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LfhA, a Novel Factor H-Binding Protein of *Leptospira interrogans*

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The early phase of leptospiral infection is characterized by the presence of live organisms in the blood. Pathogenic *Leptospira interrogans* is resistant to the alternative pathway of complement mediated-killing, while nonpathogenic members of the genus are not. Consistent with that observation, only pathogenic leptospires bound factor H, a host fluid-phase regulator of the alternative complement pathway. Ligand affinity blot analyses revealed that pathogenic *L. interrogans* produces at least two factor H-binding proteins. Through screening of a lambda phage expression library, we identified one of these as the novel membrane protein LfhA. Ligand affinity assays and surface plasmon resonance analyses of recombinant LfhA revealed specific binding of both factor H and factor H-related protein 1. Serological examination of infected humans and horses demonstrated that LfhA is expressed by *L. interrogans* during mammalian infection. LfhA may therefore contribute to the resistance of pathogenic leptospires to complement-mediated killing during leptospiremic phases of the disease.

Leptospirosis is a widespread zoonotic disease that affects humans and many species of domesticated and wild animals (36). This disease is caused by several closely related spirochetes of the genus *Leptospira*, including the species *Leptospira interrogans*. These spirochetes colonize proximal renal tubules of chronically infected carrier animals such as cattle, horses, dogs, pigs, rats, raccoons, and skunks. Carrier animals then shed leptospires in their urine, and humans acquire leptospirosis by accidental exposure to urine from infected animals or to urine-contaminated water. *L. interrogans* invades humans and other hosts through intact or injured mucous membranes and then disseminates from the site of initial infection via the bloodstream. The postentry period of approximately 10 to 14 days is characterized by a leptospiremic phase, during which leptospires persist in the blood. The primary lesion during this phase is damage to the endothelia of small blood vessels, resulting in localized ischemia in kidneys, liver, meninges, and muscles (7, 14).

The alternative complement pathway is an important component of the host innate immune defense. This pathway is initiated in the absence of specific antibodies by spontaneous hydrolysis of the thioester bond in C3 to form C3(H_2O), which allows binding of factor B, which in turn is cleaved to Ba and Bb. The C3(H_2O)Bb complex acts as a fluid-phase C3 convertase which cleaves C3, resulting in formation of the alternative pathway C3 convertase, C3bBb. The alternative pathway C3 convertase cleaves additional C3 to C3b, which binds to pathogen surfaces and unleashes a cascade of reactions resulting in formation of lytic membrane attack complexes, opsonization of pathogens, and phagocyte recruitment. Complement activation can also enhance host adaptive immune responses (9). Damage to host cells by complement activation is prevented by a number of complement-regulatory proteins, including factor H (23). Factor H, a 150-kDa plasma protein, is present in serum at a concentration of approximately 500 μg/ml and is composed of 20 short consensus repeats (SCRs) (51). Factor H binds to C3b by displacing Bb from C3 convertases and acts as a cofactor for factor I, which cleaves C3b to its inactive form, iC3b. Humans, but not all other mammals, produce a smaller protein, factor H-like protein 1 (FHL-1), by alternative splicing of the factor H gene. FHL-1 consists of the first seven SCRs of factor H plus four additional amino acids at the carboxy terminus, and it exhibits cofactor activity (62). Mammals also produce several factor H-related proteins (FHRs), from distinct genes, which share sequence similarity with the carboxy terminus of factor H (45, 62). The FHRs are not yet well characterized but appear to also possess complement-regulatory functions (20, 39, 49, 62). Terminal sialic acid moieties on vertebrate cell glycoproteins serve as receptors to bind factor H and related proteins to cell surfaces, where they serve to protect those cells against the deleterious effects of C3 activation (23, 61).

During the leptospiremic phase, the bacteria are exposed to components of the alternative pathway of complement but readily avoid complement-mediated destruction (3, 5, 10, 24–26). The ability of pathogenic leptospires to resist the alternative pathway of complement was noticed several decades ago and proposed as a virulence determinant (10, 24), although the exact mechanism underlying this resistance was not defined. Many other pathogens have evolved mechanisms to bind host factor H to their surfaces, thereby protecting themselves from

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the destructive effects of complement activation (35, 37, 62). Several bacterial pathogens of medical and veterinary importance, including Lancefield group A/B streptococci (15, 44), *Neisseria meningitidis* and *Neisseria gonorrhoeae* (48), *Borrelia burgdorferi* (1, 2, 8, 21, 30–34, 38, 56) and *Borrelia hermsii* (22), produce one or more outer surface proteins that specifically bind factor H. Given the widespread distribution of factor H-binding proteins among bacterial pathogens, we speculated that such proteins might also be responsible for resistance of *L. interrogans* to complement-mediated killing. To address this hypothesis, pathogenic leptospires were examined for the ability to bind factor H, whereupon we discovered that these bacteria produce at least two different factor H-binding proteins. During the preparation of this paper, another research group published data also indicating that pathogenic leptospires bind factor H to their surfaces (40), although the molecular nature of the interactions was not explored in that study. In this report we present data on the identification and biophysical characterization of LfhA, a factor H-binding protein of infectious *Leptospira* species.

MATERIALS AND METHODS

Bacterial strains and culture conditions. Infectious *L. interrogans* serovars Lai (strain 56601) and Copenhageni (Fiocruz L1-130) were generous gifts from David Haake (UCLA School of Medicine, Los Angeles, CA) and Albert Ko (Gonçalo Moniz Research Center, Oswaldo Cruz Foundation, Brazilian Ministry of Health, Salvador, Bahia, Brazil). Infectious *L. interrogans* serovars Pomona type kennewicki (JEN4), Pomona (Pomona) Copenhageni (M 20), Canicola (Hond Utrech IV), Grippotyphosa (Andaman), Hardjo (Hardjoprajitno), and Bratislava (Jez Bratislava) were kindly provided by Michael Donahue (Livestock Disease Diagnostic Center, University of Kentucky, Lexington). *Leptospira weilii* (Sarmin), *Leptospira inadai* (LT430), and *Leptonema illini* (Illini 3055) were presented to us by the late Faye Austin (University of Louisville, Louisville, KY). *Leptospira biflexa* serovar Biflexa was obtained from the National Veterinary Services Laboratories, Ames, Iowa. All leptospires were grown in Johnson-Harris bovine serum albumin (BSA)–Tween 80 medium (Bovuminar PLM-5 Microbiological Media; Intergen, Purchase, NY) at 30°C (63).

Sera. Normal equine sera were collected from horses serologically negative for exposure to *L. interrogans* at the University of Kentucky Equine Farms. Horse sera that tested positive for leptospirosis by the microscopic agglutination test were from clinical cases in various parts of the United States. Sera from cases of human leptospirosis were kindly provided by Renee Galloway (Centers for Disease Control and Prevention, Atlanta, GA) and were collected from patients throughout the world.

Survival of bacteria in normal serum. Fifty microliters of mid-exponential-phase cultures (approximately 2×10^8 bacteria/ml) of *L. interrogans* serovar Lai, Pomona, Copenhageni, Canicola, Grippotyphosa, Hardjo, or Bratislava or *L. biflexa* was incubated with 50 μl of different dilutions of normal equine serum in Johnson-Harris BSA–Tween 80 medium and incubated at 30°C for 1 h. All leptospires were simultaneously incubated with medium alone as controls. Intact bacteria were counted using a Petroff-Hausser counting chamber under a dark-field microscope.

Indirect fluorescent-antibody analyses. Cultured *L. interrogans* serovar Lai or Pomona or *L. biflexa* was applied to 3-aminopropyl-triethoxysilane-treated glass slides. Slides were air dried and bacteria fixed with a solution containing 25% glutaraldehyde, 10% formalin, and phosphate-buffered saline (PBS) (pH 7.4) for 15 min on ice. After two washings with PBS, blocking was performed using 2% (wt/vol) BSA in PBS. Slides were washed thrice with PBS and incubated with either 1 μg/ml purified human factor H (Calbiochem, San Diego, CA), equine serum, or PBS for 1 h at room temperature (RT) in a humidifying chamber. After three washes with PBS, slides were incubated with 1:150 dilution of factor H-specific monoclonal antibody A229 (Quidel, San Diego, CA) (27) or PBS for 1 h, followed by fluorescein isothiocyanate-conjugated goat anti-mouse antibodies (Sigma, St. Louis, MO). Slides were examined by epifluorescence using an Axioskop microscope (Zeiss, Thornwood, NY).

Factor H ligand affinity blot analyses. *L. interrogans* lysates, recombinant LfhA, and control proteins were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Proteins were then transferred to nitrocellulose membranes and blocked with 5% nonfat dry milk in Tris-buffered saline–Tween 20 (TBS-T) (20 mM Tris, 150 mM NaCl, 0.05% Tween 20 [pH 7.5]). The membranes were then incubated with either purified human factor H (0.5 μg /ml; Calbiochem) or normal equine serum for 1 h at RT. Following washing with TBS-T, membranes were incubated for 1 h at RT with monoclonal anti-factor H antibody A229 (27) (Quidel). Finally, the membranes were washed with TBS-T and incubated for 1 h at RT with horseradish peroxide-conjugated sheep anti-mouse antibodies (Amersham, Little Chalfont, United Kingdom). Bound antibodies were identified either by enhanced chemiluminescence (Pierce, Rockford, IL) or by using 12 mg 4-chloro-1-naphthol (Sigma) dissolved in 5 ml methanol, 25 ml TBS, and 30 μl of 30% hydrogen peroxide. Recombinant forms of ErpA and ErpC, two factor H-binding proteins of the spirochete *Borrelia burgdorferi* (1, 30, 32, 41, 56), were used as positive control proteins for all analyses. Carbonic anhydrase, soybean trypsin inhibitor, and lysozyme, which do not specifically bind factor H, were used as negative controls.

Library screening and sequence determination of cloned inserts. A lambda ZAP II library containing 3- to 5-kb fragments of *L. interrogans* serovar Pomona type kennewicki DNA (29) was screened to identify phage expressing gene products that bound with purified human factor H. Following propagation in *Escherichia coli* XL-1 Blue MRF′ (Stratagene, La Jolla, CA), phage plaques were transferred in duplicate to IPTG (isopropyl-β-D-thiogalactopyranoside)-saturated nitrocellulose disks and immunoblotted with either purified human factor H or equine serum, followed by monoclonal anti-factor H antibody, as described above. Plaques that bound factor H were transferred to 500 μl of SM buffer (50 mM Tris-HCl, pH 7.5, 100 mM NaCl, 8 mM $MgSO_4$, 0.01% gelatin) and allowed to elute overnight at 4°C. Plaques were rescreened until they were clonal. Plasmids were rescued from reactive phages by using ExAssist helper phage and *E. coli* SOLR (Stratagene), following the supplier's protocol.

Plasmids were isolated using QIAprep spin miniprep kits (QIAGEN, Valencia, CA). Insert DNAs were sequenced by a commercial DNA sequencing facility (Davis Sequencing LLC, Davis, CA). Sequences were compared with the *L. interrogans* serovar Lai strain 56601 and serovar Copenhageni strain Fiocruz L1-130 genomic sequences at http://www.tigr.org/. Analyses of nucleotide sequences and deduced amino acid sequences were performed using DNASIS, SignalP (6), LipoP (28), TMHMM (http://www.cbs.dtu.dk/), COILS (http://www.ch.embnet.org/index.html), and BLAST (http://www.ncbi.nlm.nih.gov/BLAST/).

Cloning and expression of recombinant LfhA. The *L. interrogans* serovar Pomona strain JEN4 *lfhA* gene, minus its signal sequence, was amplified with PCR oligonucleotide primers LfhAF (5′-GCG CTC GAG GAA GAA GAT AAT TCA GAA CTA C-3′) and LfhAR (5′-GCG GAT CCT TAC TGT TCT ACA CAG AGA AGA TT-3′). The resulting amplicon was digested with BamHI and XhoI and inserted into pET-15b (Novagen, Madison, WI). The recombinant plasmid was transformed into *E. coli* Rosetta(DE3)(pLysS) (Novagen), and expression of His_6-LfhA was induced with 1 mM IPTG. Recombinant His-tagged protein was isolated using Talon metal affinity resin (BD Biosciences Clontech, Palo Alto, CA) in buffer containing 8 M urea, following the manufacturer's recommendations. The purity of recombinant protein was confirmed by SDS-PAGE and staining with Coomassie brilliant blue.

LfhA-directed polyclonal antisera. Polyclonal LfhA antiserum was raised in a New Zealand White rabbit by a subcutaneous administration of 200 μg of recombinant LfhA adsorbed to aluminum hydroxide (Alhydrogel; Accurate Chemical & Scientific Corp., Westbury, NY) and *N*-acetylmuramyl-L-alanyl-D-isoglutamine (Sigma), followed by two subcutaneous booster injections and one intravenous booster injection. Where indicated, LfhA antiserum was affinity purified by overnight incubation with nitrocellulose membranes impregnated with 10 μg of purified recombinant LfhA protein. The affinity-purified antibodies were eluted from the nitrocellulose by incubation with 100 mM glycine (pH 2.0) for 15 min at room temperature. Purified antibody solution was neutralized by addition of 0.1 volume of 1 M Tris base (pH 8.0) and subsequently dialyzed against PBS.

Surface plasmon resonance. Protein-protein interactions were analyzed by a surface plasmon resonance technique using a Biacore 3000 instrument (Biacore AB, Uppsala, Sweden) as described earlier (21). Briefly, recombinant LfhA (40 μg/ml, dialyzed against 10 mM acetate buffer, pH 5.0) was coupled via amine coupling onto the flow cell of a sensor chip (CM5; Biacore AB) until a level of >2,000 resonance units was reached. A control flow cell was prepared by injecting buffer without protein. Purified human factor H (Calbiochem, La Jolla, CA), recombinant human FHR-1 expressed in insect cells (J. Hellwage, unpublished results), and recombinant human FHL-1 (58) were individually dialyzed against the running buffer (phosphate-buffered saline, pH 7.4, 50 μM $NiCl_2$). Each ligand was injected separately into the flow cell coupled with LfhA or into the control flow cell, using a flow rate of 10 μl/min at 25°C. Studies were also performed using recombinant forms of *B. burgdorferi* proteins, including the

factor H-binding proteins BbCRASP-3 (ErpP), OspE, and BbCRASP-1 and specific deletion mutants of BbCRASP-1 and BbCRASP-3 that do not bind factor H (unpublished results and references 21 and 31).

Triton X-114 extraction. *L. interrogans* serovar Lai was washed in PBS and then extracted with 1% protein grade Triton X-114 (Calbiochem) in PBS overnight at 4°C. The insoluble material was removed by centrifugation at 20,000 × *g* for 30 min and incubated two more times with Triton X-114. The supernatant was removed, and phase separation was performed by warming the supernatant to 37°C and subjecting it to centrifugation for 15 min at 15,000 × *g* (46). The detergent and aqueous phases were then separated, reextracted thrice, and precipitated with methanol-chloroform. The integrity of the protoplasmic cylinder was checked by immunoblot analysis of Triton X-114-extracted fragments with antiserum specific for spirochete flagella (a gift of David Blanco, University of California, Los Angeles, CA).

Southern blot analysis. Genomic DNAs of *L. interrogans* serovars Pomona, Canicola, Grippotyphosa, Hardjo, and Bratislava and of *L. biflexa*, *L. weilii*, *L. inadai*, and *Leptonema illini* were isolated from 5-ml cultures as previously described (4). Leptospiral DNAs were digested overnight with HindIII at 37°C. Digested DNAs were separated on a 0.8% agarose gel, transferred to a Hybond-N nylon membrane (Amersham, Piscataway, N.J.), and fixed by UV cross-linking.

The LfhAF and LfhAR primers were used to PCR amplify *lfhA* from *L. interrogans* serovar Pomona strain JEN4. The amplicon was labeled with digoxigenin using the DIG High Prime DNA labeling and detection kit (Roche, Indianapolis, IN). The UV cross-linked nylon membrane was subjected to prehybridization at 42°C for 30 min in DIG Easy hybridization solution (Roche). After denaturation, approximately 25 ng/ml of *lfhA* 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 buffer containing 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) and 0.1% SDS. The membrane was then washed thrice for 15 min each at 65°C in 0.5× SSC with 0.1% SDS. Next, the membrane was treated with antidigoxigenin-alkaline phosphatase, followed by the chemiluminescence substrate. DNA hybridization was detected by exposing the membrane to X-Omat AR film (Eastman Kodak Company, Rochester, NY).

Immunoblot analyses. For each analysis, a 10-mg aliquot of purified recombinant LfhA protein was applied to a single 7-cm-wide well in a polyacrylamide gel, subjected to SDS-PAGE, transferred to a nitrocellulose membrane, and blocked using 4% (wt/vol) nonfat dried milk in TBS-T. Serum samples from human or equine leptospirosis cases were diluted 1:100 in TBS-T and examined simultaneously using a Mini-Protean II Multiscreen apparatus (Bio-Rad). Polyclonal rabbit LfhA-directed antiserum, diluted 1:400, served as a positive control for all immunoblot analyses. Normal human or equine sera, diluted 1:100 in TBS-T, served as negative controls. Bound antibodies were detected using protein G conjugated to horseradish peroxidase (Zymed, San Francisco, CA) and 4-chloro-1-naphthol (Sigma).

Nucleotide sequence accession number. The DNA sequence of the *L. interrogans* serovar Pomona type kennewicki strain JEN4 library clone containing *lfhA* has been submitted to GenBank with accession number DQ370178.

RESULTS

Pathogenic leptospires bind host factor H. Pathogenic leptospires survive and multiply in the bloodstreams of animals during the initial phase of the disease, in part due to their resistance to the alternative pathway of complement (3, 5, 10, 24–26). In contrast, saprophytic leptospires are very sensitive to the killing effect of complement, presumably because their noninfectious nature has not required those bacteria to evolve complement evasion mechanisms. Those conclusions of earlier researchers were reaffirmed by the results of the present studies. Pathogenic *L. interrogans* serovars Lai, Pomona, Copenhageni, Canicola, Grippotyphosa, Hardjo, and Bratislava and the nonpathogenic *L. biflexa* were each incubated in 50% normal equine serum. After 1 h, greater than 80% of each pathogenic serovar remained intact. More than 99% of bacteria in cultures of *L. biflexa* were lysed under those conditions. *L. biflexa* was able to survive only when the concentration of normal serum was less than 5%.

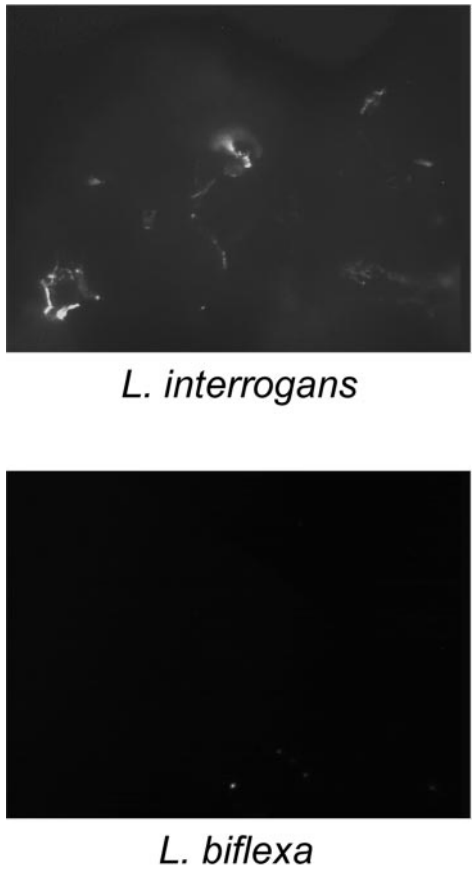

FIG. 1. Indirect immunofluorescence analysis of *L. interrogans* serovar Lai and *L. biflexa* incubated with purified human factor H, anti-factor H monoclonal antibody, and fluorescently conjugated secondary antibody. Magnification, ×100.

Many pathogens, including the spirochetes *B. burgdorferi* and *B. hermsii*, prevent activation of the alternative pathway of complement by binding factor H via specific outer membrane proteins. To examine whether a similar mechanism is used by *L. interrogans*, the ability of that bacterium to bind factor H was examined by indirect immunofluorescence analysis. *L. interrogans* exhibited substantial factor H binding (Fig. 1), while *L. biflexa* did not bind the complement regulator. Similar results were obtained when using either purified human factor H or normal equine serum. *L. interrogans* and *L. biflexa* subjected to conditions that lacked either factor H or anti-factor H monoclonal antibody did not exhibit fluorescence (not shown), indicating the specificity of this assay.

Next, we analyzed whole-cell lysates of *L. interrogans* serovars Lai and Pomona for the presence of proteins that interact with factor H. Factor H ligand affinity blot analysis of cell lysates from the two serovars indicated that both strains produced at least two factor H-binding proteins, having apparent molecular masses of approximately 30 and 50 kDa (Fig. 2A). Recombinant ErpA, a factor H binding protein of *B. burgdorferi* (56), served as a positive control (Fig. 2A). No signals were detected when either factor H or factor H-directed monoclonal antibody was omitted (data not shown).

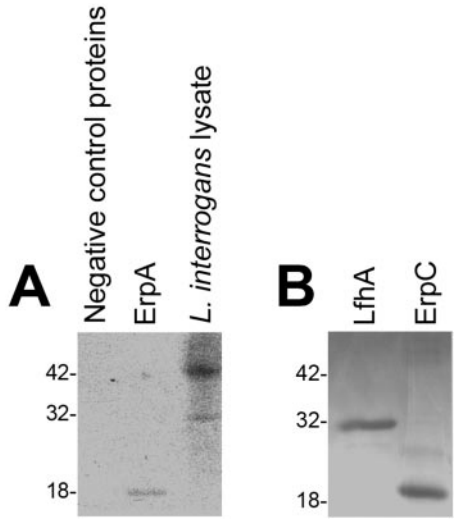

FIG. 2. Factor H ligand affinity analyses. Recombinant *B. burgdorferi* ErpA and ErpC proteins were included as positive controls (1, 30, 32, 41, 56), and carbonic anhydrase, soybean trypsin inhibitor, and lysozyme were included as negative controls. A. A lysate of *L. interrogans* serovar Pomona strain JEN4 was subjected to SDS-PAGE and examined for proteins that bind purified human factor H. Two such protein bands were identified, with apparent molecular masses of approximately 30 and 50 kDa. B. Ligand affinity blot analysis demonstrating that recombinant LfhA binds purified human factor H. Positions of molecular mass standards are indicated to the left of each panel (in kilodaltons).

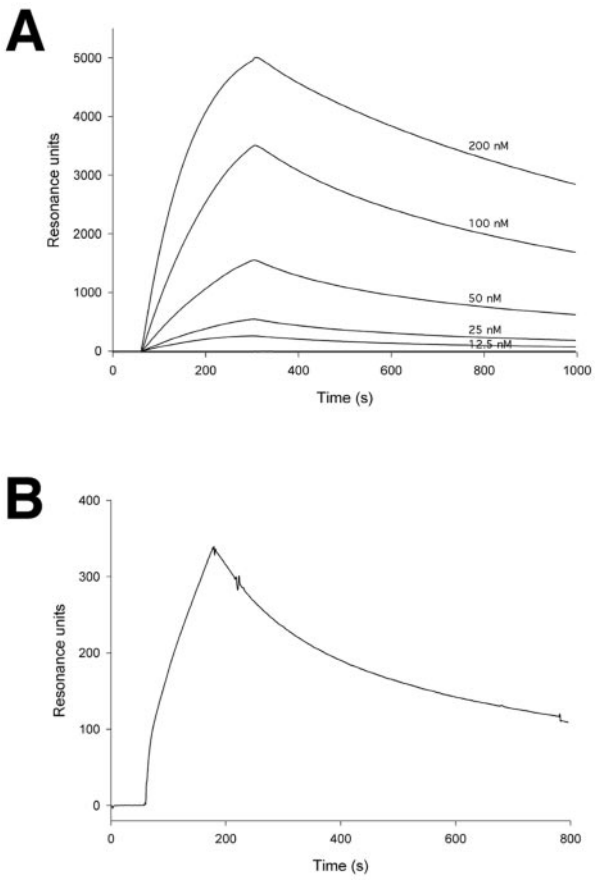

FIG. 3. Surface plasmon resonance analyses of factor H and FHR-1 binding to recombinant LfhA. A. Analyses of factor H binding, utilizing 200, 100, 50, 25, and 12.5 nM factor H. B. Analyses of FHR-1 (200 nM) binding to recombinant LfhA. Binding of proteins to the immobilized ligand is reported as relative resonance units per time.

Identification of the factor H binding protein LfhA. A lambda expression library of *L. interrogans* serovar Pomona type kennewicki strain JEN4 was screened for clones that specifically bound human factor H. Reactive phage were isolated and their inserts sequenced and compared with genomic sequences of *L. interrogans* serovars Lai (strain 56601) (50) and Copenhageni (strain Fiocruz L1-130) (43). Phagemid pOM1 contained two open reading frames (ORFs) in a 3,335-bp fragment of *L. interrogans* chromosome I DNA. The predicted amino acid sequence of one, partial ORF suggested that it encodes a 76-kDa sodium-glucose symporter. The second ORF lacked significant homology with any previously characterized protein and is allelic to ORF LA695 of *L. interrogans* serovar Lai and ORF LIC12906 of *L. interrogans* serovar Copenhageni. Those ORFs encode proteins consisting of 240 amino acid residues and predicted molecular masses of 26 kDa. The amino termini of these predicted proteins contain 28-amino-acid signal sequences followed by putative spirochetal lipoboxes, LYS ↓ C (11, 17, 53, 54). These and other sequence features suggested that the putative lipoprotein of phagemid pOM1 was likely to be an outer membrane protein (17, 53), so it was chosen for further characterization.

LfhA binds factor H and FHR-1. A recombinant His_6 tag fusion protein of the putative lipoprotein was produced and tested for the ability to bind factor H. Ligand affinity blot analyses demonstrated that the recombinant *L. interrogans* protein bound factor H (Fig. 2B), and it was therefore designated LfhA (for *Leptospira* factor *H*-binding protein *A*).

The interaction between LfhA and factor H was characterized further using real-time surface plasmon resonance (Biacore). LfhA was coupled to a sensor chip, and its interaction with free-flowing factor H was measured as relative resonance units. Factor H in increasing concentrations (12.5, 25, 50, 100, and 200 nM) was injected separately into the flow cell with LfhA or into the control flow cell. LfhA bound factor H in a dose-dependent manner (Fig. 3A). In addition, LfhA also interacted with the factor H-related protein FHR-1 (Fig. 3B), a 37- to 43-kDa plasma protein composed of five SCRs and a carboxy terminus that shares a high degree of similarity with factor H (62). LfhA did not bind FHL-1, which consists of the amino-terminal seven SCRs of factor H (data not shown). These data suggest that LfhA interacts with factor H through carboxy-terminal SCRs 18 to 20, the region it has in common with FHR-1. Control studies using known factor H-binding proteins and nonbinding proteins demonstrated the sensitivity and specificity of this technique (data not shown and references 21 and 31).

Localization of LfhA to the outer and inner membranes. As noted above, analysis of the predicted amino acid sequence of LfhA revealed features characteristic of an outer surface protein. This prediction was confirmed by solubilizing *L. interrogans* serovar Lai with Triton X-114 to obtain protoplasmic cylinder-, periplasmic protein-, and outer membrane protein-

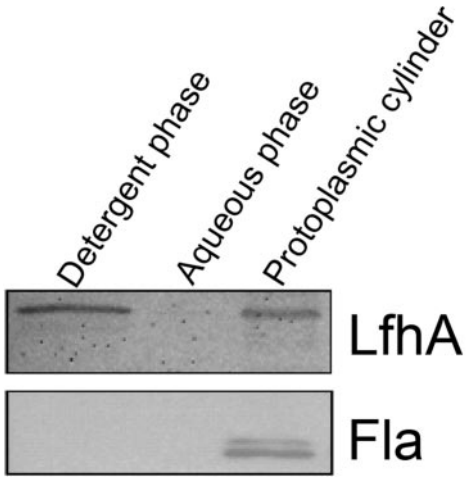

FIG. 4. Cellular localization of LfhA by cellular fractionation with Triton X-114. Fractions of the detergent phase (outer membrane fraction), aqueous phase (periplasmic fraction), and protoplasmic cylinder (inner membrane plus cytoplasm fractions) were separated by SDS-PAGE and analyzed by immunoblotting using affinity-purified rabbit antiserum directed against LfhA (top panel). As a control for fractionation purity, fractions were also analyzed using rabbit antiserum to *Treponema pallidum* FlaB, which also recognizes the *L. interrogans* endoflagellum (42) (bottom panel). Spirochete flagella are anchored to the inner membrane only and thus purify with the protoplasmic cylinder.

enriched fractions. The protoplasmic cylinder fraction consists of the cytoplasm, inner membrane, and anchored periplasmic flagella (14). Outer membrane components are found in the detergent-soluble fraction, whereas periplasmic proteins separate into the aqueous phase (13, 42, 46, 47, 55, 57). Fractions were subjected to SDS-PAGE and analyzed by immunoblotting (Fig. 4). The integrity of the protoplasmic cylinder was confirmed using polyclonal antiserum that recognizes leptospiral flagella (42). LfhA was identified in both the outer membrane and protoplasmic cylinder fractions, as has previously been reported for another *L. interrogans* protein, LipL41 (55).

Infectious *L. interrogans* contains *lfhA* loci. We next investigated the distribution of *lfhA* genes in several different pathogenic and saprophytic *Leptospira* spp. As described above, examination of the published genome sequences of *L. interrogans* serovars Lai and Copenhageni indicated that *lfhA* is present in those two serovars. The near identity of the nucleotide sequences of *lfhA* genes of *L. interrogans* isolates from the United States and China (50) and Brazil (43) suggests a high degree of conservation of the gene among *L. interrogans* strains. Southern blot analysis using a probe derived from the serovar Pomona *lfhA* gene confirmed the presence of the gene in each examined *L. interrogans* serovar (Fig. 5). Homologs of *lfhA* were not detected in *L. biflexa*, *L. inadai*, *L. weilii*, or *Leptonema illini*.

LfhA is expressed during natural infection. Next, we queried whether LfhA is expressed by *L. interrogans* during mammalian infection and would thus be poised to protect the bacterium from complement-mediated killing. To that end, recombinant LfhA was examined by immunoblotting using sera from human and equine cases of leptospirosis. Thirteen of 14 human sera and all 13 equine sera contained antibodies that

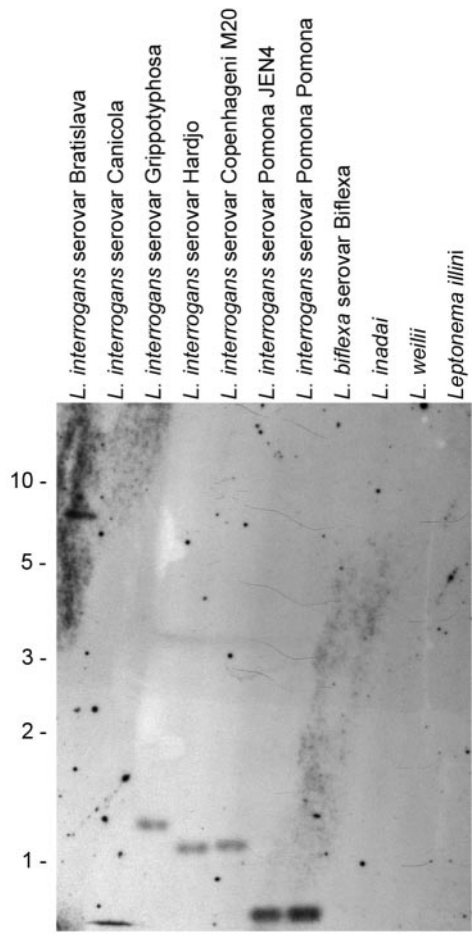

FIG. 5. Southern blot analysis of the presence of *lfhA* genes in pathogenic serovars of *L. interrogans*, other *Leptospira* spp., and *Leptonema illini*.

bound recombinant LfhA (Fig. 6). Control normal human and equine sera did not contain antibodies recognizing LfhA. Thus, not only is LfhA synthesized by *L. interrogans* during mammalian infections, but it also appears to be highly antigenic. Moreover, recognition of recombinant LfhA derived from serovar Pomona by such a wide diversity of infected humans and horses suggests extensive antigenic conservation of LfhA among many infectious leptospires.

DISCUSSION

The broad host range of pathogenic leptospires indicates an ability to overcome the antimicrobial defense systems of a wide variety of animals. Following entry through the skin or mucosa, *L. interrogans* is exposed to the innate humoral and cellular defense systems, which normally disarm and remove less virulent microbial intruders. Initial leptospiral infection is followed by bacteremia that persists through the incubation period and for 1 to 2 weeks after onset of acute disease. Multiplication in susceptible hosts is rapid, with doubling times of 8 h or less for virulent strains that cause acute fulminating disease (14). In contrast, nonpathogenic leptospires are readily killed by animal innate immune defenses. Early studies designed to explain

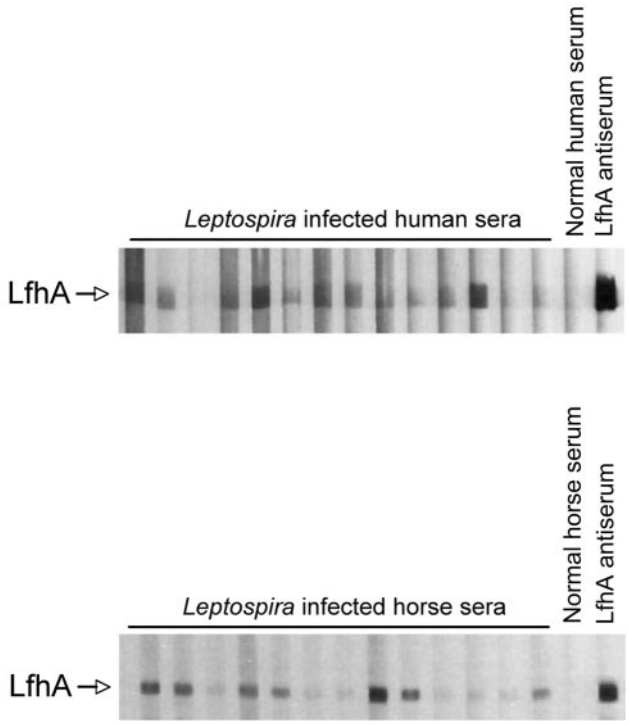

FIG. 6. Immunoblot analyses of LfhA with serum samples from humans and horses naturally infected with *L. interrogans*. Strip blots containing recombinant LfhA were probed with sera from clinically confirmed cases of human and equine leptospirosis. Normal human and equine sera and rabbit polyclonal antiserum specific for LfhA were included as controls.

the survival of pathogenic but not nonpathogenic *Leptospira* strains led to the conclusion that survival was determined by susceptibility to the alternative complement pathway cascade (3, 5, 10, 24–26). In the present study, selective binding of factor H to pathogenic leptospires was demonstrable by visual observation using indirect immunofluorescence analysis. Supporting our conclusions, similar results were reported recently by a second research group, who demonstrated that factor H bound to infectious leptospires provided cofactor activity for degradation of C3b (40). Our ligand affinity blot analyses of *L. interrogans* sonicates identified two protein bands that bound factor H, having approximate molecular masses of 30 and 50 kDa. We then isolated and characterized the novel, 26-kDa membrane protein LfhA. The identity of the larger protein is as yet unknown. LfhA bound both factor H and FHR-1 but not FHL-1, suggesting that LfhA specifically interacts with a site(s) within the carboxy-terminal SCRs 18 to 20 of factor H. The binding properties we defined for LfhA are consistent with the recent publication by Meri et al. (40), who observed that infectious leptospires bound factor H and FHR-1 but not FHL-1.

Homologs of *lfhA* were found in each of the *L. interrogans* strains we examined but were absent from other leptospires. *L. weilii* is associated with human leptospirosis in parts of the world but is genetically very distinct from *L. interrogans* (19, 60). *L. inadai* possesses many features in common with saprophytic members of the genus and has been described as largely nonpathogenic, although it is occasionally isolated from humans and other animals (16, 52, 59). Some isolates of *L. inadai* from India have proven to be resistant to serum (16). Future studies on pathogenicity and serum resistance among leptospires will determine whether those species contain *lfhA*-like genes that are undetectable by Southern blot analysis, whether some strains possess genes similar to *lfhA* while other members of the same species lack such genes, or whether they utilize alternative mechanisms of resistance to complement. In support of the last hypothesis, the *L. interrogans* strains examined in the present studies encode at least one additional factor H-binding protein. Studies to identify and characterize that protein(s) are ongoing.

Leptospiral outer membrane proteins are potential targets for inducing protective immune responses in the host, and those that are also well conserved among pathogenic serovars make attractive vaccine candidates (11, 12, 17, 18). Southern blot and preliminary sequence analyses suggest that LfhA may fit those criteria. We are presently assessing the potential use of LfhA in a vaccine for prevention of leptospirosis in humans and domestic animals. The usefulness of LfhA as a vaccinogen is strengthened by the possibility that antibodies which bind LfhA may also physically prevent factor H binding, thereby increasing the susceptibility of the bacterium to complement-mediated killing.

In conclusion, we characterized a mechanism involved in the serum resistance of pathogenic leptospires. Pathogenic leptospires bind factor H by means of LfhA, a membrane protein which is expressed during normal mammalian infection. An *lfhA* gene was detected only in pathogenic strains. These findings may be helpful in the development of advanced therapeutic and/or preventive strategies to interfere with immune evasion by pathogenic *Leptospira*.

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We dedicate this work to the memory of Faye Austin.

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