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Immuno-Protective Studies with Recombinant OMPs Against *Leptospira Interrogans* Serovar Autumanalis and Australis Infection in Swiss Albino Mice

KEYWORDS: *Leptospira*, rOMPs, immuno-protection

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ABSTRACT *Leptospirosis, a zoonosis caused by Leptospira sp., is recognized as an emergent infectious disease. Due to the lack of adequate and rapid diagnostic tools, vaccines are an attractive intervention strategy. The recombinant proteins rOmpL1, rLipL41 and rLipL32 were tested for their possible use as vaccine candidate against leptospirosis. Immunizations with the recombinant proteins were able to produce a significant immune response in Swiss albino mice individually and in combinations. Antibody titers were determined by ELISA. Best immune response was observed when all the three recombinant proteins were given combined. Immunized animals were challenged with 100 or 1000 organisms of Leptospira interrogans serovar Autumanalis and Australis to check protective efficacy of these recombinant proteins. Blood and kidney samples were checked by culture and PCR for presence of Leptospira in challenged animals at 2, 5, 7, 10, 12 day intervals from zero day of infection. The animals were protected against Leptospira infection with two combinations of recombinant proteins viz. combination of rOMPL1, LipL41 and combination of rOMPL1, LipL41, LipL32. Blood and kidney samples from immunized infected animals became negative by culture and PCR within 5-7 days post infection. The three recombinant proteins taken in the study appears to promising vaccine candidates for development of indigenous vaccine.*

INTRODUCTION

Recombinant protein vaccines have a great potential against leptospirosis. Comparison of different bacterial extracts indicates that only the protein fraction of *L. interrogans* can provide cross-protection against heterologous challenge (Faine et al., 1999). 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 (Cullen et al.,2003; Cullen et al., 2005; Haake et al., 2000; Haake et al., 1998; Shang et al., 1995), only a few attempts have been made to utilize these leptospiral antigens in a recombinant vaccine (Haake et al., 1999).

OmpL1 is a transmembrane protein (Haake et al., 1993; Shang et al., 1995) and LipL41 is an outer membrane lipoprotein (Gordon 2002). Both proteins are surface-exposed. OmpL1 and LipL41 act synergistically to induce immunoprotection in the hamster model of leptospirosis, although neither of the individual proteins induces protective immunity (Haake et al., 1999). Patients with leptospirosis have antibodies against these proteins in their sera (Haake et al., 2000; Flannery et al., 2001). LipL32 is an outer membrane lipoprotein that is conserved, both genetically and immunologically, in the various pathogenic leptospires. LipL32 antigen induces antibodies in patients with leptospirosis. LipL32 stimulates the expression of both MCP-1 and iNOS mRNAs and augments the nuclear binding of NF-kB and AP-1 transcription factors in cultured mouse proximal tubule cells (Yang, 2002). The protective characteristics of several recombinant OMP vaccines have been tested, including leptospiral outer membrane protein OmpL1, lipoprotein LipL41 (Haake et al., 1999), hemolysis-associated protein 1 (Hap1) (Branger et al., 2001) and immunoglobulin-like (Lig) protein (Palaniappan et al., 2002). These studies indicate OMPL1, LipL41 and LipL32 are quite promising to be used as vaccine candidates.

Present study was undertaken to check immuno-protective potential of recombinant OMPs viz. rOMPL1, rLipL41 and rLipL32 in Swiss albino mice, for their possible use as candidate for indigenous vaccine development.

MATERIAL AND METHODS

Leptospira reference strains and their maintenance

Pathogenic serovars of *Leptospira* species, viz, *L. interrogans* serovars Australis, Autumnalis and saprophytic species *Leptospira biflexa* serovar Patoc were obtained from the National Leptospirosis Reference Center, Regional Medical Research Center (ICMR), Port Blair India. All the standard *Leptospira* serovars were maintained in Ellinghausen McCollugh Johnson Harris (EMJH) semi-solid media by regular sub-culturing after every 15 to 30 days at 28°C.

Leptospira culture preparation for Animal challenge experiments

L. interrogans serovars Australis, Autumnalis and *Leptospira biflexa* serovar Patoc were inoculated in Fletcher's liquid medium and were incubated for 15 days at 37°C. Medium containing leptospires were centrifuged at 12,000 rpm for 20 min at 4°C. Pellets were suspended in suitable amount of PBS to give desired number of organisms/ml.

Experimental Animals

Swiss albino male mice weighing 15-20 gm were used in the study. The animals were caged in group of 4-5 separately in Institutional animal facility (Registration No. 1296/c/09/CPCSEA) and were given mice food pellets and water ad libitum.

Immune-response against recombinant proteins in BALB/c mice

Eight groups of BALB/c mice (each with 5 mice) were taken to study immune response against previously produced three recombinant proteins viz. rOMPL1, rLipL41 and rLipL32 (Jain et al., 2013). Seven different groups were given subcutaneously with 50 µg of r-proteins in various combinations as mentioned in Table 1. Each mouse was given three doses, at 0 day, 14 day and 21 day. Eighth group of mice was taken as control. Blood was collected on 0, 7,

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14, 21, 28 day for separation of serum from each mice of all groups. Serum from mice belonging to particular group was pooled. Antibody titer in pooled serum was checked by ELISA.

Animal Challenge experiments

All seven immunized groups (each with 5 mice) of BALB/c mice (~4weeks of age) were challenged i.p. with 100 or 1000 *Leptospira interrogans* serovar Autumanalis and Australis organisms, two weeks after administration of the final immunization. Eighth group was control group. Blood and kidney tissue samples were collected on 3, 5, 7, 10, 12 day of challenge experiment to prepare DNA, to estimate leptospiral load by dark field microscopy and to inoculate EMJH semisolid medium for culture.

50 µl Blood and kidney tissue homogenate in 1:10 dilution were inoculated in EMJH semisolid media supplemented with 200 µg/ml of 5-fluorouracil and 10 percent pooled rabbit sera. The tubes were incubated at 28°C for 45 days. Presence of *Leptospira* organisms in medium was checked at every one week interval by observing wet mount slides under 100X Dark field Microscope.

Genomic DNA from blood sample and kidney tissue sample was prepared using DNA isolation kits from Genei, India as per instruction manual. The primers (Forward primer 5′ CGCTGGCGGCGCGTCTTAAA 3′; Reverse primer 3′ AAGGTCCACATCGCCACTT 5′) and conditions specific for amplification of 16S RNA from pathogenic Leptospira as describe by Hookey (1992) were used for PCR. Briefly, each 25 µl PCR reaction contained 2.5 mM $MgCl_2$, 200 M dNTPs, 50 mM KCl,10mM Tris-HCl, 0.3 unit of Taq DNA polymerase, 20 p moles of primers, and 100 ng of DNA. PCR cycles consisted of Denaturation at 94°C for 1 min, annealing at 54°C for 1 min and extension at 72°C for 2 min, for 35 cycles, followed by 10 min extension at 72°C. The amplified products were detected in 0.8 % agarose by gel electophoresis.

RESULTS AND DISCUSSION

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. In the present study three recombinant OMPs (rOMPL1, rLipL41 and rLipL32) were checked for their immuno-protective potential. Swiss albino mice where immunized with recombinant proteins individually and in various combination. Antibody titers in pooled sera collected was checked by ELISA. Rise in antibodies was observed after 3 doses rOMPs and 21 days of immunization (Figure 1). Best immune response was observed when all the three recombinant proteins were given combined. Flannery et al., 2001 also observed that patients with leptospirosis have antibodies against these proteins in their sera.

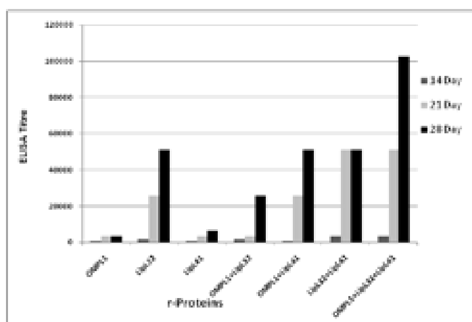

Figure 1: Titre observed in ELISA with r-proteins

Immunized Swiss albino mice were challenged i.p. with 100 or 1000 *Leptospira interrogans* serovar autumanalis and Australis organisms. Blood and kidney tissue samples were collected on 2, 5, 7, 10, 12 day of challenge experiment to perform PCR and to inoculate EMJH semisolid medium for culture. Culture and PCR results observed are mentioned in Table 1, 2, 3 and 4.

Table 1: PCR and culture results for Blood samples taken from mice challenged with *L.interogans* serovar Autumanalis

r-proteins	100 organisms								1000 organisms							
method	culture				PCR				culture				PCR			
Days	5	7	10	12	5	7	10	12	5	7	10	12	5	7	10	12
Control	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
rLipL32	+	+	-	-	+	+	+	-	+	+	+	-	+	+	+	-
rOmpL1	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
rLipL41	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
rOmpL1+rLipL41	+	-	-	-	+	-	-	-	+	-	-	-	+	-	-	-
rOmpL1+rLipL32	+	+	-	-	+	+	+	-	+	+	+	-	+	+	+	-
rLipL32+rLipL41	+	+	-	-	+	+	-	-	+	+	-	-	+	+	-	-
rOmpL1+rLipL41 +rLipL32	-	-	-	-	+	-	-	-	-	-	-	-	+	-	-	-

Table 2: PCR and culture results for Kidney tissue samples taken from mice challenged with *L.interogans* serovar Autumanalis

r-proteins	100 organisms								1000 organisms							
method	culture				PCR				culture				PCR			
Days	5	7	10	12	5	7	10	12	5	7	10	12	5	7	10	12
Control	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
rLipL32	+	+	-	-	+	+	+	-	+	+	+	-	+	+	+	-
rOmpL1	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
rLipL41	+	+	+	-	+	+	+	-	+	+	+	-	+	+	+	+
rOmpL1+rLipL41	+	-	-	-	+	-	-	-	+	-	-	-	+	-	-	-
rOmpL1+rLipL32	+	+	+	-	+	+	+	-	+	+	+	+	+	+	+	+
rLipL32+rLipL41	+	+	+	-	+	+	+	-	+	+	+	-	+	+	+	-
rOmpL1+rLipL41 +rLipL32	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-

Table 3: PCR and culture results for Blood samples taken from mice challenged with *L.interogans* serovar Australis

r-proteins method	100 organisms								1000 organisms							
	culture				PCR				culture				PCR			
Days	5	7	10	12	5	7	10	12	5	7	10	12	5	7	10	12
Control	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
rLipL32	+	+	-	-	+	+	+	-	+	+	+	-	+	+	+	-
rOmpL1	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
rLipL41	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
rOmpL1+rLipL41	-	-	-	-	+	-	-	-	+	-	-	-	+	-	-	-
rOmpL1+rLipL32	+	+	+	-	+	+	+	-	+	+	+	+	+	+	+	+
rLipL32+rLipL41	+	+	-	-	+	+	-	-	+	+	+	-	+	+	+	-
rOmpL1+rLipL41 +rLipL32	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

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Table 4: PCR and culture results for kidney samples taken from mice challenged with *L.interogans* serovar Australis

r-proteins method	100 organisms								1000 organisms							
	culture				PCR				culture				PCR			
Days	5	7	10	12	5	7	10	12	5	7	10	12	5	7	10	12
Control	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
rLipL32	+	+	-	-	+	+	+	-	+	+	+	-	+	+	+	-
rOmpL1	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
rLipL41	+	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+
rOmpL1+rLipL41	-	-	-	-	-	-	-	-	+	-	-	-	+	-	-	-
rOmpL1+rLipL32	+	+	+	-	+	+	+	-	+	+	+	+	+	+	+	+
rLipL32+rLipL41	+	+	-	-	+	+	-	-	+	+	+	-	+	+	+	-
rOmpL1+rLipL41 +rLipL32	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

Results indicated that rLip41 and rOMPL1 could not provide significant protection as organisms got cleared from blood or other tissues only 12 days of experimental infection in mice immunized with these r-proteins. However, LipL32 was seen give better protection as organisms got cleared within 10 days after infection as seen by both culture and PCR. LipL32 is an outer membrane lipoprotein that is conserved, both genetically and immunologically, in the various pathogenic leptospires. The lipL32/hap-1 gene derived from *L.interrogans* Serovar Autumnalis conferred protective immunity against a challenge with a heterologous strain of *L.interrogans* Serovar Canicola (Branger et al., 2001). LipL41 and OMPL1 gave no protection in hamster model individually (Haake et al., 1999). The animals were protected against *Leptospira* infection with two combinations of recombinant proteins viz. combination of rOMPL1, LipL41 and combination of rOMPL1, rLipL41, rLipL32. Blood and kidney samples from immunized infected animals became negative by culture and PCR within 5-7 days post infection. However, when all the three recombinant proteins given together gave higher immune response as observed in ELISA (Figure 1). Haake et al., 1999 also observed that OmpL1 and LipL41 act synergistically to induce immuneprotection in the hamster model of leptospirosis, although neither of the individual proteins induces protective immunity. *Leptospira* count for samples collected after just 2days from animals immunized with all three recombinant proteins had less than 10 leptospires/ml blood by dark field microscopy. No leptospires were observed on/after 5days of infection.

CONCLUSION

The three recombinant proteins rOMPL1, rLipL41, rLipL32 in combination appears to promising vaccine candidates for development of indigenous vaccine to provide protection against *Leptospira interrogans* serovar autumanalis and Australis organisms in human and animals.

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REFERENCE

Artiushin, S., Timoney, J.F., Nally, J., Verma, A. 2004 Host-inducible immunogenic sphingomyelinase-like protein, Lk73.5, of Leptospira interrogans. Infect. Immun. 72:742-749. | Brown, J.A., LeFebvre, R.B., Pan, M.J. 1991, Protein and antigen profiles of prevalent serovars of Leptospira interrogans. Infect. Immun. 59:1772-1777. | Branger, C.. Sonrier, C., Chatrenet, B., Klonjkowski, B., Ruvoen-Clouet, N., Aubert, A, Andre-Fontaine, G., Eloit, M. 2001. Identification of the hemolysis- associated protein 1 as a cross-protective immunogen of Leptospira interrogans by adenovirus-mediated vaccination. Infect. Immun., 69 : 6831-6838. | Cullen, P. A., D. A. Haake, D. M. Bulach, R. L. Zuerner, and B. Adler. 2003. LipL21 is a novel surface-exposed lipoprotein of pathogenic Leptospira species. Infect. Immun. 71:2414–2421. | Cullen, P. A., X. Xu, J. Matsunaga, Y. Sanchez, A. I. Ko, D. A. Haake, and B. Adler. 2005. Surfaceome of Leptospira spp. Infect. Immun. 73:4853–4863. | Dai, B., Chen, Z., Yan, H., Zhao, H., Li, S. 1997, PCR amplification, molecular cloning, DNA sequence analysis and immuno/protection in BALB/C mice of the 33 kDa endoflagellar protein of L. interrorgans serovar lai. Chin. Med. Sci. J. 12:15-21. | Faine, S., B. Adher, C. Bloin, and P. Perolat. 1999. Leptospira and leptospirosis, 2nd ed. MedSci, Melbourne, Australia. | Flannery, B., Costa, D., Carvalho, F. P., Guerreiro, H.; Matsunaga, J., Da Silva, E. D. 2001. Evaluation of recombinant Leptospira antigen-based enzyme-linked immunosorbent assays for the serodiagnosis of leptospirosis. J. Clin. Microbiol. 39: 3303-3310. | | Haake, D. A., C. Martinich, T. A. Summers, E. S. Shang, J. D. Pruetz, A. M. McCoy, M. K. Mazel, and C. A. Bolin. 1998. Characterization of leptospiral outer membrane lipoprotein LipL36: downregulation associated with latelog- phase growth and mammalian infection. Infect. Immun. 66:1579–1587 | Haake, D. A., G. Chao, R. L. Zuerner, J. K. Barnett, D. Barnett, M. Mazel, J. Matsunaga, P. N. Levett, and C. A. Bolin. 2000. The leptospiral major outer membrane protein LipL32 is a lipoprotein expressed during mammalian infection. Infect. Immun. 68:2276–2285. | Haake, D. A., M. K. Mazel, A. M. McCoy, F. Milward, G. Chao, J. Matsunaga, and E. A. Wagar. 1999. Leptospiral outer membrane proteins OmpL1 and LipL41 exhibit synergistic immunoprotection. Infect. Immun. 67:6572–6582. | Hookey JV. Detection of Leptospiraceae by amplification of 16S ribosomal DNA. FEMS Microbiol Lett 1992; 90 : 267-274 . | Jain, R., Katare, N. and Shrotri, C.K. 2013. Cloning and expression of Leptospira introgans serovar Australis ompl1, lipl32 and lipl41 outer membrane protein genes in Escherichia coli and immunoreactivity of recombinant proteins. National Journal of Life Science. 10(2) : 257-261 | Palaniappan, R.U., McDonough, S.P., Divers, T.J., Chen, C.S., Pan, M.J., Matsumoto, M., Chang, Y.F. 2006 Immunoprotection of recombinant leptospiral immunoglobulin-like protein A against Leptospira interrogans serovar Pomona infection. Infect. Immun. 74:1745-1750. | Shang, E. S., M. M. Exner, T. A. Summers, C. Martinich, C. I. Champion, R. E. Hancock, and D. A. Haake. 1995. The rare outer membrane protein, OmpL1, of pathogenic Leptospira species is a heat-modifiable porin. Infect. Immun. 63:3174–3181. | Shang, E.S., Exner, M.M., Summers, T.A., Martinich, C., Champion, C.I., Hancock, R.E., Haake, D.A. 1995, The rare outer membrane protein, OmpL1, of pathogenic Leptospira species is a heat-modifiable porin. Infect. Immun. 63:3174-3181. | Silva, E.F., Medeiros, M.A., McBride, A.J., Matsunaga, J., Esteves, G.S., Ramos, J.G., Santos, C.S., Croda, J., Homma, A., Dellagostin, O.A., Haake, D.A., Reis, M.G., Ko, A.I. 2007 The terminal portion of leptospiral immunoglobulin- like protein LigA confers protective immunity against lethal infection in the hamster model of leptospirosis. Vaccine. 25:6277-6286. |