Identification of the Hemolysis-Associated Protein 1 as a Cross-Protective Immunogen of *Leptospira interrogans* by Adenovirus-Mediated Vaccination

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New vaccine strategies are needed for the prevention of leptospirosis, a widespread human and animal disease caused by pathogenic leptospires. Our previous work determined that a protein leptospiral extract conferred cross-protection in a gerbil model of leptospirosis. The 31- to 34-kDa protein fraction of *Leptospira interrogans* serovar autumnalis was shown sufficient for this purpose. In the present study, N-terminal sequencing of a 32-kDa fraction and Southern blotting of genomic DNA with corresponding degenerated oligonucleotide probes identified two of its constituents: hemolysis-associated protein 1 (Hap1) and the outer membrane *Leptospira* protein 1 (OmpL1). Adenovirus-mediated Hap1 vaccination induces significant protection against a virulent heterologous *Leptospira* challenge in gerbils, whereas a similar OmpL1 construct failed to protect the animals. These data indicate that Hap1 could be a good candidate for developing a new generation of vaccines able to induce broad protection against leptospirosis disease.

Leptospirosis is an infectious disease in both humans and animals, caused by spirochetes belonging to the genus *Leptospira*. Pathogenic leptospires were formerly classified as members of the species *Leptospira interrogans*, which was divided into numerous serogroups. Leptospirosis in mammals is transmitted by direct contact with infected animals or by exposure to water, moist soil, or vegetation contaminated by the urine of infected animals (9, 10, 26). Although leptospirosis has a worldwide distribution, it is most common in tropical and rural areas (9, 10, 21, 35). This acute febrile disease can progress to hepatic and renal dysfunction and hemorrhagic disorders that cause death in 5 to 10% of cases in humans (9, 10, 21) and are more lethal in dogs. In livestock, *Leptospira* infection is a frequent cause of abortion, stillbirth, infertility, milk drop syndrome, and (occasionally) death (4, 13, 23, 25, 31).

The medical and economic losses caused by such forms of the zoonotic disease justify the use of *Leptospira* vaccines in human or animal populations at risk. However, available vaccines confer only short-lasting immunity (6 to 12 months) and induce a lipopolysaccharide-directed immune response that is serogroup specific. As these bacteria provide no cross-protection between the different serogroups, several inactivated strains are incorporated into current vaccines to cover the spectrum of the most probable contaminants. However, these vaccines generally provide protection against the lethal onset of the disease but do not prevent persistent shedding of spirochetes from infected animals.

The identification of the common immunogenic proteins of *Leptospira* would be a major step toward the development of purer, better-defined, and probably more-efficient vaccines. Such vaccines would confer cross-protection against a wide range of pathogenic *Leptospira* strains. Our previous work showed that a whole proteic extract prepared from pathogenic *L. interrogans* serovar autumnalis provided significant cross-protection against a heterologous challenge, whereas no protective effect was induced by the same extract prepared from saprophytic *Leptospira biflexa* strain Patoc (32, 33). The purpose of the present study was to investigate the protective effect of proteic antigens common to the different serogroups of the pathogenic species *L. interrogans* but not identified or poorly expressed in the saprophytic species (11). We first studied the protective potency of the highly antigenic 31- to 34-kDa protein fraction recognized by analysis of sera from animals immunized with the pathogenic strains and identified the major proteins that constitute the 31- to 34-kDa fraction. Finally, the effects of both proteins found in the 32-kDa fraction were tested in laboratory animals vaccinated with recombinant adenoviruses.

MATERIALS AND METHODS

*Leptospira* strains. *L. interrogans* serovar autumnalis (strain 32) was isolated from the liver of a dead dog. This strain, which was selected based on the acute clinical effects observed in infected dogs, lost its virulence following several subcultures.

*L. interrogans* serogroup Icterohemorrhagiae (serovars copenhageni strain M20 and icterohaemorrhagiae strain RGA), *L. interrogans* serovar canicola (strain Hond Utrecht), and *L. biflexa* strain Patoc were kindly provided by the Pasteur Institute (Paris, France).

A virulent strain of *L. interrogans* serovar canicola was kindly provided by the Pasteur Institute (Paris, France) and was used for challenge. The virulence of the strain was maintained by regular passages (twice a year) into gerbils in the laboratory.

Leptospires were cultured in EMJH enriched medium at 29°C (7) and grown to 10⁸ leptospires/ml, as estimated by turbidimetry with a Hach apparatus cali-
brane or previously described (33). Typically, 100 turbidimetric units were equal-

Preparation of protein extracts from leptospires and amino acid sequencing. Leptospira serovar autumnalis proteins were separated by chloroform-methanol-water extraction, as previously described (1), and sodium dodecyl sulfate-poly-

Preparation of tissue cultures from mice. Three groups of gerbils received two intraperitoneal injections (with a 2-week interval between injections) of 400 μg of protein extract in ground sonicated acrylamide-bis acrylamide gel. For their welfare, animals received 20 mg of acetylsalicilic acid (Vetalgine ND)/kg of body weight at each immunization.

(iii) Recombinant adenosine-immunized animals. Blood sampling was done on days 0, 14, 21, 35, and 65, and specific serum antibodies were measured by ELISA. Briefly, 96-well microtitre plates (Nunc) were coated overnight at 4 °C with 4 μg of recombinant Hap1 (rHap1) or CsCl-purified Ads at 10^9 PFU per well. Plates were washed three times with TE-T (50 mM Tris [pH = 7.6], 50 mM EDTA, 0.05% Tween 20) and then blocked with TE-T containing 1% (wt/vol) gelatin at 37 °C for 30 min. One hundred microliters of diluted serum (1:50 to 1:2,000) was incubated at 37 °C for 30 min. After five washes with TE-T, Streptavidin-horseradish peroxidase conjugate (Amer-

RESULTS

Protection against heterologous challenge in animals vaccinated with 26- to 31- and 31- to 34-kDa protein fractions. By Western blot analysis, dog sera with leptospirosis have been shown to differentially react against proteins from pathogenic (L. interrogans) and nonpathogenic (L. biflexa) leptospira strains. In particular, those sera specifically react against frac-

 protects animals against a 10^6 LC50 challenge of the virulent strain of L. interrogans serovar canicola. Gerbil mortality was recorded daily after challenge, and surviving animals were sacrificed after 30 days for serum analysis by enzyme-linked immunosorbent assay (ELISA).

Serological analysis. (i) Protein fraction (26- to 31- and 31- to 34-kDa)-immunized animals. Blood samples were drawn on the day of the challenge and controlled by microscopic agglutination test (MAT) performed with serovar autumnalis, icterohaemorrhagiae, copenhageni, and canicola cultures. Western blot analysis was performed with total serovar autumnalis extract, as previously described (11).

(ii) Recombinant adenosine-immunized animals. Blood samples were drawn on days 0, 14, 21, 35, and 65, and specific serum antibodies were measured by ELISA. Briefly, 96-well microtitre plates (Nunc) were coated overnight at 4 °C with 4 μg of recombinant Hap1 (rHap1) or CsCl-purified Ads at 10^9 PFU per well. Plates were washed three times with TE-T (50 mM Tris [pH = 7.6], 50 mM EDTA, 0.05% Tween 20) and then blocked with TE-T containing 1% (wt/vol) gelatin at 37 °C for 30 min. One hundred microliters of diluted serum (1:50 to 1:2,000) was incubated at 37 °C for 30 min. After five washes with TE-T, Streptavidin-horseradish peroxidase conjugate (Amer-

Nucleotide sequence accession number. The nucleotide sequence of the hap1 gene from L. interrogans serovar autumnalis strain 32 has been deposited in GenBank database under accession no. AF566366.
tions of 26 to 31 kDa and 31 to 34 kDa from serovar autumnalis not found in L. biflexa. To further characterize the immunogenic properties of these fractions, an immunization assay was conducted with a gerbil model of leptospirosis with extraction products from serovar autumnalis fractions of 26 to 31 and 31 to 34 kDa. Two groups (n = 10) of animals were immunized with 26- to 31- or 31- to 34-kDa fractions, and a control group (n = 14) was immunized with a L. biflexa whole-protein extract. Animals were challenged with the pathogenic serovar canicola strain. Ten days after challenge, all animals in the L. biflexa control group had died, whereas 4 of 10 animals immunized with 31- to 34-kDa L. interrogans proteins and 2 of 10 immunized with 26- to 31-kDa L. interrogans proteins were still alive at the end of the trial. The survival rate for animals vaccinated with 31- to 34-kDa proteins was significant (Yates chi-square test; P < 0.05).

Serological controls by MAT and Western blot analyses showed that animals immunized with the 31- to 34-kDa fraction failed to produce agglutinating antibody but only antibodies recognizing two adjacent but well-defined bands in the Western blots. Serological control analyses of the sera of animals immunized with 26- to 31-kDa protein remained negative for MAT but exhibited a smear with Western blotting, indicating that the 26- to 31-kDa protein extract was still mildly contaminated by lipopolysaccharide.

Identification of the major components of the 32-kDa fraction. Preparative electrophoresis was performed only with the 31- to 34-kDa fraction in order to separate the major proteins found at 32 and 34 kDa. The purified 32- and 34-kDa fractions were then selected for amino acid sequencing. Peptide sequencing performed with the 32-kDa fraction identified two sequences: TFLPGVSVINYGYVK (peptide A) and TYAIVGFLQDLG (peptide B). According to the BLAST analysis, peptide A showed 93% identity to a part of Hap1 from L. interrogans serovar lai (GenBank accession no. AAB68646, aa 49 to 64). Peptide B was identical to a peptide present in OmpL1 from Leptospira kirschneri (GenBank accession no. AA74591, aa 26 to 39). To ensure that these peptides were not part of other unrelated proteins, degenerated oligonucleotide probes were designed (probes A and B, corresponding to peptide A and peptide B) to identify other potential genes coding for these peptides. The hybridization results for probes A and B are presented in Fig. 1. Hybridization with peptide A allowed detection of fragments at 0.9 to 1, 2.3, and >6 kb (we considered only the most intensive fragment), and hybridization with peptide B allowed detection of fragments at 0.8 to 0.9, 2.7 to 2.9, and >6 kb for genomic DNA digested, respectively, by DpnI, EcoRI, and BglII. The sizes of these fragments were concordant with those obtained from maps described for hap1 and ompL1. Therefore, it was concluded that the 32-kDa antigenic fraction contained at least two proteins: Hap1 and OmpL1. For technical reasons, amino acid sequencing of the 34-kDa fraction was unsuccessful.

Production and purification of rHap1. Upon E. coli production, rHap1 was detected in both bacteria and the culture medium. Western blot analysis of the bacterial lysate identified three bands between 28 and 32 kDa, whereas a single band at 31 kDa was detected in the culture medium. Affinity chromatography (Hi-Trap chelating column) allowed selective purification of one of the three proteins found in the bacterial lysate (Fig. 2). As this purified protein comigrated with the recombinant protein found in the culture medium, it was used as the antigen in ELISA.

Gerbil antibody response to adenovirus-mediated immunization. Recombinant adenovirus was chosen as an in vivo eukaryotic expression system. Two adenoviral constructs were generated, Ad-hap1 and Ad-ompL1. Both recombinant adenoviruses are designed as a replication-defective adenovirus expressing a Hap1 or OmpL1 open reading frame under the control of the IE gene promoter from human cytomegalovirus. An immunization assay was conducted using both recombinant adenoviruses Ad-hap1 and Ad-ompL1.

The humoral immune response of gerbils immunized with either Ad-hap1 or Ad-ompL1 or a combination of both recombinant adenoviruses was analyzed by IgG ELISA against the adenovirus vector itself (Fig. 3). After the first injection, antiadenovirus response increased rapidly, reaching a plateau that was boosted neither by a second injection nor after the challenge. Moreover, there was no significant decrease in response throughout the whole experiment.

The humoral response against Hap1 was determined by ELISA against recombinant rHap1 (Fig. 4). No antibody response against Hap1 was detected in the control after immunization. As expected with group 2 (Ad-ompL1), no significant antibody response against Hap1 was developed after immunization. In groups 1 (Ad-hap1) and 3 (Ad-hap1 plus Ad-ompL1), there was a significant increase in the antibody titer after the first injection, which was not boosted by the second immunization. Furthermore, heterologous challenge induced an immune response against Hap1 in the group control (Ad-null) and a strong increase in immune response in the surviving gerbils of the other groups. It is noteworthy that the highest boost effect of the response against Hap1 was obtained with group 1 (Ad-hap1).

Protective effect against challenge. Gerbils were challenged with L. interrogans serovar canicola 2 weeks after the last adenovirus injection, and mortality rates were recorded 30 days after the challenge. The experiments were performed twice, with controls receiving either PBS or Ad-null (Table 1). During the two trials, no significant difference was observed between controls receiving PBS and Ad-null (8 of 16 survivals in the first trial versus 9 of 17 in the second 30 days postchallenge). The consistency of results in the control groups led us to analyze the combined results of the two experiments. In the two trials, mortality rates were not significantly different between animals vaccinated with recombinant adenovirus expressing OmpL1 (Ad-ompL1) and controls (11 of 30 versus 17 of 33). In the first trial, 13 of 15 Ad-hap1-vaccinated animals survived versus 8 of 16 controls (exact chi-square unilateral test; P < 0.03). Similar results were observed in the second trial: 13 of 15 versus 9 of 17. The survival rate of gerbils vaccinated with Ad-hap1 was significantly higher than those of both controls (P < 0.01) and gerbils vaccinated with Ad-ompL1 (P < 0.001). Survival of animals vaccinated with both adenovirus-expressed proteins was 9 of 14 versus 8 of 16 in the control group for the first trial and 13 of 15 versus 9 of 17 for the second. This difference was only statistically significant for the second trial (P < 0.01). Analysis of the combined results from both experiments showed no significant protection with Ad-hap1 and Ad-ompL1 together 30 days after challenge.
Statistical analysis of mortality incidence (log rank test) for the two trials is shown in Table 2. Animals immunized by the two proteins expressed by adenovirus, compared to control animals, showed a significant survival rate for the second trial \((P < 0.005)\) but not for the first. When these animals were compared with those immunized with Ad-ompL1, the difference was statistically significant for both experiments \((P < 0.02\) and \(P < 0.005)\). Moreover, when the results for the two experi-
iments were combined, this difference was significant compared to the control or the group immunized by Ad-ompL1 (P < 0.01 and P < 0.005). Table 1 shows that the group immunized by Ad-ompL1 had a lower rate of survival (5 of 15; 6 of 15), regardless of the experiment. Figure 5 indicates that death occurred earlier in this group than in the others. In terms of the rate of death, there was significant difference between this group and the control group over all the experiments.

**DISCUSSION**

Our previous work showed that leptospire protein extracts can induce cross-protection within pathogenic strains of *Leptospira* in the gerbil model (32, 33). The purpose of the present study was to provide a more precise definition of the proteins involved in this response, i.e., to identify those proteins common to pathogenic strains that would be most suitable as vaccines, providing cross-protection against several *Leptospira* serovars. Previous results obtained by Gitton et al. (11, 12) and Haake et al. and Zuerner et al. (15, 37) showed that a 31- to 34-kDa protein fraction of *L. interrogans* was highly antigenic, i.e., the sera of infected animals were strongly recognized regardless of the infecting serovar. Moreover, this fraction seemed to be conserved in *L. interrogans* (pathogenic) but not in *L. biflexa* (saprophytic) (11, 15). Thus, this fraction appears...
to contain one or more proteins that could be useful for vaccine development (32). This led us to isolate the 31- to 34-kDa protein fraction from serovar autumnalis allowed the identification of at least one of these proteins in protection remained to be demonstrated.

These proteins were first expressed in E. coli to use them as pure immunogens in protective trials and as soluble antigens for ELISAs. Expression of OmpL1 in E. coli gave results similar to those previously described by Haake et al. (14, 30). Three 28- to 32-kDa bands appeared when an antihistidine antibody or a polyclonal rabbit antisera against leptospire was used in Western blotting (data not shown). This recombinant protein could not be produced in large amounts because of its high toxicity in E. coli (30). Hap1 extracted from bacterial bodies and probed with the same sera gave three bands (28, 31, and 32 kDa), and this protein was also found as a single band (31 kDa) in the culture medium (data not shown). This result is not concordant with a previous report (16) of incomplete processing of Hap1 in E. coli, indicating that the recombinant protein was distributed equally between the membrane and the cytoplasm. No mention was made of its presence in culture medium. These data were interpreted as evidence of inefficient cleavage of the peptide signal in E. coli (16). Hap1 was described by Lee et al. (24) as a hemolysin secreted into the medium, which is in agreement with our results (data not shown).

As most potential cross-protective immunogens should have conserved sequences, the coding regions of these two proteins isolated from L. interrogans serovar autumnalis were sequenced and compared with the deduced amino acid sequences of counterparts isolated from other serovars. The Hap1 protein of L. interrogans serovar Iai (GenBank accession no. AAB68646) and that of serovar autumnalis (GenBank accession no. AF366366) were found to be identical. Comparison of the Hap1 protein of L. interrogans serovar Iai or serovar autumnalis with L. kirschneri serovar grippotyphosa (GenBank accession no. AAF60198) showed the same amino acid sequence except a prolin (Res-215), which generally plays a role in protein conformation. The partial sequence of the OmpL1 protein from L. interrogans serovar autumnalis was identical to that of L. kirschneri serovar grippotyphosa, except for 6 aa (not shown), 2 of which belonged to the same side chain group.

The direct protective effect of these common proteins (Hap1 and/or OmpL1) obtained from serovar autumnalis was then tested against lethal onset after a heterologous challenge. Due to the very low production yield of recombinant protein OmpL1 in E. coli, this antigen was not involved in vaccination trials. rHap1 produced in E. coli was tested in vaccination trials but showed no evidence of direct protection (data not shown).

### Table 1: Protective effect of immunization with Ad-hap1, Ad-ompL1, or a combination of both, followed by intraperitoneal heterologous challenge

<table>
<thead>
<tr>
<th>Treatment group</th>
<th>Surviving animals/group</th>
<th>Expt 1</th>
<th>Expt 2</th>
<th>Total (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ad-hap1</td>
<td>13/15</td>
<td>13/15</td>
<td>26/30</td>
<td>87%</td>
</tr>
<tr>
<td>Ad-ompL1</td>
<td>5/15</td>
<td>6/15</td>
<td>11/30</td>
<td>36.6%</td>
</tr>
<tr>
<td>Ad-hap1 + Ad-ompL1</td>
<td>9/14</td>
<td>13/15</td>
<td>22/29</td>
<td>75.8%</td>
</tr>
<tr>
<td>Ad-null</td>
<td>9/17</td>
<td>17/33</td>
<td></td>
<td>51%</td>
</tr>
<tr>
<td>PBS</td>
<td>8/16</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*Animals were vaccinated twice at 3-week intervals and challenged with L. interrogans serovar Canicola 2 weeks after the last vaccine injection.

*Values on the number of surviving animals 30 days after challenge divided by the number of animals challenged.

### Table 2: Statistical analysis of the surviving curves produced by the log rank test

<table>
<thead>
<tr>
<th>Group(s)</th>
<th>Expt 1</th>
<th>Expt 2</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ad-hap1/control</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ad-hap1/Ad-ompL1</td>
<td>6.64</td>
<td>&lt;5.10^-2</td>
<td></td>
</tr>
<tr>
<td>Ad-hap1 + Ad-ompL1/control</td>
<td>8.9</td>
<td>&lt;5.10^-3</td>
<td>9.22 &lt;5.10^-3</td>
</tr>
<tr>
<td>Ad-ompL1</td>
<td>1.34</td>
<td>NS</td>
<td>8.54  &lt;5.10^-3</td>
</tr>
<tr>
<td>Ad-hap1 + Ad-ompL1/Ad-ompL1</td>
<td>5.48</td>
<td>&lt;5.10^-2</td>
<td>8.62 &lt;5.10^-3</td>
</tr>
<tr>
<td>Ad-ompL1/control</td>
<td>4.59</td>
<td>&lt;5.10^-2</td>
<td>3.87  &lt;5.10^-3</td>
</tr>
</tbody>
</table>

*Statistical analysis was performed with the log rank test. Significance was determined by comparison to the control group or the Ad-ompL1-immunized group.

*NS, not significant.
Therefore, it would seem likely that the mode of production and/or extraction of this recombinant protein and/or its presentation to the immune system are unsuitable for the elicitation of an appropriate immune response. Replication-defective recombinant adenoviruses expressing Hap1 or OmpL1 were designed to facilitate direct gene transfer of these proteins into gerbils (20). Ad-hap1 induced significant protection against a challenge, whereas Ad-ompL1 induced no protection in immunized gerbils. The survival rate of gerbils vaccinated with Ad-hap1 was significantly higher than that of both controls and animals vaccinated with Ad-ompL1.

Haake et al. (17) found that the recombinant proteins OmpL1 and LipL41, alone or together, afforded no protection during virulent homologous challenge in the hamster model of leptospirosis, whereas significant protection was obtained when animals were immunized with the membrane-associated forms of these two proteins. This author deduced that they provide synergistic protection, although the way in which OmpL1 and LipL41 associate with membrane is an important determinant for immunoprotection. However, in our experiments, the group immunized by Ad-ompL1 afforded no significant protection compared to the control groups; moreover, the analysis of the combined experiments by the log rank test showed that the OmpL1 protein expressed by adenovirus had a negative effect on the two experiment groups versus the control group ($P < 0.05$). When taken together, the results for both experiments showed the most negative effect by Ad-ompL1 ($P < 0.01$). Therefore, it seemed of interest for us to test immunization with both Ad-hap1 and Ad-ompL1. Survival of animals immunized with both proteins expressed in adenovirus and that of the control group was statistically lower only in the second experiment ($P < 0.05$). But combining the data from both experiments improved the significance of the results from $P < 0.05$ to $P < 0.01$, indicating that the protective effect by Hap1 occurred throughout both experiments. This rate of survival, compared to that of the Ad-hap1 group alone, was not as significant. As the association of the two proteins had a negative effect when protection was significant with Ad-hap1 alone, it is likely that the OmpL1 protein expressed by adenovirus was responsible for this negative effect. However, it is unclear which mechanism or mechanisms facilitate the effect elicited by OmpL1.

As previously demonstrated for many genes (including those coding for proteins targeted to the cell nucleus), recombinant adenoviruses can elicit not only T-cytotoxic responses but also a strong antibody response against the foreign gene product (19). It is noteworthy that these eukaryotic vectors can be used against bacterial diseases, for which antibody response remains the main specific effector of immune response.

It has been suggested that hemolysins (28) play an important role in the virulence and pathogenesis of many bacteria and leptospires in particular (3, 29, 34). Moreover, Hap1 is only produced by pathogenic leptospires (16), which might reflect its role in virulence. The protective effect induced by Ad-hap1 immunization inhibits the virulent activity of the pathogenic strains. Moreover, this protein appears to be a powerful immunogen, as in the case of other bacteria for which hemolysins have been used as the main vaccine component (18). Follow-up studies are needed to define how the immunity resulting from Ad-hap1 immunization can be improved. Moreover, determination of the immunity mechanism and of the location of immunoprotective epitopes could make it possible to use other systems of immunization.

In conclusion, our results show that the cross-protective effect within pathogenic strains of *Leptospira* is shared by Hap1 protein mediated by an adenovirus vector. This finding should facilitate the design and development of new generations of vaccines.
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REFERENCES


