Immunoprotection of Recombinant Leptospiral Immunoglobulin-Like Protein A against *Leptospira interrogans* Serovar Pomona Infection

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We previously reported the cloning and characterization of leptospiral immunoglobulin-like proteins LigA and LigB of *Leptospira interrogans*. LigA and LigB are conserved at the amino-terminal region but are variable at the carboxyl-terminal region. Here, we evaluate the potential of recombinant LigA (rLigA) as a vaccine candidate against infection by *L. interrogans* serovar Pomona in a hamster model. rLigA was truncated into conserved (rLigAcon) and variable (rLigAvar) regions and expressed in *Escherichia coli* as a fusion protein with glutathione-S-transferase (rLigA). Golden Syrian hamsters were immunized at 3 and 6 weeks of age with rLigA (rLigAcon and rLigAvar) with aluminum hydroxide as an adjuvant. Hamsters given recombinant glutathione-S-transferase (rGST)–adjuvant and phosphate-buffered saline–adjuvant served as nonvaccinated controls. Three weeks after the last vaccination, all animals were challenged intraperitoneally with 10⁶ *L. interrogans* serovar Pomona bacteria (NVSL 1427-35-093002). All hamsters immunized with recombinant LigA survived after challenge and had no significant histopathological changes. In contrast, nonimmunized and rGST-immunized hamsters were subjected to lethal doses, and the hamsters that survived showed severe tubulointerstitial nephritis. All vaccinated animals showed a rise in antibody titers against rLigA. Results from this study indicate that rLigA is a potential vaccine candidate against *L. interrogans* serovar Pomona infection.

Leptospirosis is a serious worldwide zoonotic disease caused by infection with *Leptospira* spp., gram-negative spirochetes that comprise 24 serogroups and more than 250 serovars (3, 25). Infection of animals or people occurs through direct or indirect contact with contaminated urine or, less frequently, by exposure to infected animal tissues. An infected animal can remain asymptomatic and shed infectious organisms in the urine throughout its life (18). In most cases of human leptospirosis, patients develop an influenza-like illness, while diarrhea, vomiting, meningitis, or uveitis may occur in some cases (11, 12). In 5 to 15% of cases, severe multisystemic complications may develop, including renal failure, jaundice, and occasionally pulmonary failure (3). Recently, a case of acute respiratory failure with lethal pulmonary hemorrhage has been reported (5). In animals, leptospirosis infection is a frequent cause of kidney and liver failure (dogs), abortion, stillbirth, infertility (cattle, pigs, and horses), uveitis (horses), hemolytic anemia (sheep and cattle), and occasionally death (15–17, 37).

The worldwide distribution of this potentially fatal zoonotic infection and its association with autoimmune disease (12, 23, 35) provide the impetus to develop an effective and safe vaccine. Prevention of leptospirosis in dogs is accomplished to some extent by inoculation with bacterins that contain the most commonly encountered serovars. Although leptospiral bacterins may protect dogs from developing clinical signs of the disease, they are ineffective in preventing leptospiromia and renal shedding (2). In contrast, a monovalent leptospiral vaccine can prevent renal colonization and urinary shedding in cattle challenged with *Leptospira borgpetersenii* serovar Hardjo, but with minor interstitial nephritis (4). Immunity in vaccinated cattle is reportedly mediated by a type 1 (Th1) cell-mediated immune response to serovar Hardjo infection (8, 29, 30). Comparison of different bacterial extracts indicates that only the protein fraction of *L. interrogans* can provide cross-protection against heterologous challenge (18). Efforts to develop recombinant leptospiral vaccines have therefore focused on the outer membrane proteins of the spirochetes. Despite the identification of leptospiral antigens such as OmpL1, LipL41, LipL36, LipL32, and LipL21 (13, 14, 20, 21, 36), only a few attempts have been made to utilize these leptospiral antigens in a recombinant vaccine (22). Most studies exploring the pathogenicity of *Leptospira* have employed hamsters or guinea pigs as animal models because the mouse is not considered an ideal model (28). However, Lig protein has been reported to protect mice against *L. interrogans* serovar Manila infection (24). Recently, 3- or 6-week-old C3H/HeJ and C3H/SCID mice have been employed to study the lethality with *L. interrogans* serovar Copenhageni, where acclimatization and immunization including a booster require at least a 6- to 7-week period (31). However, *L. interrogans* serovar Pomona infection is not lethal to mice (1). Therefore, the hamster is an ideal alternative animal model for a leptospiral vaccine trial against *L. interrogans* serovar Pomona infection.

Leptospiriae survive both in the environment and inside the host. Thus, it seems likely that spirochetes adapt to diverse environments by selective gene expression (19). Therefore, identification of leptospiral antigens that are expressed only during infection may provide new strategies for construction of novel vaccines. We recently described two closely related outer
surface proteins, termed leptospiral immunoglobulin-like proteins LigA and LigB, from *L. interrogans* serovar Pomona that are upregulated during infection (33, 34). LigA and LigB are identical at the amino terminus but vary at the carboxyl terminus (33). These leptospiral immunoglobulin-like proteins are not present in the nonpathogenic *L. biflexa* serovar Patoc (33). The lack of lethality of leptospiral infection in hamsters by high-passage *L. interrogans* and *L. kirschneri* strain RMS2 and culture-attenuated strains is associated with loss of LigA and LigB expression (26, 27). Although these homologous proteins are expressed at very low levels in low-passage strains, expression could not be detected in rats immunized with a killed virulent strain and whole-cell preparations from the low-passage strain (26, 27). Therefore, most vaccines, which rely on whole-cell lysates or bacterins, likely do not contain the Lig proteins. In this study, we report that vaccination with recombinant LigA (rLigA) induced protective immunity against challenge in a hamster model. Thus, recombinant Lig protein is a promising candidate for an effective vaccine to prevent leptospirosis.

**MATERIALS AND METHODS**

**Bacterial strains, media, and plasmids.** *L. interrogans* serovar Pomona was isolated from naturally infected dogs (34). Leptospires were maintained on EMJH medium at 30°C (34). To isolate low-passage cultures of leptospires, hamsters were experimentally infected with a sublethal dose of *L. interrogans* serovar Pomona (NVSL 1427-35-093002). Infected hamster tissues were harvested aseptically and homogenized with sterile phosphate-buffered saline (PBS), and the lysates were inoculated into EMJH medium. Growth was monitored aseptically and homogenized with sterile phosphate-buffered saline (PBS), and the lysates were inoculated into EMJH medium. Growth was monitored using dark-field microscopy.

**Fifty-percent lethal dose (LD₅₀) value of *L. interrogans* in hamster.** Forty-eight 8- to 10-week-old Golden Syrian hamsters (Harlan Sprague-Dawley) were divided into six equal groups. Each group was infected intraperitoneally with 10⁶ to 10⁷ low-passage (three to four passages) *L. interrogans* serovar Pomona (NVSL 1427-35-093002). One group of hamsters received only PBS (control). The animals were monitored daily. Tissues were collected aseptically from animals that died due to infection and subjected to culture and histopathological analysis.

**Cloning, expression, and purification of LigA as a GST fusion protein.** Attempts to express the entire open reading frame of LigA (with/without signal sequences) led to toxicity in *Escherichia coli*, resulting in weak expression. Therefore, LigA was expressed as conserved and variable regions of LigA. Cloning and expression of LigA was carried out as previously described (33, 34). Briefly, LigA was truncated into conserved (Con) (the N-terminal 599 amino acids without the signal sequences) and variable (VarA) (the C-terminal 599 amino acids) regions. The regions were amplified using PCR with the following primers: ligConF, GTCTCGAGAGGTGGCTCCGTTTTAAT; ligConR, CCCTCGAGAATATCCGTAATAGA; VariAF, CCCCCGGGCTTACCGTTCC; and VariAR, CCCTCGAGCCGGGGTCAATATCCGGTG. The amplified PCR products were cloned into the pCR2.1 vector (Invitrogen, CA). The amplified PCR products were transformed into BL21(DE3), and expression was induced by adding isopropyl-β-D-thiogalactopyranoside (IPTG) to a final concentration of 0.5 mM at 30°C for 4 weeks. Growth was monitored using dark-field microscopy.

**Preparation of anti-rLigA antiserum in rabbits.** Sera from hamsters given rLigA, rGST, and bacterins were collected aseptically from each animal, homogenized in 0.5 ml EMJH medium, transferred into 20 ml EMJH medium, and maintained at 30°C for 4 weeks. Growth was monitored using dark-field microscopy.

**Histopathology.** Hamster tissues were collected and fixed in 10% neutral buffered formalin. The fixed tissues were sectioned at 5 μm, stained with hematoxylin and eosin, and examined by light microscopy. The renal lesions for hamsters were graded on a scale with 0 as normal, 1 as mild, 2 as moderate, and 3 as severe. Statistical analysis was performed based on the scores for the overall experiments between control and rLigA-immunized animals by use of Statistix 7.0 software.

**KELA with recombinant antigens.** Sera from hamsters given rLigA, rGST, and PBS were evaluated for the presence of specific immunoglobulin G (IgG) by use of a *kinetic enzyme-linked* immunosorbent assay (KELA) with recombinant antigens (33). A checkerboard titration was followed to optimize the concentrations of reagents for the KELA as previously described (27, 29, 30). Briefly, the optimum concentrations of recombinant antigens (rLigAcon-GST, rLigAvar-GST, and rGST) were diluted in 0.1 M bicarbonate buffer, coated onto a 96-well microtiter plate (Nunc, Denmark), rocked for 1 h, and then incubated overnight at 4°C. The plates were washed three times with 0.1 M PBS containing 0.05% Tween 20 (PBST). Next, 100 μl of hamster serum (primary antibody) diluted 1:100 in PBST was added to each well and incubated for 1 h at 37°C in a humid chamber. The plates were washed three times as described above with PBST and incubated with 100 μl of a 1:1,000

FIG. 1. Expression and purification of conserved and variable regions of LigA. The conserved and variable regions of LigA were cloned into pGEX-4T-2 and expressed as GST fusion proteins as described in Materials and Methods. GST fusion protein was purified by affinity chromatography and subjected to SDS-PAGE followed by Coomassie blue staining. (A) Expression of the conserved region of LigA. (B) Expression of the variable region of LigA. Lane 1, *E. coli* with vector and pGEX-4T-2 only (control); lane 2, unduced *E. coli* with recombinant construct; lane 3, IPTG-induced *E. coli* with recombinant construct; lane 4, affinity chromatography-purified GST fusion proteins (~1.5 μg). M represents the molecular size marker in kilodaltons (Bio-Rad).
Expression and purification of LigA as truncated GST fusion proteins. We truncated LigA into conserved (rLigAcon) and variable (rLigAvar) regions and expressed them as GST fusion proteins in E. coli. The purified GST fusion proteins (rCon-GST and VarA-GST) appeared as a single band by SDS-PAGE analysis (Fig. 1A and B).

**LD₅₀ value for L. interrogans serovar Pomona in a hamster model.** A dose of 10⁸ leptospires was uniformly lethal, whereas five of eight hamsters that received a dose of 10⁷ leptospires survived. Thus, the LD₅₀ for *L. interrogans* serovar Pomona infection was approximately 10⁷.

**Antibody responses to recombinant antigens.** In order to assess whether hamsters given rLigA (rCon-GST and rVarA-GST), rGST, and PBS (control) developed IgG antibody response against the antigens, sera collected from the animals on days 0, 21, 42, and 71 (29 days after challenge) were tested by KELA using rCon-GST, rVarA-GST, and rGST. The results indicate that hamsters vaccinated with rLigA developed IgG antibodies to rCon and rVarA (Fig. 2A and B). Hamsters also developed antibodies to GST, which suggests that hamsters mounted an immune response to both the GST portion and the recombinant protein. Reactivity of rCon and rVarA was determined by subtracting GST activity. No detectable IgG response was found with the group of hamsters administered PBS-adjuvant.

After challenge with leptospires (day 79), sera collected from all hamsters contained IgG antibodies to rLigA. These results support previous observations that leptospires upregulate Lig expression only upon infection.

**rLigA confers protective immunity in a hamster model.** Control and rLigA-immunized hamsters were challenged using a single LD₅₀ inoculum. As shown in Table 1, 13 of 16, 4 of 5, and 4 of 7 challenged control animals survived in three separate experiments, respectively. By comparison, all animals immunized with rLigA survived after challenge, indicating the immunoprotective potential of LigA (Table 1). The difference between survival rates of rLigA-immunized and control animals for overall experiments was statistically significant (*P* = 0.01), indicating the immunoprotective effect of rLigA after challenge. The effectiveness of immunoprotection was further evaluated by histopathology and culture.

**Histopathological analysis.** Except for insignificant interstitial lymphocytic infiltrates in the kidneys of three hamsters, no changes were detected in any tissues obtained from hamsters infected with rLigA. There were no histopathological lesions in the rLigA-immunized animals, which is in contrast to results for control groups.

**RESULTS**

<table>
<thead>
<tr>
<th>Expt and inoculum</th>
<th>Total no. of animals</th>
<th>No. of animals that survived</th>
</tr>
</thead>
<tbody>
<tr>
<td>Expt 1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>rLigA</td>
<td>8</td>
<td>8*</td>
</tr>
<tr>
<td>rGST</td>
<td>8</td>
<td>7</td>
</tr>
<tr>
<td>PBS</td>
<td>8</td>
<td>6</td>
</tr>
<tr>
<td>Expt 2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>rLigA</td>
<td>5</td>
<td>5*</td>
</tr>
<tr>
<td>rGST</td>
<td>5</td>
<td>4</td>
</tr>
<tr>
<td>Expt 3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>rLigA</td>
<td>8</td>
<td>8*</td>
</tr>
<tr>
<td>rGST</td>
<td>7</td>
<td>4</td>
</tr>
</tbody>
</table>

* A chi-square test was performed with Statistix 7.0 software to determine if there was a statistically significant difference in mortality between the control and rLigA-immunized animals from all experiments. *P* (0.01) was found, indicating the protective efficacy of rLigA against challenge.

* A chi-square test was performed with Statistix 7.0 software to determine if there was a statistically significant difference in mortality between the control and rLigA-immunized animals from all experiments. *P* (0.01) was found, indicating the protective efficacy of rLigA against challenge.
immunized with rLigA (Fig. 3A and B). In contrast, chronic tubulointerstitial nephritis was found in hamsters given GST-adjuvant and PBS-adjuvant (Fig. 3C and D). Prominent interstitial infiltrates of lymphocytes were accompanied by a few heterophils and occasional macrophages mixed with lower numbers of plasma cells. Occasionally, tubules were mildly dilated and distended with hyaline material that contained rare sloughed epithelial cells. The lining epithelium was also moderately attenuated. Rarely, desquamated epithelial cells and heterophils mixed with hyaline material formed casts within the lumina of proximal convoluted tubules. In some cases, prominent hyaline casts were accompanied by widespread moderate interstitial fibrosis. Glomerular changes were generally mild and considered to be secondary to the tubulointerstitial nephritis. Occasional glomeruli had shrunken glomerular tufts, while others had patchy increases in the amount of mesangial matrix and mild hypertrophy of the parietal epithelium lining Bowman’s capsules. Bowman’s capsules of some glomeruli in regions of severe tubular atrophy and fibrosis were dilated, and the uriniferous spaces were filled with a faintly eosinophilic lacy material. The renal lesions of hamsters were graded, and statistical analysis of results for control and rLigA-immunized animals was performed. The difference in graded renal lesions between control and rLigA-immunized animals was statistically significant, indicating the protective efficacy of rLigA (Table 2).

Leptospira culture from tissues. Kidney, liver, urinary bladder, lungs, and spleen were evaluated for the presence of leptospires by culture of tissues from both immunized and control groups. Except for the kidneys from two rLigA-immunized animals from the third experiment, all tissues from rLigA-immunized hamsters were negative. In contrast, all tissue samples collected from the control groups were culture positive.

Passive-immunization assay. Although the hamsters that received 300 μl of rabbit serum (group D) to LigA survived after challenge, all had severe kidney lesions, similar to results for control groups, as revealed by histopathological analysis. The
results indicate that passive immunization with rabbit serum failed to provide protection against leptospirosis infection (Table 3).

**DISCUSSION**

The main focus of our research is to identify leptospiral antigens that can be used for the development of more-effective vaccines for leptospirosis. Since outer membrane and surface proteins of bacteria mediate the primary interaction with the host, efforts to develop recombinant vaccines based on these proteins hold great promise. The outer membrane proteins of *Leptospira* are of special interest because *Leptospira* survives outside (contaminated water or soil) as well as inside the host and expression of some of these proteins is regulated by temperature (32). Currently available vaccines provide only short-term immunity and afford little cross-protection against different serovars (6, 7, 38). Therefore, identification of leptospirosis antigens that are uniquely expressed during infection may not only help in the development of an ideal vaccine but also aid in studies about the pathogenesis of leptospirosis.

*E. coli* containing a plasmid with full-length *ligA* expressed rLigA only at very low levels because of its high toxicity (34). Attempts to express recombinant LigA in a PET system with a His tag fusion protein also failed (our unpublished data). Since GST represents one of the carrier systems for vaccination (33), we truncated Lig proteins and utilized the conserved regions of LigA and LigB as GST fusion proteins. Recently, the utility of recombinant LigA and LigB proteins for serodiagnosis was established (33, 34). Based on the NCBI database, Lig proteins (NCBI accession no. AF368236, AF219120, and AY327260) show homology to cell binding proteins such as invasin of *Yersinia* spp. (23% identity and 40% similarity) and invasin of *Clostridium* spp. (31% identity and 45% similarity). These Lig proteins contain an immunoglobulin-like domain as found in intimin of *E. coli* and invasin of *Yersinia* spp. (34). However, the role of Lig proteins in adhesion still needs to be characterized. Since the attachment of bacteria to host cells triggers various virulence genes, the use of surface proteins such as Lig proteins as vaccine candidates may prevent attachment and abrogate colonization.

In order to develop an effective vaccine, immunization with the recombinant LipL41 and OmpL1 provided only partial immunoprotection in a hamster model (22). Adenovirus-mediated vaccination with Hap1, a hemolysis-associated protein of *Leptospira* that shows 93% homology with LipL32, provides approximately 87% protection against challenge in a gerbil model (6, 7). However, recombinant Hap1 or LipL32 purified from *E. coli* failed to induce protective immunity (7). Therefore, rLigA is the first leptospirosis protein that confers protective immunity in a hamster model. Although there is variability in the lethality rates between experiments, the overall difference in survival rate between control and rLigA-immunized animals was statistically significant, indicating the protective efficacy of rLigA. Further, all of the surviving control animals showed severe tubulointerstitial nephritis, indicating the end stage of infection. In contrast, all of the rLigA-immunized animals showed no signs of lesion, which was also statistically significant, indicating the protective efficacy of recombinant LigA. We demonstrated earlier that sera collected from animals given commercial vaccines have no reactivity to Lig proteins (33). Since Lig proteins are not present in culture-attenuated strains and high-passage cultures of *Leptospira* (26), it may be important to include the Lig proteins in the vaccine preparation. Hamsters and guinea pigs are good models for studying leptospirosis, but susceptibility to infection depends on age and weight of the animal. Koizumi and Watanabe indicated that *ligA* from *L. interrogans* Pomona (97% and 68% similarity with conserved and variable regions of LigA, respectively), and the recombinant Lig proteins have been shown to confer protective immunity in a mouse model (24, 34). Since McBride et al. indicated that the mouse is not an ideal model for leptospirosis (28), we studied the protective immunity of Lig proteins in a hamster model. Further, a number of different serovars of *Leptospira* are pathogenic in hamsters and, therefore, cross-protective efficacy can be studied with the hamster model. The immunogenicity of Lig proteins and their effective use in diagnosis of canine and equine leptospirosis is evident (33, 34). This confirms that Lig proteins are expressed or upregulated during infection. We previously showed that a single recombinant outer surface protein, OspA of *Borrelia burgdorferi*, protects dogs and horses against infection (9, 10). However, the immunoprotective potential of rLigA in domestic animals or humans needs to be studied.

Passive protection with rLigA antiseraum failed to provide hamsters with complete protection against leptospirosis infection. Although it has recently been reported that a monovalent leptospiral vaccine could induce a type 1 protective immune response (30), the immunological mechanism responsible for protective immunity in hamsters immunized with rLigA is not known. Since leptospires are considered invasive and not facultative intracellular pathogens (39), cell-mediated immunity may play an important role in protection against leptospirosis infection. The present study indicates that vaccination with rLigA in a hamster model not only prevented fatalities but also

### Table 2. Immunoprotective potential of rLigA based on histopathological analysis

<table>
<thead>
<tr>
<th>Score</th>
<th>No. of control animals</th>
<th>No. of rLigA-immunized animals</th>
</tr>
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<tbody>
<tr>
<td>0</td>
<td>0</td>
<td>17</td>
</tr>
<tr>
<td>1</td>
<td>0</td>
<td>3</td>
</tr>
<tr>
<td>2</td>
<td>4</td>
<td>0</td>
</tr>
<tr>
<td>3</td>
<td>17</td>
<td>0</td>
</tr>
</tbody>
</table>

* The renal lesions in hamsters were graded on a scale of severity, with 0 as normal, 1 as mild, 2 as moderate, and 3 as severe. Statistical analysis of results for control and rLigA-immunized animals was performed using the Wilcoxon rank sum test with Statistix 7.0 software. The difference between overall results for control and rLigA-immunized animals was statistically significant (*P* < 0.001), indicating the protective efficacy of rLigA.

### Table 3. Passive-protection study with polyclonal antibodies to rLigA

<table>
<thead>
<tr>
<th>Group</th>
<th>Amt of anti-rLigA (µl)</th>
<th>Total no. of animals</th>
<th>No. of animals that survived*</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>50</td>
<td>3</td>
<td>1</td>
</tr>
<tr>
<td>B</td>
<td>100</td>
<td>3</td>
<td>1</td>
</tr>
<tr>
<td>C</td>
<td>200</td>
<td>3</td>
<td>2</td>
</tr>
<tr>
<td>D</td>
<td>300</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>Control</td>
<td>300</td>
<td>3</td>
<td>2</td>
</tr>
</tbody>
</table>

* All animals that survived had severe histopathological lesions, as seen with the control animals.
prevented histopathological lesions in the kidney and prevented growth of the organisms significantly in vivo. Thus, recombinant LigA represents a strong candidate for an effective expanded-spectrum vaccine to prevent leptospirosis. Although LD₅₀ is generally used to evaluate leptospirosis vaccine efficacy, our results clearly demonstrate severe renal disease in the control group survivors. Thus, it is crucial to include renal histopathology when evaluating vaccine efficacy against leptospiral challenge. Further studies to understand the mechanism of immunoprotection and/or cross-protection of rLigA against various serovars are in progress.

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