A Prime-Boost Strategy Using the Novel Vaccine Candidate, LemA, Protects Hamsters against Leptospirosis

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Toward developing an effective vaccine capable of conferring heterologous protection, the putative lipoprotein LemA, which presents an M3 epitope similar to that of Listeria, was evaluated as a vaccine candidate in the hamster model of leptospirosis. LemA is conserved (>70% pairwise identity) among the pathogenic Leptospira spp., indicating its potential in stimulating a cross-protective immune response. Using different vaccination strategies, including prime-boost, DNA vaccine, and a subunit preparation, recombinant LemA conferred different levels of protection in hamsters. Significant protection against mortality was observed for the prime-boost and the DNA vaccine strategies, which showed 87.5% (P < 0.01) and 62.5% (P < 0.05) efficacy, respectively. Although the subunit vaccine preparation protected 50.0% of immunized hamsters, the level of protection was not significant. None of the hamsters in the control groups survived challenge with a virulent strain of Leptospira interrogans serogroup Icterohaemorrhagiae. Characterization of the immune response found that the strongest antibody response was stimulated by the subunit vaccine preparation, followed by the prime-boost strategy. The DNA vaccine failed to elicit an antibody response in immunized hamsters.

Leptospirosis is a widespread zoonosis caused by pathogenic Leptospira spp. that can infect almost all mammals (1–3), and it is an emerging public health problem, with an estimated 500,000 cases per year (4). Humans are generally infected through direct or indirect contact with the urine of infected animals, primarily rats in urban leptospirosis (1, 5). Symptoms range from a mild influenza-like illness to a severe infection, such as Weil’s disease or leptospirosis-associated pulmonary hemorrhage syndrome (LPHS), which has a case fatality rate of >50% (6, 7).

Currently available vaccines are based on inactivated whole bacteria or membrane preparations from pathogenic leptospires that require annual booster immunizations and do not confer cross-protective immunity (8). There are more than 270 pathogenic serovars of Leptospira spp., and this antigenic diversity has been attributed to the distribution and composition of the lipopolysaccharides (LPS) in the outer membrane (OM) (9), thereby precluding their use in multivalent vaccine preparations.

Several leptospiral vaccine candidates have been evaluated in animal models, as reviewed in reference 8. However, the majority of the subunit vaccines tested to date do not induce significant protection against mortality (10). Many of these antigens, e.g., OmpL1, Lpl41, LipL32, and the Lig proteins, are localized in the OM, are highly conserved among the pathogenic species (11, 12), and are important for the development of vaccines that can offer heterologous protection (10, 13). The OM contains a diverse range of proteins, including lipoproteins, predicted by genome-sequencing projects (14–17), and some are unique to the pathogenic serovars (14). Spirochete lipoproteins represent the most-abundant proteins in the OM (18). Furthermore, several lipoproteins, such as LipL41, LipL32, LIC10325, and LIC13059, have been shown to stimulate a protective immune response against leptospirosis, indicating their potential as vaccine candidates (19–21). The hypothetical lipoprotein LemA (LIC11058), which presents an M3 epitope similar to that of Listeria, contains a transmembrane helix with an extracellular amino terminus, and its subsequent modification releases an epitope that is presented on the surface of phagocytes (22). The biological function of this protein remains unknown.

DNA vaccines stimulate both humoral and cell-mediated immunity in different animal models, and this may be a promising strategy in the development of new vaccines against leptospirosis. DNA-type vaccine candidates have been evaluated in several leptospirosis vaccine studies (23–25), including prime-boost strategies (26). In a previous study, we reported that the LemA subunit vaccine preparation induced a strong humoral immune response in immunized mice and that it was recognized by antibodies present in sera of patients with severe leptospirosis (27). The aim of the present study was to determine, using prime-boost, DNA, and protein-based immunizations, whether LemA could induce a protective immune response in hamsters against challenge with a virulent strain of Leptospira interrogans. The current paper demonstrates that LemA is a good candidate to compose a vaccine for leptospirosis control.

MATERIALS AND METHODS

Bacterial strains and culture conditions. The L. interrogans strains were cultured in Ellinghausen-McCullough-Johnson-Harris (EMJH) liquid medium (Difco; BD, Franklin Lakes, NJ, USA) supplemented with Leptospira enrichment EMJH (Difco) at 30°C (28). The strains used in this study included L. interrogans serovars Autumnalis, Bataviae, Bratislava, Bratislava, Canicola, Djasiman, Hebdomadis, Icterohaemorrhagiae, Muenschen, and Pomona, Leptospira borgpetersenii serovars Ballum, Castellonis, Javanica, and Pomona, and Leptospira kirschneri Mini, Poi, and Sejroe. Leptospira kirschneri serovars Cynopteri and Grippotyphosa, and Leptospira santarosai serovar Pomona. The Escherichia coli

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TABLE 1 Protection conferred by immunization with different LemA vaccination strategies against lethal challenge in the hamster model

<table>
<thead>
<tr>
<th>Vaccine preparation</th>
<th>% Protection (no. of survivors/total no. challenged)</th>
<th>% Culture positive (no. positive/total no. tested)</th>
<th>% Positive for indicated lesion (no. with lesion/total no. examined)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Lung alveolar hemorrhage</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Kidney</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Cell degeneration and necrosis</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Lymphocyte infiltration</td>
</tr>
<tr>
<td>rLemA</td>
<td>50 (4/8)</td>
<td>0.08</td>
<td>100 (4/4)</td>
</tr>
<tr>
<td>pTARGET-lemA</td>
<td>62.5 (5/8)</td>
<td>0.03</td>
<td>100 (5/5)</td>
</tr>
<tr>
<td>pTARGET-lemA + rLemA</td>
<td>87.5 (7/8)</td>
<td>0.005</td>
<td>71.4 (5/7)</td>
</tr>
<tr>
<td>Bacterin</td>
<td>100 (6/6)</td>
<td>&lt;0.001</td>
<td>ND</td>
</tr>
<tr>
<td>Alhydrogel</td>
<td>0 (0/6)</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>pTARGET</td>
<td>0 (0/6)</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

a Two-tailed P value was determined by Fisher exact test in comparison to the result for the relevant control.
b Data are for immunized hamsters that were protected against lethal challenge.
c ND, not determined.

strains TOP10 and TOP10F (Invitrogen, São Paulo, Brazil) were grown at 37°C in Luria-Bertani (LB) medium (1% tryptone, 0.5% yeast extract, 0.5% NaCl, and 2% agar) with the addition of ampicillin to 100 μg ml⁻¹.

Presence and conservation of lemA in Leptospira spp. The presence of lemA among Leptospira spp. was confirmed by PCR amplification using genomic DNA from 17 serovars, including serovars of L. borgpetersenii, L. interrogans, L. kirschneri, and L. santarosai. The PCR product was amplified with the primers used to clone the LemA coding region, as described previously (27). The 16S gene was amplified using the primer pair fD1_F and rP2_R (29) to confirm the identity of the DNA template. The coding sequences were aligned with Geneious (Biomatters Ltd.), based on the ClustalW algorithm, with the default settings. Protein sequences were generated by translation of the coding sequences (CDS) and were aligned using the default settings.

Amplification, expression, and purification of recombinant LemA. The lemA coding sequence was amplified using oligonucleotides based on the lemA gene sequence from L. interrogans serovar Copenhageni strain Fiocruz L1-130 genome. Cloning into the vector pQE30 and the expression and purification of recombinant LemA (rLemA) protein were performed as previously described (27). The protein concentration was determined by using a bicinchoninic acid (BCA) protein assay kit (Pierce; Thermo Scientific, Rockford, IL, USA).

For construction of the DNA vaccine, lemA was amplified with the primer pair lemApTARGET_F (5′-ATGGATTTAAGAAGATGTA) and lemApTARGET_R (5′-GGAGAGCTTGTAACGTTGTA) and cloned into the mammalian expression vector pTARGET (Promega, Madison, WI, USA). The identity and orientation of the cloned inserts was confirmed by DNA sequencing. E. coli TOP10 competent cells were transformed with the pTARGET-lemA construct, and plasmid DNA was purified using the Prefectprep Plasmid Maxi kit (Eppendorf, Hamburg, Germany). The plasmid DNA concentration was determined using a Qubit fluorometer (Invitrogen, São Paulo, Brazil).

To confirm the expression of rLemA in the pTARGET-lemA construct, Vero cells were transfected with either the lemA construct or pTARGET (control), using the transfection reagent Lipofectamine (Invitrogen). Briefly, Vero cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 100 IU ml⁻¹ penicillin, 100 μg ml⁻¹ streptomycin, and 10% (vol/vol) heat-inactivated fetal bovine serum. When 60 to 75% confluence was reached, the cells were transfected with 15 μg of plasmid DNA precondensed with Lipofectamine in serum-free DMEM. Forty-eight hours after transfection, the cells were removed from the flasks, washed with phosphate-buffered saline (PBS), and suspended in DMEM. The transfected cells were suspended in lysis buffer (PBS containing 4% [vol/vol] NP-40) and incubated on ice for 30 min. The expression of rLemA by cell lysates was analyzed by Western blot (WB) assay using mouse anti-LemA serum (1:100 in PBS) (27). The reactions were developed with ECL WB detection reagents (GE Healthcare, São Paulo, Brazil).

Immunization and challenge of hamsters. The vaccine preparations used in this study were rLemA-Alhydrogel, pTARGET-lemA, pTARGET, PBS-Alhydrogel, and heat-killed whole leptospires (bacterin). Six or 8 female hamsters aged 4 to 6 weeks were used for protection experiments (Table 1). The various doses for the vaccination strategies were as follows: subunit, rLemA (2 × 100 μg); prime-boost, pTARGET-lemA (100 μg) plus rLemA (100 μg); DNA vaccine, pTARGET-lemA (2 × 100 μg); and bacterin, 10⁶ heat-killed whole leptospires. The second dose of each strategy was administered 21 days after the first. Blood samples were collected from the retroorbital venous plexus before each immunization and challenge, and the sera were stored at −20°C. Forty-two days after the first immunization, the hamsters were challenged intraperitoneally with a dose of 10 leptospires, equivalent to 5 times the 50% lethal dose (LD₅₀) (19, 31), of the L. interrogans serogroup Icterohaemorrhagiae strain Spool (32). Hamsters were monitored daily and were euthanized upon the appearance of clinical symptoms of leptospirosis. Animal experiments described in this study were carried out in strict accordance with the recommendations approved by the Ethics Committee in Animal Experimentation, Federal University of Pelotas, Brazil (permit number 2348). All surgery was performed under sodium pentobarbital anesthesia, and all efforts were made to minimize suffering.

Evaluation of the humoral immune response in hamsters. The antibody responses were monitored by indirect enzyme-linked immunosorbent assay (ELISA) using rLemA as previously described (27). Briefly, each well was coated with 50 ng of rLemA diluted in carbonate-bicarbonate buffer, pH 9.6. The ELISA plates were washed three times with PBST (PBS with 0.05% [vol/vol] Tween 20) and blocked. Hamster serum diluted 1:50 was added for 1 h at 37°C, and then the plates were washed 3 times with PBST. Peroxidase-conjugated anti-golden Syrian hamster IgG antibody (Rockland), 1:8,000 dilution, was added, the plates were incubated at 37°C for 1 h and washed 5 times with PBST, and the reaction was visualized with o-phenylenediamine dihydrochloride (Sigma-Aldrich) and hydrogen peroxide. The reaction was stopped by the addition of 0.1 M sulfuric acid, and absorbance was determined at 492 nm using a Multiskan MCC/340 ELISA plate reader (Titer tek Instruments, Huntsville, AL, USA).

Culture, histopathology, and imprint detection of leptospires. The presence of leptospires in the kidney and lung tissue samples was determined by culture, histopathology, and imprint in survivors. For histopathological studies, kidney and lung tissues samples were fixed in 10% formalin (pH 7.0) and embedded in paraffin. Six sections, 5 to 6 μm, were stained with hematoxylin and eosin and examined by a qualified pathologist for evidence of interstitial nephritis or pulmonary hemorrhage (19). Kidney samples were used to confirm sterilizing immunity by culture as described previously (28). The direct detection of leptospires in the kid-

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ays of hamsters was evaluated by the imprint method as previously described (33, 34).

Statistical analysis. The Fisher exact test and the Wilcoxon log-rank test were used to determine significant differences for protection against mortality and for survival, respectively, using Epi Info 6 (U.S. Centers for Disease Control) and Prism 5 (Graphpad, La Jolla, CA, USA) software packages. Variance analysis was used to determine significant differences between the assay results. The Student t test was used to determine significant differences in the serological assays. Differences were considered significant at a P value of ≤0.05.

RESULTS

Distribution and conservation of lemA among Leptospira spp. The PCR assay found that the lemA gene was present in L. interrogans (nine serovars), L. borgpetersenii (six serovars), L. kirschneri (two serovars), and L. santarosai (one serovar). Only L. interrogans serovar Hebdomadis was negative in the PCR assay. Furthermore, the genome sequences available in GenBank indicated that lemA is present in all Leptospira spp. sequenced to date. The overall pairwise identity of the DNA sequences was 70.8%, compared to 68.0% for the amino acid sequences. The L. interrogans lemA sequences were highly conserved, showing 99.9 and 100% pairwise identities for the DNA and protein sequences, respectively, and in a between-species comparison with the L. santarosai sequences, the pairwise identities were 93.4% and 93.5%, respectively. The Leptospira noguchii sequences reduced the mean pairwise identities to 90.6 and 96.0% for the DNA and amino acid sequences, respectively. Similarly, comparison to the Leptospira weilii sequences resulted in pairwise identities of 89.2 and 94.5%, respectively. Of note, the Leptospira biflexa genome contains a lemA ortholog, although the pairwise identities of the DNA and amino acid sequences with the pathogenic Leptospira spp. were 61.5 and 56.3%, respectively.

Subunit and DNA vaccine preparation. Even though rLemA was expressed as a truncated fragment (17 kDa) rather than as the full length of the native protein (22.6 kDa) and was engineered to avoid potential hydrophobic regions (27), the recombinant protein was insoluble when expressed in E. coli TOP10F'. The rLemA protein was recovered from the E. coli pellet, solubilized in 8 M urea, and purified by immobilized-metal affinity chromatography (IMAC), and the yield of rLemA was >40 mg liter⁻¹ (Fig. 1A). The expression of LemA in the pTARGET construct was confirmed by detection of rLemA, with the expected molecular mass of 17 kDa, in transfected Vero cells (Fig. 1B).

Humoral immune response in rLemA-immunized hamsters. To quantify the humoral antibody response, an ELISA was performed with the sera collected on days 0, 21, and 42 postimmunization (p.i.). Significant levels of circulating anti-rLemA antibodies were detected (P < 0.05) (Fig. 2). At 21 and 42 days p.i., there was a significant induction of IgG in hamsters immunized with the rLemA subunit preparation compared to that in the negative-control group (PBS-Alhydrogel) (P < 0.05). The IgG response in hamsters immunized using the prime-boost strategy differed significantly from that in the negative-control group on day 42 p.i. (P < 0.05). In the group immunized with the DNA vaccine, there were no detectable levels of IgG (Fig. 2).

Efficacy of the LemA vaccine preparations. The protective efficacy of the LemA vaccine preparations was evaluated. The prime-boost vaccination strategy significantly protected 87.5% of the hamsters against lethal challenge (P < 0.01), and 62.5% of hamsters immunized with the DNA vaccine survived (P < 0.05) (Fig. 3 and Table 1). The rLemA subunit vaccine preparation protected 50.0% of vaccinated animals (P = 0.08). Of note, among the hamsters immunized with the rLemA subunit preparation that died following challenge, death occurred significantly later than for animals in the control group (P < 0.05) (Fig. 3).

Histopathological analysis of the organs collected from hamsters 34 days postinfection revealed evidence for acute leptospirosis with pulmonary and kidney lesions (Table 1). Surviving animals immunized using the DNA (100%), prime-boost (71.4%), or subunit (30.0%) vaccine strategy were culture positive, indicating a lack of sterilizing immunity (Table 1), and the imprint evaluation confirmed these results (data not shown). In contrast, none of the hamsters immunized with bacterin vaccine showed evidence of the presence of leptospires (culture or imprint).

DISCUSSION

The identification of leptospiral OM proteins (OMPs) represents an important step in the discovery and development of novel vaccine candidates for leptospirosis. The sequencing and annotation of the bacterial genomes of pathogenic L. interrogans serovars Lai (16) and Copenhageni (15) and L. borgpetersenii (17) resulted in the identification of many putative OMPs. Further bioinformatics analysis of the L. interrogans serovar Copenhageni genome identified more than 200 proteins containing motifs suggesting localization to the OM (27). A major limitation in the field of leptospirosis has been the identification of membrane-associated proteins through conventional laboratory methods. However, the post-genomic age has made it possible to characterize new targets for vaccine candidates and diagnostic tests for leptospirosis. Recently, our group characterized the immunogenic and antigenic potential of eight putative OMPs from L. interrogans serovar Copenhageni strain Fiocruz L1-130 (27). Bioinformatics was used to identify genes predicted to encode OMPs. The recombinant proteins from seven of these genes induced significant IgG responses in mice, and they were recognized by sera collected from patients with severe leptospirosis. In the present study, we evaluated the im-
mune-protective potential against lethal challenge of the LemA recombinant protein in the hamster model.

Using the lemA sequences available in GenBank, sequence analysis demonstrated that lemA was conserved at the DNA and protein levels (70% overall pairwise identity) in L. interrogans, L. noguchii, L. santarosai, and L. weilii strains. An ortholog of lemA was also identified in the L. biflexa genome, albeit with less homology to the gene found in the pathogenic serovars. The absence of lemA in the Hebdomadis serovar is difficult to explain, as lemA is conserved in all serovars tested to date. It is possible that the PCR failed, possibly due to significant differences in the level of identity of lemA in the genome. The genome of L. interrogans serovar Hebdomadis is currently being sequenced at the J. Craig Venter Institute, and once it is available, this issue will be resolved.

In leptospirosis, a major effort has been to find a potent and effective immunotherapy. However, the available vaccines do not confer cross-protection against heterologous Leptospira serovars. The presence of the lemA gene in different serovars indicates that it has a potential role as a vaccine candidate. In Leptospira spp., LemA is a putative lipoprotein and has similarities with orthologous proteins described in other bacteria. This protein belongs to a family that is predicted to contain a transmembrane helix with an extracellular amino-terminal region. However, the biological function of LemA and its involvement in the pathogenesis of leptospirosis is unknown. At the time of writing, only one leptospiral virulence factor, Loa22, has fulfilled Koch’s molecular postulates (35). Experimentally, only a few proteins have been shown to be present on the leptospiral surface (36), and therefore, our knowledge of the superficial layer of leptospires remains limited.

Immune strategies using LemA (subunit, DNA vaccine, and prime-boost) were evaluated using the hamster model of lethal leptospirosis. The subunit vaccine preparation included Alhydrogel as an adjuvant. This is regularly used in commercial animal vaccines and is approved for use in human vaccines (37). Animals immunized with the subunit vaccine demonstrated the highest IgG response; however, only 50.0% of hamsters survived the challenge. The subunit vaccine had a significant impact on survival time (Fig. 3). There were no significant differences observed between the surviving and dead hamsters except on day 21 p.i., when the IgG response was found to be higher among the susceptible animals. Even though the hamsters immunized using the prime-boost strategy produced significantly smaller amounts of IgG, this significantly protected the hamsters (87.5%). Of note, the susceptibility of the only animal that did not survive the challenge dose could not have been predicted from the IgG response observed. Furthermore, while there was no significant IgG response in the hamsters immunized using the DNA vaccine, a significant number were protected against mortality (62.5%). Several studies have shown the effectiveness of DNA vaccines and reported that protection was associated with a strong antibody response (23–25).
This is contrary to the results presented in the current study that suggest the involvement of a cellular immune response in protection, although this is mere speculation since cytokine levels were not determined. However, there are reports in the literature that support the involvement of a cellular immune response in protection against leptospirosis (38–41).

Bacterin vaccines have been shown to induce sterilizing immunity in previous studies (42). In our study, none of the rLemA vaccine strategies except the bacterin induced 100% sterilizing immunity, as there was evidence of lesions in the lungs and kidneys among all of the surviving hamsters. In hamsters immunized using the prime-boost or subunit vaccine strategy, 28.5 and 50.0% immunity, as there was evidence of lesions in the lungs and kidneys.

This study reports on the successful evaluation of a novel vaccine candidate that offers significant protection against mortality in the hamster model of leptospirosis. Furthermore, due to the considerable level of conservation of LemA among Leptospira spp., it is a potential candidate for the development of a vaccine that could generate cross-protection against a wide range of Lep-
tospira serovars; however, this needs to be better evaluated in trials of heterologous challenge.

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