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

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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(H₂O), which allows binding of factor B, which in turn is cleaved to Ba and Bb. The C3(H₂O)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
the destructive effects of complement activation (35, 37, 62). Several bacterial pathogens of medical and veterinary importance, including Lancelotia group A/B streptococci (15, 44), Neisseria meningitidis and Neisseria gonorrhoeae (48), Borrelia burgdorferi (1, 2, 8, 21, 30–34, 38, 56) and Borrelia henselii (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 biochemical 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 (FioCorp 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 kennewickii (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 weltei (Sarmint), Leptospira inadai (Sarmin), and Leptospira biflexa was incubated with medium alone as controls. Intact L. biflexa and Copenhageni (Fiocruz L1-130) were generously gifts from field microscope.

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factor H-binding proteins BbCRASP-3 (ErpP), OsG, 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 antisera 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. welshii*, *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 submitted to GenBank with accession number DQ370178.

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**RESULTS**

Pathogenic leptospires bind host factor H. Pathogenic leptospires survive and multiply in the bloodstream 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, Copenageni, 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%.

**Southern blot analysis.** Genomic DNAs of *L. interrogans* serovars Pomona, Canicola, Grippotyphosa, Hardjo, and Bratislava and of *L. biflexa*, *L. welshii*, *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.

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**RESULTS**

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. 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.

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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 \( \downarrow \) 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 proteoliposomal cylinder-, periplasmic protein-, and outer membrane protein-
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 Leptomonas 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 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
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 *Leptosira* 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 *Leptosira* 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 *Leptosira*.

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We dedicate this work to the memory of Faye Austin.

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**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.


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