Identification of a novel antigen of pathogenic *Leptospira* spp. that reacted with convalescent mice 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 mice 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 beyond 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 Philippines in 1998 and was kindly provided by Dr Yanagihara. *L. interrogans* serovar icterohaemorrhagiae strain NIID-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 at 30°C (Faine *et al.*, 1999).

Challenge experiments. 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

Abbreviations: IP, intraperitoneal; TLR, Toll-like receptor.

The GenBank/EMBL/DBJ accession number for the nucleotide sequence of the *lag42* gene from *L. interrogans* serovar manilae strain UP-MMC is AB091092.

ranged from 1×10^1 – 10^7 leptospire, respectively. Liver and kidney tissues were removed from dead animals, minced and inoculated with Korthof's medium to demonstrate infection with leptospire. Mice that were still alive at 28 days after challenge were killed and their sera were harvested for subsequent experiments. Liver and kidney tissues were removed and inoculated into medium for the isolation of leptospire.

Isolation of genes that encode leptospiral 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 partially digested with *Sau*3AI, purified by sucrose gradient centrifugation and fragments that ranged from 2 to 6 kbp were isolated and ligated to *Bam*HI-digested ZAP Express vector (Stratagene). *Escherichia coli* XL-1 Blue MRF' (Stratagene) was used as host strain to make a phage library. About 1×10^5 library clones were screened with the convalescent sera from mice that were still alive at 28 days after challenge and peroxidase-conjugated secondary antibody; positive clones were then detected using ECL Western blotting detection systems (Amersham Biosciences). A positive clone (IS44) was selected, plaque-purified and a plasmid (pBK-CMV; Stratagene) that contained insert DNA was auto-excised according to the supplier's instructions. Sequencing was performed by the dideoxynucleotide chain-termination method, using the BigDye Terminator Cycle Sequencing Ready Reaction kit, version 2.0 (Applied Biosystems).

SDS-PAGE and immunoblotting experiments. Samples were subjected to SDS-PAGE using the buffers described by Laemmli (1970) and Western blotting with the Tris/glycine/methanol buffer of Towbin *et al.* (1979). The blot was incubated in 900-fold-diluted convalescent mice sera or 10 000-fold-diluted anti-Lag42 serum and then detected as described above.

Expression and purification of recombinant Lag42 and 44-3/GST fusion proteins. The region of clone IS44 that encoded the mature part of the Lag42 protein was amplified with *Taq* DNA polymerase (Ex *Taq*, TaKaRa Biotech) by using the upstream primer Lag42-F (5'-GGCGCGGATCCGTCCCAGAAAGGAGAGAAACG-3') and the downstream primer Lag42-R (5'-GGCCGCTCGAGCTATTCTCCCAACTCCTTGATG-3'); the primers contained a *Bam*HI site and an *Xho*I site, respectively, near their 5' ends (underlined). After an initial 25 s denaturation step at 94 °C, the reaction mixture was subjected to 30 cycles of denaturation at 94 °C for 10 s, annealing at 50 °C for 30 s and extension at 72 °C for 2 min. DNA fragments corresponding to the N-terminal (Lag42N) and C-terminal (Lag42C) of the Lag42 protein were amplified by using the upstream primer Lag42-F with the downstream primer Lag42-NR (5'-GGCCGCTCGAGCGAAGCGGTAGGACGAGTCAG-3'), and the upstream primer Lag42-CF (5'-GGCGCGGATCCACTCCGGATAAAGGAGTCAG-3') with the downstream primer Lag42-R, respectively. A DNA fragment corresponding to the 44-3 protein was amplified by using the upstream primer 44-3F (5'-GGCGCGGATCCACACAGTCCATCATCAAGG-3') and the downstream primer T7 (5'-GTAATACGACTCACTATAGGGC-3'), located on pBK-CMV. Amplification conditions were identical to those described above except that the extension time was reduced to 1 min. The PCR products were purified with a High Pure PCR Product Purification kit (Roche), digested with *Bam*HI and *Xho*I (for Lag42, Lag42N and Lag42C) or *Bam*HI only (for 44-3) and cloned into the GST vector pGEX-6P-1 (Amersham Biosciences). To express GST fusion proteins, the plasmids of GST/Lag42, GST/Lag42N, GST/Lag42C and GST/44-3 fusion proteins were transformed into *E. coli* strain JM109. GST fusion proteins were expressed in *E. coli* JM109 clones that harboured each plasmid and purified using Glutathione Sepharose 4B (Amersham Biosciences), according to the supplier's instructions.

Production of anti-Lag42C serum. The Lag42C protein was cleaved from the GST/Lag42C fusion protein with PreScission protease (Amersham Biosciences) and purified according to the supplier's instructions. Antiserum against Lag42C was raised by intramuscular injection of 100 µg purified Lag42C into a male Japan white rabbit (SLC, Shizuoka, Japan) with complete Freund's adjuvant, and giving booster injections of the same amount of protein with incomplete Freund's adjuvant 2 and 4 weeks later.

PCR. For amplification of the *lag42* gene from various *Leptospira* strains, their genomic DNA was purified by using a High Pure PCR Template Purification kit (Roche). PCR was conducted with Ex *Taq* by using the upstream primer Lag42s-F (5'-ATCCCCAAGCGCCTCTCGAAG-3') and the downstream primer Lag42-R under the conditions of an initial denaturation step at 94 °C for 25 s, then 30 cycles of denaturation at 94 °C for 10 s, annealing at 50 °C for 30 s and extension at 72 °C for 1 min. Reference strains were obtained from Institut Pasteur, Paris, France (CZ 214 K, H 6, 1342 K, Veldrat Semarang 173, Celledoni and Jez Bratislava) and WHO, Geneva, Switzerland (Salinem, Veldrat Batavia 46, Castellon 3, Moskva V, 3522 C, Patoc I, CH 11, Hond Utrech IV, Pomona and Hardjoprajitno) and maintained at NIID. Other strains (Ictero No. 1, Akiyami A, Akiyami B, Akiyami C and Shibaura) had been isolated in Japan and maintained at NIID.

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 so susceptible to infection with a highly virulent *Leptospira* strain that it is difficult to analyse host immune responses against highly

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

Strain (dose*)	Host	No. animals	Survival rate†	Time to death (days)
UP-MMC				
10 ⁷	Syrian hamsters	5	1	5, 5, 5, 8
10 ⁵		5	0	6, 6, 6, 6, 7
10 ³		5	1	8, 8, 8, 8
10 ¹		5	1	9, 9, 9, 11
10 ⁸	C3H/HeJ mice	5	0	4, 4, 4, 4, 4
10 ⁷		5	3	5, 9
10 ⁶		5	5	—
NIID-7				
10 ⁸	C3H/HeJ mice	3	0	4, 4, 6
10 ⁷		3	0	9, 9, 10

*Leptospire were inoculated into animals intraperitoneally.

†Number of animals that survived until day 28.

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 1×10^8 and 1×10^7 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* NIID-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 1×10^8 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 gene encoding Toll-like receptor (TLR) 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 mice sera [Fig. 1(a and b), lane 1]. Sequence analysis of clone IS44 revealed that three open reading frames, genes 44-1, lag42 and 44-3, were encoded by the insert. 44-3 was fused to 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

Fig. 1. Analysis of clone IS44 by (a) SDS-PAGE and (b) immunoblotting. *E. coli* JM109 harbouring clone IS44 or its derivatives was cultured overnight at 37 °C; 100 µl was then transferred to 1 ml fresh medium and cultured for a further 1 h at 37 °C. Then, 0.1 mM IPTG (final concn) was added to the culture, which was further incubated for 3 h at 37 °C. *E. coli* cells were pelleted and suspended in 100 µl $1 \times$ SDS-PAGE sample buffer. Ten microlitres of the sample was subjected to SDS-PAGE and Western blotting with the convalescent mice sera. Lanes: 1, *E. coli* harbouring clone IS44; 2, *E. coli* harbouring the plasmid of GST/44-3, IPTG (-); 3, *E. coli* harbouring the plasmid of GST/44-3, IPTG (+); 4, *E. coli* harbouring the plasmid of GST/LAg42, IPTG (-); 5, *E. coli* harbouring the plasmid of GST/LAg42, IPTG (+). Arrowheads indicate bands of each recombinant protein. (c) About 5×10^8 cells of *L. interrogans* strain UP-MMC were suspended in 100 µl $1 \times$ SDS-PAGE sample buffer. Ten microlitres of the sample was subjected to SDS-PAGE and Western blotting with anti-LAg42C serum. Lanes: 1, SDS-PAGE; 2, immunoblotting. Asterisk indicates a non-specific band, which was detected with secondary antibody alone.

Online. We propose the TTG codon at positions 406–408, not the ATG codon at positions 562–564, as the translational start codon of LAg42, because a consensus –35 and –10 promoter element and a consensus ribosome-binding site were identified upstream of the predicted TTG start codon (see supplementary data in JMM Online). The predicted molecular mass (approx. 41 537 Da) is also consistent with LAg42 expressed in *L. interrogans* UP-MMC (Fig. 1c). BLAST searches revealed that the N-terminal of LAg42 showed homology with the N-terminal of various hypothetical proteins in many pathogens, while the C-terminal of LAg42 did not significantly match any sequences in GenBank (data not shown).

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 (Jez 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

Fig. 2. Reactivity of convalescent mice sera to the N- and C-terminals of the LAg42 protein. *E. coli* JM109 harbouring each plasmid was cultured overnight at 37 °C and 100 µl aliquots were transferred to 1 ml fresh medium and cultured for 1 h at 37 °C. Then, 0.1 mM IPTG (final concn) was added to the culture, which was further incubated for 3 h at 37 °C. *E. coli* cells were pelleted and suspended in 100 µl 1× SDS-PAGE sample buffer. Ten microlitres of each sample was subjected to (a) SDS-PAGE and (b) Western blotting with convalescent mice sera. Lanes: 1, *E. coli* harbouring clone IS44; 2, *E. coli* harbouring the plasmid of GST/LAg42N; 3, *E. coli* harbouring the plasmid of GST/LAg42C. Arrowheads indicate each recombinant protein.

Fig. 3. Presence of the *lag42* gene among various leptospiral strains by PCR. Samples (amplified as described in Methods) were run on 0.9% agarose gel. Lanes: 1, λ -EcoT14I digest; 2, *L. interrogans* serovar icterohaemorrhagiae (Ictero No. 1); 3, *L. interrogans* autumnalis (Akiyami A); 4, *L. interrogans* hebdomadis (Akiyami B); 5, *L. interrogans* australis (Akiyami C); 6, *L. interrogans* pyrogenes (Salinem); 7, *L. interrogans* copenhageni (Shibaura); 8, *Leptospira borgpetersenii* javanica (Veldrat Batavia 46); 9, *L. borgpetersenii* castellanis (Castellon 3); 10, *Leptospira kirschneri* grippotyphosa (Moskva V); 11, *L. kirschneri* cynopteri (3522 C); 12, *Leptospira noguchii* panama (CZ 214 K); 13, *Leptospira inadai* malaya (H 6); 14, *L. biflexa* patoc (Patoc I); 15, *L. biflexa* andaman (CH 11); 16, *Leptospira santarosai* shermani (1342 K); 17, *L. meyeri* semaranga (Veldrat Semarang 173); 18, *Leptospira weilii* celledoni (Celledoni); 19, *L. interrogans* manilae (UP-MMC).

LAg42 is immunogenic (Fig. 2), but does not show significant homology with any sequences in GenBank, whereas BLAST searches revealed that the N-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 heterologous challenge in an experimental animal model (Sonrier *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 (Haake *et al.*, 1999). Adenovirus-mediated vaccination of Hap1 (also known as LipL32) induced protection in gerbils (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.

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