GmbH, Heidelberg, Germany) in line with standard laboratory procedures. All embedding steps were performed manually to prevent material loss. Transmission electron microscopy was performed on all up to 10 epon blocks of each vitreous, using 70-nm sections, even if only vitreal fibrils, cellular debris and no intact inflammatory cells were seen in semithin sections.

The fixed liquid fraction was centrifuged at 1500 rpm for 10 min (Biofuge A, 1987, Heraeus Sepatech GmbH, Osterode, Germany), the supernatant decanted and the residual fluid pipetted onto a colloidal-coated copper grid (Stork Veco, B.V., Eerbeek, the Netherlands). After 2 min, the surplus liquid was removed and the grids were contrasted with 2% phosphorwolfram-acid (Sigma-Aldrich Chemie GmbH, Taufkirchen, Germany) and examined under a transmission electron microscope Zeiss EM10 (Carl Zeiss AG, Oberkochen, Germany) using calibrated magnifications.

The cultured control leptospire belonging to serovar Grippotyphosa were fixed in 6.5% glutaraldehyde (Serva GmbH) for 24 h at room temperature and centrifuged at 1500 rpm for 10 min (Biofuge A, 1987, Haereus Sepatech GmbH). The supernatant was decanted and the residual bacterial pellet prepared like the compact vitreous fraction described above.

Results

Vitreous samples from 17 horses suffering from ERU were examined. All displayed liquefaction of the normally gelatinous vitreous structure, leaving only a small remaining fraction of the more solid material. A striking yellow colour was noted in a large number of the vitreous samples.

In the MAT, high vitreal antibody titres against leptospire were detected, ranging from 1:200 to 1:51 200 (16/17 samples). The antibodies were mostly directed against serovar Grippotyphosa (15/17), followed by serovar Pomona (2/17), serovar Bratislava (1/17), serovar Copenhageni (1/17) and serovar Javanica (1/17) (Table 1). Four samples included antibodies against two different serovars. Horses with a positive culture result had variable vitreal antibody titres ranging from 1:200 (horse no. 12) to 1:3200 (horse no. 9).

Leptospire were detected by culture in 9 of 12 examinations (Table 1). As an additional confirmation of the serovar would have been redundant, the serovars of the cultured leptospire were assumed to be the same as that determined in the MAT as previously shown by Wollanke (2002).

Polymerase chain reaction was positive for leptospire in all samples (16/16), including the two cases negative in culture and the five cases where culture examinations were not performed (Table 1).

Spirochetal bacteria were detected by transmission electron microscopy in 24% (4/17) of the vitreous samples (horse nos 1, 2, 10 and 17). All four horses had suffered at least two reported acute episodes. The last acute episode was seen between 10 days

Table 1. Summary of all results and clinical data

Horse no.	EM	Clinical data –			Age (years)	Vitreal antibodies serovar/titre (microscopic agglutination test)	Culture	PCR
		A: no. of acute episodes;	B: period between last acute episode and vitrectomy	Sex				
1	Positive	A: 4; B: 14 days; eye was already painful	Female	7	Grippotyphosa 1:3200	Contamination	Positive	
2	Positive	A: 6; B: 10 days	Female	7	Grippotyphosa 1:400	No culture	Positive	
3	Negative	A: 2; B: 7 days	Female	3	Grippotyphosa 1:100	Positive	Positive	
4	Negative	A: several; B: 21 days	Gelding	8	Grippotyphosa 1:51 200	Negative	Positive	
5	Negative	A: 5; B: 17 days	Gelding	9	Grippotyphosa 1:800 Pomona 1:1600	Positive	Positive	
6	Negative	A: 4; B: 15 months	Gelding	7	Grippotyphosa 1:1600	Positive	Positive	
7	Negative	A: 2; B: 24 days	Female	8	Grippotyphosa 1:400 Pomona 1:800	Negative	Positive	
8	Negative	A: 2; B: 24 days	Gelding	5	Microagglutination test negative	No culture	Positive	
9	Negative	A: 2; B: 1 month	Gelding	9	Grippotyphosa 1:400 Javanica 1:3200	Positive	Positive	
10	Positive	A: 2; B: 4 months	Female	5	Grippotyphosa 1:3200	No culture	Positive	
11	Negative	A: 4; B: 21 days	Gelding	17	Copenhageni 1:400, Bratislava 1:400	Positive	Positive	
12	Negative	A: not reported; impaired vision for the past year	Female	7	Grippotyphosa 1:200	Positive	Positive	
13	Negative	A: 2; B: 2 months	Gelding	18	Grippotyphosa 1:1600	Positive	Positive	
14	Negative	A: 3; B: 14 days	Female	10	Grippotyphosa 1:1600	Positive	Positive	
15	Negative	A: 5; B: 7 days	Female	5	Grippotyphosa 1:200	Positive	Positive	
16	Negative	A: 3; B: 10 days	Male	3	Grippotyphosa 1:400	No culture	Positive	
17	Positive	A: 3; B: 21 days	Female	9	Grippotyphosa 1:400	No culture	No PCR	

EM, Electron microscopy; PCR, Polymerase chain reaction.

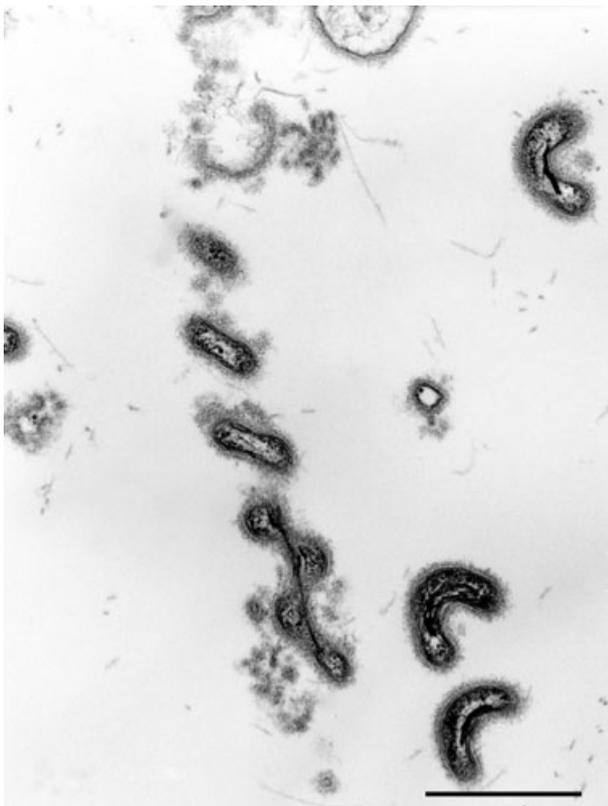


Fig. 1. Vitreous; horse no. 1. Electron micrograph of vitreal leptospire with typical morphologic features and presenting an osmiophilic envelope on the outer sheet; bar 0.5 μm .

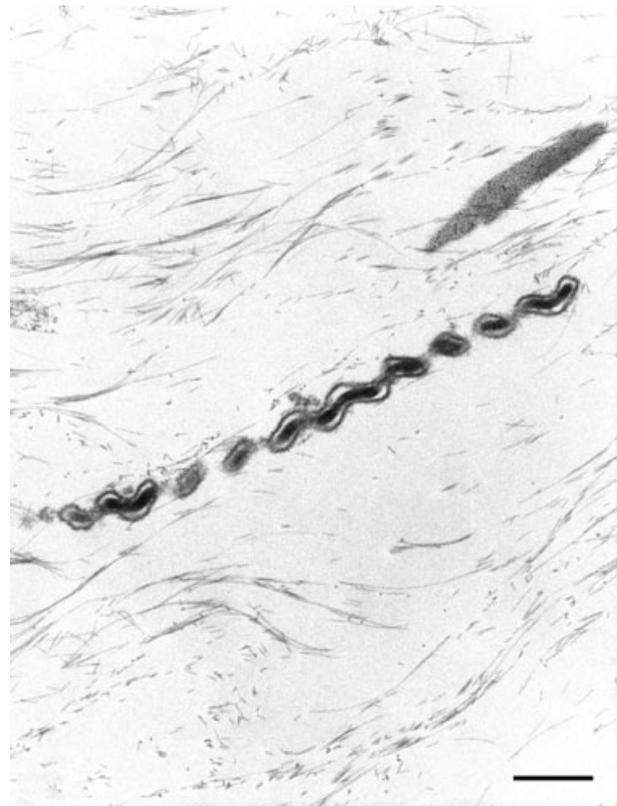


Fig. 2. Vitreous; horse no. 2. Electron micrograph of a vitreal leptospire with surrounding collagen fibrils; bar 0.5 μm .

and 4 months before vitrectomy. The bacteria were found as well in epon sections (horse nos 1 and 2) (Figs 1 and 2) as in negative-stained preparations (horse nos. 10 and 17) (Fig. 3). In

three out of four samples, only one free spirochete was found (horse nos 2, 10 and 17), in contrast to the fourth positive sample (horse no. 1), which contained numerous free spirochetes.

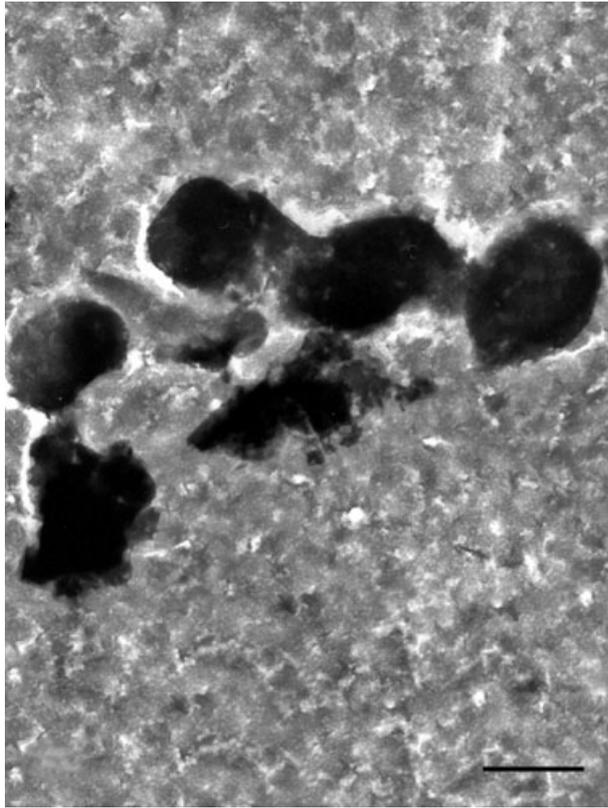


Fig. 3. Vitreous; horse no. 10. Electron micrograph of a vitreal leptospire in negative stain, recognizable by their spiral configuration; bar 0.5 μm .

Morphologically, such spirochetes varied from 2.0 to 4.0 μm in length, with a diameter of 0.12–0.15 μm (Fig. 1–3). The bacteria consisted of a single, straight, central axial filament with a protoplasmic cylinder wrapped around it. The cytoplasm was enclosed by a cell wall, surrounded by a periplasmic space and an outer sheet (Figs 1 and 2). In all cases, the spirochetes were covered by an osmiophilic, fine granular material adherent to the outer cell wall surface (Figs 1 and 2) in contrast to cultured forms, which did not exhibit this granular coat (Fig. 4). At least one spirochete was found by a phagocyte, likely a neutrophil, in the sample of horse no. 1 (Fig. 5). The intraphagocytic spirochete was located within a phagolysosome and exhibited markedly reduced surrounding osmiophilic material when compared with the free spirochetes (Fig. 5). Additional intracytoplasmic phagolysosomes in the same cell contained up to three osmiophilic, fine granular spheroids, up to 2 μm in diameter, without recognizable internal structures. Several identical spheroids were present on the surface of this cell being about to be phagocytosed (Fig. 5). A precise determination of the phagocyte was not possible because the nucleus was out of the section plane. But the small osmiophilic, homogeneous, 0.26 \times 0.13 μm -wide, membrane-bound, osmiophilic granules within the cytoplasm could suggest a neutrophil (Fig. 5).

All samples contained a variable number of vitreal collagen fibrils and a small number of macrophages, lymphocytes and plasma cells, mostly degenerated, and free cell organelles. The sample containing numerous free spirochetes (horse no. 1) was the only one to also include a large number of intact inflammatory cells, consisting of macrophages in various

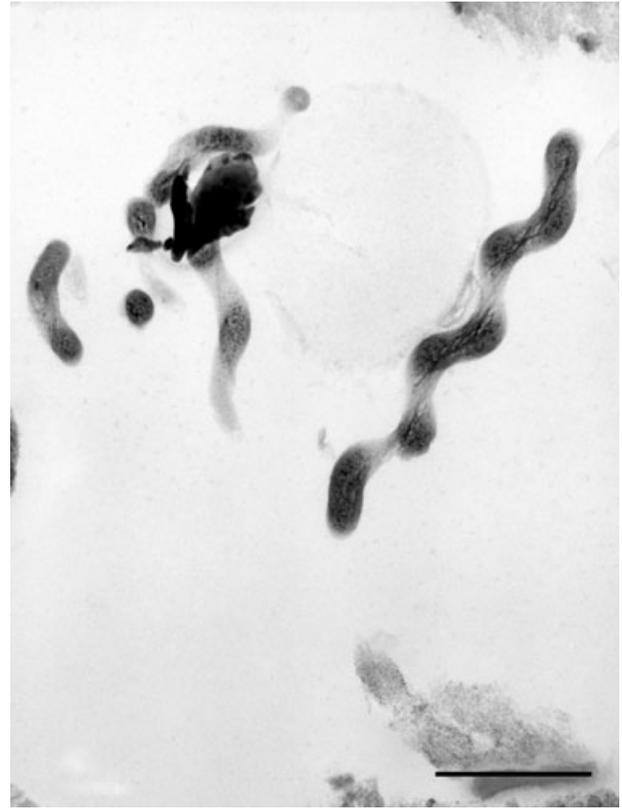


Fig. 4. Culture of leptospires. Electron micrograph of a cultured leptospire. Notice the clear outer surface; bar 0.5 μm .

activation states, and a few neutrophils, plasma cells and lymphocytes and a large amount of cell debris.

Discussion

This study presents the results of vitreal examinations from horses with ERU.

Macroscopic vitreal examination revealed a striking yellow colour in a large number of the samples. This colour change generally results from an inflammation-induced breakdown of the blood–vitreous barrier, allowing serum to enter into the vitreous, causing the vitreous to shrink and liquefy (Spencer, 1985). The size of this more compact fraction depended on the duration and degree of intraocular inflammation, the inflammatory infiltrates and the extent of shrinkage of the collagen frame.

Polymerase chain reaction revealed a positive result for leptospires in all samples examined (16/16), even in horses with a negative culture examination. In these cases, the question arises of whether PCR detected only leptospiral fragments or whether there were viable leptospira in the vitreous but not in the material inoculated to the transport medium for culture examination.

Almost all horses in this study demonstrated vitreal antibody titres against leptospires. In our material, as in previous studies, serovar Grippotyphosa was predominant in horses from central Europe (Brem et al., 1999; Hartskeerl et al., 2004), in contrast to North America, where serovar Pomona is found most frequently (Halliwell et al., 1985). All antibody titres were considered to be significant for leptospiral infection (Wollanke, 2002). Antibody titres were even detected in clinically healthy eyes, but are considered to be also caused by a leptospiral

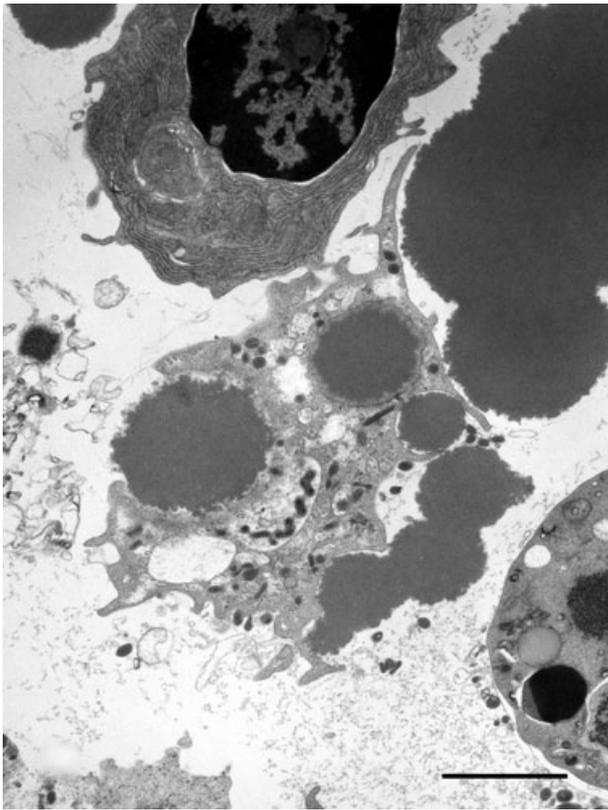


Fig. 5. Vitreous; horse no. 1. Electron micrograph of a phagocyte with incorporated leptospire in phagolysosoma and phagocytized osmiophilic spheres; bar 2 μ m.

infection (Wollanke et al., 2001). Significant antibody titres in the four horses with ultrastructurally detected spirochetes suggested that they were leptospire. Seventy-five per cent (9/12) of the cases in this study demonstrated viable leptospire in culture examinations along with high vitreal antibody titres, as also previously described by Brem et al. (1998). Thus, we concluded that the antibodies alone are unable to eliminate all of the leptospira present in the vitreous.

Four of 17 vitreous samples (horse nos 1, 2, 10 and 17) in our study were leptospira positive in transmission electron microscopy. Given the small amount of vitreal material available for examination (following division of the sample for multiple analyses) and the expectedly low number of leptospire, this result is surprisingly high. As far as we know, this is the first time entire and complete leptospire have been detected ultrastructurally in vitreous. Fragments of leptospire-like structures had been demonstrated ultrastructurally in vitreous by Niedermaier (2002). Culture detection of leptospire had already been successfully performed in various groups (Hartwigk and Stoebbe, 1952; Kathe, 1955; Brem et al., 1999). In this study, vitreal leptospire were surrounded by an osmiophilic protein coat of variable thickness, which was reduced in the phagocytized leptospira. There are two possible explanations for this osmiophilic protein coat. First, the protein coat may be the result of opsonization accelerating monocytic phagocytosis. If this were the case, the coat would consist of opsonins such as antibody Fc fragment, C3b and collectins (Picker, 1999). Experimentally, leptospire are phagocytized rapidly by macrophages – whether they are opsonized or non-opsonized (Faine et al., 1964). Secondly, the explan-

ation might lie in leptospiral masking by host protein (Faine et al., 2000). This might explain the failure of the antibodies to eliminate the leptospire. Morphologically, we could not distinguish between these two options and this question could be the subject of further studies. Beside the intact leptospire, phagocytes also incorporated ‘osmiophilic spheres’. On the basis of their osmiophilia and with no recognizable structures in them, they may be interpreted as proteinaceous masses. The nature of these proteins may also trigger further investigations.

Generally, inflammatory cells quickly become necrotic in the avascular vitreous (Spencer, 1985). Correspondingly, in our study, most samples contained only few, mostly degenerated, macrophages, lymphocytes and plasma cells and free cell organelles, as also reported by Niedermaier et al. (2006). In contrast, Niedermaier et al. (2006) found additionally lymphoblasts, fibroblasts and fibrocytes. This might be due to the fact that the cases there were more chronic. One vitreous sample (horse no. 1), however, contained a large number of intact inflammatory cells, consisting of macrophages and neutrophils with a lesser number of lymphocytes and plasma cells and numerous free leptospire. This is likely the result of sampling during an acute inflammatory episode.

To our knowledge, vitreous has never before been examined in the acute phase of ERU. This may be due to the fact that conservative therapies are applied at first, and vitrectomy is usually performed, if at all, only after the acute, painful episode. Also all previous histological examinations of eyes with ERU had been performed in symptom-free periods or in chronic stages in which a dominating T-cell population was observed in the uvea (Romeike et al., 1998; Gilger et al., 1999; Deeg et al., 2002). Our finding of vitreal plasma cells and previous descriptions of plasma cells migrating through the ciliary body epithelium might explain the local antibody production (Cooley et al., 1990; Dubielzig et al., 1997).

In the four horses with the ultrastructurally detected leptospire and in the nine horses with positive culture examination, two or more acute episodes were reported. We conclude that leptospire are present not only in the first acute episode but also in the second or third. One possible explanation for the repeated acute episodes might be the vitreal persistence of leptospire, with renewed local reactivation of leptospiral growth. As discussed before, the detected antibodies were unable to eliminate all of the leptospira present in vitreous. If we consider leptospiral persistence as a possible explanation, how do the leptospire manage to protect themselves against the immune response? One explanation might be a variation in leptospiral surface. Experimentally, leptospire have been seen to form large bubbles as a distension of the cytoplasm. Occasionally, the whole organism rolls up inside such a bubble, giving the appearance of an encysted spirochete (Swain, 1955). During renal leptospirosis, leptospire can, at any rate, change superficial molecules situated on the outer membrane, depending on the stage of infection and intrarenal localization (Barnett et al., 1999).

In the light of our own and previous results, there can be no doubt about the involvement of leptospiral infection as the cause of the repeated episodes in ERU. As a consequence of the intraocular inflammation, several secondary reactions occur. In chronic stages, because of the predominance of CD4-positive uveal T-lymphocyte infiltrates, some of them arranged in follicles with a minor B-cell population, a delayed hypersensitivity reaction has been discussed (Romeike et al.,

1998; Gilger et al., 1999). As a consequence of intraocular inflammation, numerous cells begin to express class II antigen, i.e. the retinal pigment epithelium, which can process and present S-antigen to T-helper cells (Percopo et al., 1990). Autoantibody production against retinal S-antigen, for example, can be explained in this way. Cross-reactions between intraocular structures and antibodies directed against leptospire (molecular mimicry) may also explain the perpetuation of intraocular inflammation (Parma et al., 1992; Wildner and Diedrichs-Mohring, 2003).

Prevention may consist in antileptospiral vaccination. However, it should be noted that vaccination cannot be used to treat established ERU (Rohrbach et al., 2005), and should be administered to prevent the systemic leptospirosis that precedes ocular invasion by the leptospire.

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