Leptospirosis is a zoonosis caused by pathogenic species of Leptospira that affects humans, wildlife, and many domesticated animals. The disease in humans varies from a mild flu-like form to a more severe syndrome involving multiorgan failure, whereas in horses the infection is mainly associated with spontaneous abortion and recurrent uveitis. Equine recurrent uveitis (ERU), also known as moon blindness or periodic ophthalma, is a major cause of blindness in horses and is characterized by episodes of intraocular inflammation that develop weeks to months after an initial uveitic episode and recur at regular intervals (11). Leptospira interrogans serovar Pomona and Leptospira kirschneri serovar Grippotyphosa have been incriminated as the most common infectious causes of the disease in North America and Europe, respectively (19, 21). The association of ERU with pathogenic leptospires has been established by high titers of leptospiral agglutinins in the blood and aqueous humor (19) and by isolation of established by high titers of leptospiral agglutinins in the blood.

The association of ERU with pathogenic leptospires has been incriminated as the most common infectious causes of the disease in North America and Europe, respectively (19, 21). Leptospira interrogans serovar Pomona and Leptospira kirschneri serovar Grippotyphosa have been incriminated as the most common infectious causes of the disease in North America and Europe, respectively (19, 21). The association of ERU with pathogenic leptospires has been established by high titers of leptospiral agglutinins in the blood and aqueous humor (19) and by isolation of Leptospira from ocular fluids of uveitic horses (5, 9, 21). Typically, ERU appears as a late sequela of leptospiral infection that generally appears months to years after a naturally acquired or experimentally induced infection (33, 42, 47).

ERU is widely considered to be an immune-mediated disease, and eyes with ERU exhibit infiltration of lymphocytes, plasma cells, and macrophages into the ciliary body and iris, thereby constituting morphological evidence of breach of immune privilege. CD4+ T lymphocytes are the most abundant infiltrating cells in the anterior uveal tracts of uveitic horses. The T-lymphocyte response in such horses has a Th1 bias based on quantitative reverse transcription-PCR (RT-PCR), which showed significantly greater interleukin-2 (IL-2)/gamma interferon than IL-4-specific mRNA (11). Also, peripheral blood leukocytes of chronically uveitic horses do not exhibit a Th1 response, consistent with an independent local response (11).

Pathogenic Leptospira spp. respond to environmental stimuli such as temperature (34), osmolarity (32), and other, unknown cues in the body of the host (1, 32, 37) by altering expression of many proteins. The eye, which is filled with a very dilute aqueous solution of albumin, chloride, bicarbonate, neutral amino acids, and small amounts of insoluble proteoglycans, poses unique challenges to the adaptability of Leptospira to a nutrient-poor environment (10). Design of effective therapies for management of the uveitis is dependent upon an understanding of how Leptospira spp. survive in the eye and initiate pathological changes. Although there is well documented evidence of an association of infection with Leptospira and ERU, the pathogenesis of the resulting uveitis is largely unknown. One reason for this is a lack of information regarding antigenic leptospiral proteins expressed during uveitis. The present study was undertaken to identify leptospiral proteins expressed during ocular infection and has led to the identification of two novel immunoreactive lipoproteins with possible roles in ERU pathogenesis.
Following PCR amplification of \textit{lruA} (Table 2), specific for of \textit{Leptospira interrogans} serovar Pomona, the chromosomal DNA of \textit{L. interrogans} serovar Pomona type kennecwicki (JEN4), Pomona (Pomona) Copenhageni (M 20), Canicola (Haydon Ultech IV), Grippotyphosa (Andaman), Hardjo (Hardjoprajitno), and Bratislava (Bratslava) were kindly provided by Mike Donahue (Livestock Disease Diagnostic Center, University of Kentucky, Lexington). \textit{Leptospira biflexa} serovar Biflexa was obtained from the National Veterinary Services Laboratories, Ames, Iowa. Leptospira were grown in Johnson-Harris bovine serum albumin–Tween 80 medium (Bovuminar PLM-5 Microbiological Media; Intergen, Purchase, NY) at 30°C unless otherwise indicated.

Eye fluids and companion sera from horses of varied age, breed, and origin were obtained from a commercial horse slaughter plant in North America. Eyes with gross evidence of uveitis were enucleated after slaughter, and aqueous humor was removed with a 10-ml syringe and stored at −20°C. The eyes were placed in 10% formaldehyde for subsequent embedding, sectioning, and staining with hematoxylin and eosin for histologic examination. Eye fluids and sera were assayed for antibodies to serovars Pomona, Canicola, Icterohaemorrhagiae, Hardjo, Bratislava, and Grippotyphosa in the microscopic agglutination test (MAT) (Table 1). Extracts were prepared from the ciliary body, cornea, lens, and retina of a normal eye from a young horse serologically negative for \textit{Leptospira} (38).

Library screening and plasmid rescue. A lambda ZAP II library containing 3- to 5-kb fragments of \textit{L. interrogans} serovar Pomona type kennecwicki DNA (23) was screened to identify phage expressing gene products reactive with pooled eye fluids from uveitic horses. Following propagation on \textit{Escherichia coli} XL-1 (MRP) (Stratagene, La Jolla, CA), plasmid DNA was isolated and purified using a QIAprep spin miniprep kit (Qiagen, Valencia, CA) and digested with restriction enzymes according to the manufacturer’s protocol.

DNA sequencing and analysis. Plasmid DNA was isolated using a QIAprep spin miniprep kit (Qiagen, Valencia, CA) and sequenced in a commercial sequencing facility (Davis Sequencing LLC, Davis, CA) using T7, T3, and custom-designed primers (Table 2). Sequences were edited and connected using Chromas 1.61 (Technelysium Pty. Ltd., Queensland, Australia) and DNASIS SOLR (Stratagene, La Jolla, CA) according to the manufacturer's protocol. The secondary antibody was horseradish peroxidase (HRP)-labeled protein G (Jackson ImmunoResearch, West Grove, PA) and incubated on ice overnight. Twenty-five microliters of EZ View Red Immunocapture Protein G Agarose beads (Thermo Scientific, Rockford, IL) was added, and the mixture was mixed for 2 h at 4°C. The antibody-antigen complexes bound to protein A were recovered by centrifugation at 15,000 × g for 8 s, washed twice with 800 μl immunoprecipitation buffer (50 mM Tris-Cl, pH 8.0, 100 mM NaCl, 2 mM EDTA, and 0.2% SDS) and resuspended in 100 μl 2 × SDS sample buffer. Samples of 5 μl were loaded per lane. The gel was run at 120 V for 20 min and transferred to nitrocellulose filters. Filters were blocked with 5% nonfat dry milk (Calbiochem, La Jolla, CA) in 1 × Tris-buffered saline containing 0.1% Tween 20 (Sigma) and incubated at room temperature for 1 h. Blots were washed 3 times with 50 ml of 1× TBST and probed with 10 μl of alkaline phosphatase-labeled secondary antibody (Sigma). Blots were washed 3 times with 50 ml of 1× TBST and developed using the ECL chemiluminescence detection system (Amersham Life Science, Piscataway, NJ) and exposed to X-ray film (Kodak Biomax AL). The secondary antibody was horseradish peroxidase (HRP)-labeled protein G (Jackson ImmunoResearch, West Grove, PA) and incubated on ice overnight. Twenty-five microliters of EZ View Red Immunocapture Protein G Agarose beads (Thermo Scientific, Rockford, IL) was added, and the mixture was mixed for 2 h at 4°C. The antibody-antigen complexes bound to protein A were recovered by centrifugation at 15,000 × g for 8 s, washed twice with 800 μl immunoprecipitation buffer (50 mM Tris-Cl, pH 8.0, 100 mM NaCl, 2 mM EDTA, and 0.2% SDS) and resuspended in 100 μl 2 × SDS sample buffer. Samples of 5 μl were loaded per lane. The gel was run at 120 V for 20 min and transferred to nitrocellulose filters. Filters were blocked with 5% nonfat dry milk (Calbiochem, La Jolla, CA) in 1 × Tris-buffered saline containing 0.1% Tween 20 (Sigma) and incubated at room temperature for 1 h. Blots were washed 3 times with 50 ml of 1× TBST and probed with 10 μl of alkaline phosphatase-labeled secondary antibody (Sigma). Blots were washed 3 times with 50 ml of 1× TBST and developed using the ECL chemiluminescence detection system (Amersham Life Science, Piscataway, NJ) and exposed to X-ray film (Kodak Biomax AL).
tation wash buffer (10 mM Tris-Cl, pH 8.0, 0.4 M NaCl, 0.01% Triton X-100), and washed once with 800 μl no-salt wash buffer (10 mM Tris-Cl, pH 8.0, 0.01% Triton X-100). Pellets were re suspended with 100 μl final sample buffer containing 0.25 M phenylmethanesulfonyl fluoride. Ten microliters of each sample was subjected to electrophoresis (12% PAGE-SDS gel; C amenbray, N.J.), and fixed by UV cross-linking according to the manufacturer’s protocol.

Detection of genes in different *Leptospira* spp. and serovars. DNAs of *L. interrogans* serovars Pomona, Canicola, Grippotyphosa, Hardjo, and Bratislava; *L. biflexa* serovar Biloxa; *Leptospira weilii* (Sarmin); *Leptospira immitis* (LT430); and *Leptotrichia illini* (Illini 3055) were isolated from 5-mL cultures as previously described (1). Leptospiral DNAs were digested overnight with HindIII at 37°C. Digested DNAs were separated on a 0.8% agarose gel for 4 h at 50 V, transferred to a Hybond-N nylon membrane (Amersham, Piscataway, N.J.), and fixed by UV cross-linking according to the manufacturer’s protocol.

Primers a1 1R and a5-1R plus b5-1R (Table 2), specific for of lruA and lruB, were used in the PCR to amplify the lruA and lruB genes and were labeled with digoxigenin by using the DIG High Prime DNA labeling and detection kit (Roche Applied Science, Indianapolis, IN). Prior to digoxigenin labeling, the lruA PCR amplicon was digested with HindIII and the larger fragment extracted from the gel. The UV-cross-linked nylon membrane was subjected to prehybridization at 42°C for 30 min in DIG Easy Hybridization Solution. After denaturation, approximately 25 ng/ml of probe was mixed with DIG Easy Hybridization solution and incubated with the membrane at 42°C with gentle agitation. The next day, the membrane was washed for 15 min at room temperature with three changes of the buffer containing 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) and 0.1% SDS, and then washed thrice for 30 min each time with 0.1× SSC containing 0.1% SDS. Prior to stringency washes, the membrane was treated with anti-digoxigenin-alkaline phosphatase, followed by a chemiluminescent substrate (DIG High Prime DNA labeling and detection kit). Hybridization was detected by exposing the membrane to an X-ray film (Fierce, Rockford, IL).

**RESULTS**

Identification and analysis of lruA and lruB. Screening of approximately 10^5 plaques of a lambda library of *L. interrogans* serovar Pomona type kenneewickii with pooled eye fluids from uveitic horses revealed 14 reactive plaques. Plasmds rescued from these phages were sequenced and compared with *L. interrogans* serovar Lai strain 56601 (41) and *L. interrogans* serovar Copenhageni Fiocruz L1-130 (36) genomic sequences. Homologies to eight different regions of the *L. interrogans* genome were demonstrated. These regions encode the previously described leptospiral proteins LigA/LigB (31, 37) (two phagemids), LigC (one phagemid), GrpE/DnaK/DnaJ (2) (two phagemids), and QlpA2 (30, 35) (two phagemids), plus two novel proteins. Phagemids pA1, pD1, and pB5, which encode these two proteins, were selected for further analysis.

Sequencing of pA1, pD1, and chromosomal DNA of JEN4 using primers T3, T7, a1-1R, a1-1R, a1-1R, a1-1R, and a1-1R (Table 2) revealed an open reading frame positioned in a 2,243-bp fragment of JEN4 chromosomal DNA (GenBank accession no.AY741529). This open reading frame encoded a protein (designated LruA) of 555 amino acids with a predicted molecular mass of 62 kDa. The amino terminus of LruA consisted of a 22-amino-acid signal peptide with a putative lipoprotein signal peptidase II cleavage site, FSN (20), followed by a 243-bp fragment of JEN4 chromosomal DNA (GenBank accession no. AY741529). This open reading frame encoded a protein of 555 amino acids with a predicted molecular mass of 62 kDa. The amino terminus of LruA consisted of a 22-amino-acid signal peptide with a putative lipoprotein signal peptidase II cleavage site, FSN (20).

Phagemid pB5 contained a 1,936-bp insert (GenBank accession no. AY741530) that encoded two proteins, pL13 and LruB. Analysis of the nucleic acid sequence demonstrated ribosomal binding sites for both genes but a putative promoter for pL13 and a transcriptional terminator for LruB only, suggesting translation of pL13 and a 48-kDa protein, LruB, from a polycistrionic mRNA. The N-terminal 22 amino acid residues of LruB resembled a signal peptide sequence with a potential lipoprotein signal peptide II cleavage site, FSN (20). A con-
served domain, IrpA (PSSM-Id 12815), spanning from Q_{42} to T_{351} was predicted for LruB. Proteins with this conserved domain have been described for several bacteria (24, 29) and are apparently associated with iron metabolism regulation. LruB showed 94.1% (25 amino acid substitutions) and 97.7% (10 amino acid substitutions) identity with LA3469 of *Leptospira interrogans* serovar Lai (41) and LIC10713 of *L. interrogans* (36), respectively. These amino acid substitutions were clustered between residues 155 and 176 for LIC10713 of *L. interrogans* and from amino acid residues 155 to 182 and 216 to 236 for LA3469 of *Leptospira interrogans* serovar Lai.

**Acylation of LruA.** Intrinsic labeling of *L. interrogans* with $^{14}$C]palmitate resulted in acylation of lipopolysaccharide and previously identified lipoproteins, including LipL32 (16), LipL36 (15), and LipL41 (43). Only some of the bands observed by Coomassie blue staining of the gel were labeled with $^{14}$C]palmitate, thus confirming the selectivity of the procedure (data not shown). Because LruA is a leptospiral inner membrane protein, immunoprecipitation of LruA was performed with total membrane lysate rather than with Triton X-100 extract (15, 16, 43). Immunoprecipitation of the total membrane lysate of $^{14}$C]palmitate-labeled *L. interrogans* by using LruA antiserum confirmed that LruA is acylated by *L. interrogans* (Fig. 1). Antiserum to LipL41 was included as a positive control for immunoprecipitation.

**Distribution of lruA and lruB among Leptospira spp.** The distribution of *lruA* and *lruB* in a number of pathogenic and saprophytic strains of *Leptospira* was examined by Southern blotting. *lruA* was found in *L. interrogans* serogroup Icterohemorrhagiae serovar Copenhageni and serovars Pomona (strains pomona and JEN4), Canicola, Hardjo, Bratislava, and Grippotyphosa and in *L. weili* but not in *L. biflexa, L. inadai*, or *Leptonema illini* (Fig. 2). *lruB* was also found to be present in all *L. interrogans* serovars but not in saprophytic *L. biflexa*, the intermediate pathogen *L. inadai*, or the non-*L. interrogans* pathogenic *L. weili*. (Fig. 2). *lruA* and *lruB* appear to be restricted to pathogenic *Leptospira* species, since they were not detected in saprophytic *L. biflexa*. *lruB* seems to be present only in *L. interrogans* and not in other species (Fig. 2).

**Expression and cellular localization of LruA and LruB.** Since culture temperature can affect expression of several *L. interrogans* genes, transcription of *lruA* and *lruB* in *L. interrogans* serovar Pomona grown at 30°C or 37°C was examined by RT-PCR. *lruA* and *lruB* transcripts were clearly detectable in cultures grown at 30°C or 37°C (data not shown). Reactions without reverse transcriptase yielded no product, indicating purity of the RNA preparations. Immunoblotting of whole-cell lysates from cultures grown at 30°C or 37°C with *LruA* and *LruB*-specific rabbit antiserum showed equivalent expression levels of each protein (data not shown).

Trigon X-114 was used to separate hydrophobic outer membrane proteins, hydrophilic periplasmic proteins, and the protoplasmic cylinder (Fig. 3), using antisera to known outer and inner membrane proteins LipL32 and LipL31, respectively (16, 18). Trigon X-114 fractions immunoblotted with antisera specific for *LruA* and *LruB* revealed a pattern of fractionation similar to that of LipL31 in both cases. This indicated that...
LruA and LruB are found largely, if not exclusively, in the leptospiral inner membrane.

**LruA- and LruB-specific antibody levels in sera and eye fluids of uveitic horses.** Eye fluids and sera from uveitic horses and sera from aborted mares were tested by ELISA, using LruA and LruB as antigens (Fig. 4). High levels of LruA- and LruB-specific IgG and IgA antibodies were detected in uveitic eye fluids but not in companion sera (Fig. 4). Aborted mares with high serum MAT titers showed only moderate serum levels of LruA- and LruB-specific IgG. IgA specific for LruA and LruB was detected only in uveitic eye fluids. Normal eye fluids did not contain detectable levels of LruA- and LruB-specific IgG and IgA antibodies (not shown).

**Cross-reactivity of LruA and LruB with equine ocular tissue.** Possible roles of LruA and LruB in autoimmunity were investigated by immunoblotting extracts of ciliary body, lens, and retina of a normal equine eye with specific antisera. LruA antiserum reacted with a ~22-kDa band in lens extract and a ~65-kDa band in ciliary body extract (Fig. 5A and B, respectively). Antiserum to LruB reacted very strongly with a ~30-kDa band of equine retinal extract (Fig. 5C). Preimmunization rabbit sera served as controls for nonspecific reactivity. Antiserum to LK73.5 (1), an immunoreactive, host-inducible sphingomyelinase of *L. interrogans*, showed no reactivity with eye tissue extracts (not shown).

**DISCUSSION**

Recurrent uveitis is a well-established sequela of natural and experimentally induced *Leptospira* infection in the horse and typically appears months to years following natural or experimental exposure (8, 42, 47). Culture of the organism from uveitic fluids indicates an ability to adapt to conditions in aqueous and vitreous humors. Compared to serum, with which it is isosmolar, aqueous humor contains an excess of chloride, bicarbonate, ascorbate, lactate, and neutral amino acids (10). Our study for the first time provides information on leptospiral proteins expressed in this environment and which stimulate local antibody responses. In this study, screening of an expression library using eye fluids from uveitic horses identified a number of known and novel lipoproteins. Here we describe LruA and LruB, novel immunogenic lipoproteins of *Leptospira* that are expressed in the eyes of uveitic horses and cross-react with equine ocular tissue.

LruA and LruB have Phe$^-_1$-Ile$^-_2$-Ser$^-_3$ and Phe$^-_3$-Ser$^-_4$-Asn$^-_5$-Cys$^-_6$ as potential signal peptidase cleavage sites, respectively, which conform to the consensus lipobox sequence of spirochetal lipoproteins (17). $[^{14}C]$palmitate radiolabeling experiment confirmed that, in *L. interrogans*, the FIS$^-_1$C$^-_2$ sequence in the LruA signal peptide is a lipoprotein signal peptide cleavage site as predicted by the LipoP algorithm (22). This is the first experimental evidence that serine is allowed in the $^-_1$ position of leptospiral lipoproteins. We were unable to immunoprecipitate sufficient quantities of LruB to demonstrate palmitate labeling, possibly due to the low level of LruB expression or to degradation during immunoprecipitation. However, LruB is likely to be a lipoprotein, because the FSN$^-_1$C sequence in the LruB signal peptide is a predicted lipoprotein signal peptide cleavage site by the LipoP algorithm and because asparagine in the $^-_1$ position has been demonstrated for the leptospiral lipoprotein LipL41 (43).

Evidence for intraocular expression of both proteins is the much higher level of specific antibodies in uveitic ocular fluids than in companion sera. The higher ocular levels may be explained by local synthesis, an intact blood-eye barrier, and lack of degradation. The very low levels of specific IgG and IgA in companion sera suggest that systemic antibody responses to LruA and LruB in uveitic horses are suppressed or poorly expressed during the bacteremia that preceded ocular invasion. Lack of expression during infection is a less likely explanation of very low antibody levels in sera of uveitic horses, since LruA- and LruB-specific IgG and IgA levels are significantly ($P < 0.001$) higher in sera of recently aborted mares. The low levels are a manifestation either of a deviant systemic immune response or of a systemic response that has waned over the possibly long interval since systemic immune stimulation following initial infection. A previous comparison (46) of amounts and isotypes of immunoglobulin in uveitic and normal equine vitreous humor revealed significantly larger amounts of IgA in uveitic fluids and absence of IgM from normal and uveitic fluids. Amounts of IgGa, IgGb, IgGc, and IgG(T) in uveitic fluids are about 1,000-fold less than those in companion sera but in the same proportions. It was therefore concluded that there is local IgA synthesis in uveitic eyes but that IgA and other immunoglobulins in normal fluids were derived from plasma. Although quantitation of IgG isotypes was not done...
for the uveitic fluids in the present study, the significantly \( P < 0.001 \) lower levels of LruA- and LruB-specific IgG in companion sera indicate that local synthesis of IgG and not diffusion from plasma is the source of specific antibody in eye fluids of horses with ERU.

Prolonged intraocular survival of *Leptospira* spp. in the face of antibody responses to bacterial proteins indicates an absence of cells and molecules involved in the innate immune response and bacterial clearance. This may be explained by the immunosuppressive and anti-inflammatory effects of transforming growth factor β2 and other factors responsible for ocular immune privilege (13, 44, 45). Although unproven, it is likely that *Leptospira* spp. which enter the ocular compartment induce anterior chamber-associated immune deviation (ACAID), an important manifestation of immune privilege resulting in an inability of the host to display delayed hypersensitivity reactivity to *Leptospira* antigens and to produce *Leptospira*-specific complement-fixing antibodies (25, 26). A third potential outcome of ACAID is the induction and differentiation in the spleen of regulatory T cells that suppress *Lepto-

FIG. 4. LruA- and LruB-specific IgG (left panels) and IgA (right panels) levels in eye fluids and companion sera of 12 uveitic horses and in sera of 10 recently aborted mares. Error bars indicate standard deviations. OD\(_{490}\) optical density at 490 nm.

FIG. 5. Cross-reactivity of LruA and LruB with equine ocular tissue. (A and B) Immunoblots showing reactivity of rabbit antiserum to LruA with extracts of normal equine lens and ciliary body. (C) Reactivity of LruB antiserum with extracts of normal equine retina. Preimmunization sera from rabbits were used as controls for specificity of binding. The arrows and asterisk indicate cross-reactive bands to LruA- or LruB-antiserum in normal equine ocular tissues.
spira-specific Th1 and Th2 responses. Once induced, ACAID is long lasting, and so its effects would be expected to persist into the later stages of ERU.

Although direct Leptospira-mediated injury to the eye structure cannot be ruled out in explaining the pathogenic mechanisms of ERU, there is a growing body of evidence that autoimmune responses to ocular tissue components play a significant role in pathogenesis (6, 11, 38, 39, 40). Cross-reactivity between leptospiral lysates and the cornea or lens has been previously reported (28, 38). Antibodies and T lymphocytes specific for retinal S antigen and interphotoreceptor retinoid binding protein (IRBP) have also been observed in eyes of uveitic horses (6). Moreover, injection of IRBP with complete Freund’s adjuvant induced a disease similar to ERU (7).

Intraocular immune responses that are not proinflammatory, it is possible that the early phase of the immunopathogenesis of ERU involves production of non-complement-fixing antibody and non-DTH T lymphocytes specific for LruA and LruB. Reactivity of these molecules and cells with their targets in ocular tissue then initiates a process that leads to desequestration of IRBP and other ocular autoantigens. Future experiments, including characterization of cross-reacting eye antigens, will explore these hypotheses and lead toward a better understanding of the pathogenesis of leptospiral uveitis.

ACKNOWLEDGMENTS

This work was funded by the Keeneland Association. A. Verma was funded by a Paul Mellon Fellowship in Equine Studies. The work was also supported by Public Health Service grant AI-34431 (to D.A.H.) from the National Institute of Allergy and Infectious Diseases and VA Medical Research Funds (to J.M. and D.A.H.).

We thank Mike Donahue for providing Leptospira isolates, Raksha Tiwari for helpful comments, and Brian Stevenson for critical review of the manuscript.

REFERENCES


29. Marchler-Bauer, A., J. B. Anderson, P. F. Cherukuri, C. DeWeese-Scott,


