Identification of a novel antigen of pathogenic Leptospira spp. that reacted with convalescent mouse sera

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The virulence of leptospires isolated from human patients against C3H/HeJ mice was investigated. Infection with clinical isolates from patients with severe leptospirosis was lethal to C3H/HeJ mice, suggesting that C3H/HeJ mice are suitable as an acute lethal model of severe leptospirosis. Using this model, a novel antigen of pathogenic Leptospira spp. (named LAg42), which reacted with convalescent mouse sera, was identified. LAg42 is a 42 kDa inner-membrane protein and its immunogenic region is located in the C-terminal region. The gene for LAg42 is conserved among pathogenic leptospires but not among non-pathogenic leptospires, which suggests its involvement in virulence.

INTRODUCTION

Leptospirosis, caused by infection with pathogenic Leptospira spp., is an important zoonotic disease that is distributed worldwide. There are more than 230 serovars among the pathogenic leptospires (Faine et al., 1999); this diversity of serovars among leptospires is attributed to differences in LPS carbohydrate composition. Leptospiral LPS is a protective immunogen that is generally specific for each serovar (serogroup) (Faine et al., 1999). In contrast to LPS, however, protein extracts prepared from a pathogenic Leptospira isolate can induce protective immunity against challenge with a heterologous serovar in an experimental animal model (Sonrier et al., 2000). This result indicates that leptospiral proteins are potential candidates for a new vaccine that generates broad cross-protection between serovars. Identification of immunogenic proteins of leptospires has thus been attempted and some leptospiral proteins, such as LipL41, OmpL1 and Hap1 (also known as LipL32), are found to be protective immunogens that are conserved among pathogenic leptospires (Branger et al., 2001; Haake et al., 1999).

So far, laboratory guinea pigs and hamsters have usually been used as experimental animal models for leptospirosis. However, the high susceptibility of hamsters to highly virulent leptospire strains makes analyses difficult (Haake et al., 1999; this study). It has been reported that an inbred strain of mice (C3H/HeJ), when infected with leptospires, exhibited histopathological lung and kidney changes that were similar to severe human leptospirosis (Pereira et al., 1998). In this study, we aimed to confirm that C3H/HeJ mice are suitable as an acute lethal model of severe leptospirosis and to identify a novel antigen of pathogenic Leptospira spp.

METHODS

Animals. Female, specific pathogen-free C3H/HeJ mice and golden Syrian hamsters (SLC, Shizuoka, Japan) were used in this study. All animal experiments were approved by the animal research committee of the National Institute of Infectious Diseases (NIID).

Leptospira strains. Leptospira interrogans serovar manilae strain UP-MMC was isolated from the blood of a human patient with severe leptospirosis at the University of the Philippines in 1998 and was kindly provided by Dr Yanagihara. L. interrogans serovar icterohaemorrhagiae strain NID-7 was isolated from the blood of a human patient, whose symptoms included jaundice and acute renal failure, at NIID in 2001. L. interrogans serovar hebdomadis strain OP84 was isolated from the blood of a human patient with mild leptospirosis at Okinawa Prefectural Institute of Health and Environment, Japan, in 1999 and was kindly provided by Dr Nakamura. To maintain virulence, leptospires were inoculated into hamsters, recovered from kidney tissues of dead or moribund hamsters and stored at −80 °C. Leptospira strains mentioned above and other reference strains were cultivated in liquid modified Korthof’s medium with 10% rabbit serum. Each Leptospira strain that had been passaged fewer than three times was used for intraperitoneal (IP) inoculation of mice or hamsters. The number of leptospires was counted by using a Thoma counting chamber (depth, 0.01 mm) modified for dark-field microscopy. Nine-week-old mice and hamsters, in groups of five, were subjected to IP inoculation with a tenfold graded dose that ranged from 1 × 10^6–10^8 leptospires and with a 100-fold graded dose that...
Purification kit (Roche), digested with enzymes described above except that the extension time was reduced to 1 min.

Isolation of genes that encode leptospiiral protein antigens.

Genomic DNA was prepared from *L. interrogans* serovar manilae strain UP-MMC using the cetyltrimethylammonium bromide (CTAB) precipitation method (Ausubel et al., 1993). The DNA was digested with SacII, purified by sucrose gradient centrifugation and fragments that ranged from 2 to 6 kb were isolated and ligated to BamHI-digested ZAP Express vector (Stratagene). Escherichia coli XL-1 Blue MRF (Stratagene) was used as host strain to make a phage library. About $1 \times 10^8$ library clones were screened with the convalescent sera from mice that were still alive at 28 days after challenge and peroxidase-conjugated rabbit anti-mouse IgG were detected by using ECL Western blotting detection systems (Amersham Biosciences). A positive clone (IS44) was selected, plaque-purified and a plasmid (pPK-CMV; Stratagene) that contained insert DNA was autoexcised according to the supplier’s instructions. Sequencing was performed by the dye-deoxynucleotide chain-termination method, using the BigDye Terminator Cycle Sequencing Ready Reaction kit, version 2.0 (Applied Biosystems).

**Table 1. Virulence of *L. interrogans* strains UP-MMC and NID-7 against experimental animals**

<table>
<thead>
<tr>
<th>Strain</th>
<th>Host</th>
<th>No. animals</th>
<th>Survival rate†</th>
<th>Time to death (days)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>UP-MMC</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10⁴</td>
<td>Syrian hamsters</td>
<td>5</td>
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<td></td>
</tr>
<tr>
<td>10³</td>
<td></td>
<td>5</td>
<td>6, 6, 6, 6, 5</td>
<td></td>
</tr>
<tr>
<td>10²</td>
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<td>5</td>
<td>8, 8, 8, 8, 8</td>
<td></td>
</tr>
<tr>
<td>10¹</td>
<td></td>
<td>5</td>
<td>9, 9, 9, 9, 9</td>
<td></td>
</tr>
<tr>
<td>10⁰</td>
<td></td>
<td>5</td>
<td>3</td>
<td>4, 4, 4</td>
</tr>
<tr>
<td>NID-7</td>
<td></td>
<td>5</td>
<td></td>
<td>5</td>
</tr>
<tr>
<td>10⁴</td>
<td>CHJ/He mice</td>
<td>5</td>
<td>4, 4, 4, 4, 4</td>
<td></td>
</tr>
<tr>
<td>10³</td>
<td></td>
<td>3</td>
<td>9</td>
<td></td>
</tr>
<tr>
<td>10²</td>
<td></td>
<td>3</td>
<td></td>
<td>9, 9, 9, 9, 9</td>
</tr>
</tbody>
</table>

*Leptospires were inoculated into animals intraperitoneally.
†Number of animals that survived until day 28.

**RESULTS AND DISCUSSION**

Animal models for infection with *L. interrogans*

IP inoculation of *L. interrogans* serovar manilae strain UP-MMC into 9-week-old hamsters resulted in a high percentage of lethal infection (Table 1). Although 3 of 20 hamsters survived to day 28 after the challenge, the time before death decreased as the challenge dose increased. The 50 % lethal dose for strain UP-MMC given by IP inoculation in this experiment was <10 cells. Thus, hamsters are not susceptible to infection with a highly virulent *Leptospira* strain that it is difficult to analyse host immune responses against highly virulent *Leptospira*.
virulent *Leptospira* or the mechanism(s) of leptospiral pathogenesis. Thus, we tried to use mice as a model animal instead of hamsters. In 9-week-old C3H/HeJ mice, infection with $1 \times 10^8$ and $1 \times 10^7$ UP-MMC cells was 100 and 40% lethal, respectively (Table 1). The animals showed jaundice and subcutaneous haemorrhages; leptospires were recovered from liver and kidney tissues of dead animals. Another clinical isolate, *L. interrogans* NID-7, isolated from a patient with severe leptospirosis, also showed lethal infection in C3H/HeJ mice (Table 1). However, *L. interrogans* OP-84, a clinical isolate from a patient with mild leptospirosis, did not kill any C3H/HeJ mice, even when $1 \times 10^7$ organisms were inoculated into 6-week-old mice (data not shown). Results obtained here and in a previous study (Pereira et al., 1998) indicate that C3H/HeJ mice are suitable animals for an acute lethal model of severe leptospirosis caused by highly virulent strains. C3H/HeJ mice possess a defective mutation in the *tlr4* gene encoding Toll-like receptor 4, the inflammatory signalling receptor for LPS (Poltorak et al., 1998, Qureshi et al., 1999). C3H/HeJ mice are known to be highly resistant to LPS-induced shock but extremely susceptible to infection with the LPS-bearing pathogen *Salmonella typhimurium* (O’Brien et al., 1980). Leptospires produce an atypical LPS that differs from enterobacterial LPS in several biochemical, physiological and biological properties (de Souza & Koury, 1992). A recent report demonstrated that leptospiral LPS activated target cells through a TLR2-dependent mechanism (Werts et al., 2001). Therefore, susceptibility of C3H/HeJ mice to leptospiral infection may not be attributed to TLR4; further study is required.

**Isolation of the leptospiral gene that encodes the LAg42 protein**

A leptospiral expression library from genomic DNA of *L. interrogans* serovar manilae strain UP-MMC was constructed and screened with convalescent sera from C3H/HeJ mice, as described above. A single clone (IS44) reacted positively; its plasmid DNA was purified and transformed into *E. coli* JM109. Immunoblot analysis (Fig. 1) showed that clone IS44 expressed a 42 kDa protein that reacted with the convalescent sera. The LAg42 protein was also expressed in *E. coli* harboring clone IS44 or its derivatives was subjected to SDS-PAGE and Western blotting with the convalescent sera (Fig. 1a and b). Thus, the LAg42 gene encoded a protein of the same molecular mass (approx. 41 537 Da) is also consistent with the C-terminal of lacZ of the vector. Molecular masses of both the LAg42 and 44-3/LacZ fusion proteins were about 42 kDa. Therefore, in order to determine which protein reacted with the sera, GST/LAg42 and GST/44-3 fusion proteins were expressed in *E. coli* and probed with the convalescent sera. Both fusion proteins were expressed in *E. coli* but only the GST/LAg42 fusion protein reacted with the convalescent sera (Fig. 1a and b). Thus, the lag42 gene corresponds to the 42 kDa protein that reacted with the convalescent sera. The LAg42 protein was also expressed in *L. interrogans* UP-MMC, with the same molecular mass (Fig. 1c).

The nucleotide and deduced amino acid sequences of the lag42 gene are available as supplementary data in JMM Online.

![Fig. 1.](image)

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Novel antigen of pathogenic *Leptospira* spp.
LAG42 protein C-terminal is immunogenic

Next, we tried to determine the immunogenic region in LAG42. The deduced amino acid sequence of LAG42 indicates that a transmembrane region is located at amino acid positions 107–123. Thus, we constructed GST fusion proteins with the N-terminal (amino acid positions 1–95) and the C-terminal (amino acid positions 168–371) of LAG42 (see Methods) and performed immunoblot analysis. The result of the immunoblotting is shown in Fig. 2. The convalescent sera reacted with only the C-terminal of LAG42, which indicates that the C-terminal of LAG42 induces the production of antibodies in mice.

Distribution of the lag42 gene among leptospires

The presence of the lag42 gene among pathogenic and non-pathogenic leptospires was examined by PCR. A total of 18 Leptospira strains was evaluated. A PCR product of lag42 (0.89 kbp) was amplified from all pathogenic leptospires tested, but not from non-pathogenic Leptospira biflexa or Leptospira meyeri (Fig. 3). We also revealed the presence of the lag42 gene in L. interrogans serovars bratislava (Iez Bratislava), canicola (Hond Utrecht IV), pomona (Pomona) and hardjo (Hardjoprajitno) (data not shown). The absence of the lag42 gene in L. biflexa was proved by Southern blot analysis (data not shown).

The novel antigen LAG42 identified in this study does not appear to possess a signal sequence and has a transmembrane region, which suggests that this protein is an inner membrane protein with a molecular mass of 42 kDa. The C-terminal of LAG42 protein C-terminal is immunogenic (Fig. 2), but does not show significant homology with any sequences in GenBank, whereas BLAST searches revealed that the C-terminal of LAG42 has similarity with the N-terminal of hypothetical proteins found in various pathogens. PCR results show that the lag42 gene is conserved among pathogenic leptospires but not among non-pathogenic strains (Fig. 3). These results suggest that LAG42 may be involved in virulence and may also be a target for protective immune response. It has been demonstrated that whole protein extracts from a pathogenic Leptospira strain confer protection against homologous and heterogeneous challenge in an experimental animal model (Sonnier et al., 2000). Thus, the identification of protein antigens that are conserved among the pathogenic leptospires would lead to the development of new vaccines. OmpL1 and LipL41 have been shown to induce synergistic immunoprotection in hamsters (Branger et al., 2001). The LAG42 protein may be one of the candidates for such new vaccines; however, further studies are required to evaluate its relevance.

Conclusions

In conclusion, we found that clinical isolates from patients who showed manifestations of severe, but not mild, leptospirosis produced lethal infection in C3H/HeJ mice. Our results and a previous report (Pereira et al., 1998) indicate that C3H/HeJ mice can be used as an acute lethal animal model of severe leptospirosis. By using convalescent sera from this animal model, we identified a novel antigen of pathogenic leptospires, designated LAG42.
ACKNOWLEDGEMENTS

We are grateful to H. Kawabata for helpful suggestions throughout this study. We also thank Y. Yanagihara, G. Baranton and M. Nakamura for providing *Leptospira* strains. This work was supported by a grant from the Ministry of Health, Labour and Welfare (H12-Shinkou-29).

REFERENCES


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