Vaccine 29 (2011) 3413–3418

Contents lists available at ScienceDirect

Vaccine

journal homepage: www.elsevier.com/locate/vaccine

Recombinant LipL32 and LigA from *Leptospira* are unable to stimulate protective immunity against leptospirosis in the hamster model

Deanna S. Deveson Lucas[a,d,1], Paul A. Cullen[a,1], Miranda Lo[a], Amporn Srikram[b], Rasana W. Sermswan[c], Ben Adler[a,d,*]

[a] *Department of Microbiology, Monash University, VIC 3800, Australia*
[b] *Melioidosis Research Center, Faculty of Medicine, Khon Kaen University, Khon Kaen 40002, Thailand*
[c] *Department of Biochemistry, Faculty of Medicine, Khon Kaen University, Khon Kaen 40002, Thailand*
[d] *Australian Research Council Centre of Excellence in Structural and Functional Microbial Genomics, Department of Microbiology, Monash University, VIC 3800, Australia*

ARTICLE INFO

Article history:
Received 2 November 2010
Received in revised form 28 January 2011
Accepted 24 February 2011
Available online 9 March 2011

Keywords:
LipL32
LigA
Leptospira spp.
Immunity

ABSTRACT

The major antigenic component of pathogenic *Leptospira* spp. is lipopolysaccharide (LPS). However, due to the specificity of the immune response generated towards LPS and the diversity in leptospiral LPS carbohydrate structure, current commercial vaccines stimulate protection only against homologous or closely related serovars. Vaccines that confer heterologous protection would enhance protection in vaccinated animals and reduce transmission to humans. Several studies have investigated the potential of various leptospiral outer membrane proteins to stimulate protective immunity against pathogenic *Leptospira* species. These include the surface-exposed lipoproteins LipL32 and LigA. However, consistent protection from infection has proved difficult to reproduce. In this study we assessed the protective capacity of recombinant LipL32, the six carboxy-terminal unique Ig-like repeat domains of LigA (LigANI) and a LipL32–LigANI fusion protein in hamsters against infection with *Leptospira interrogans* serovar Manilae. Despite all of the proteins eliciting antibody responses, none of the hamsters was protected against infection.

© 2011 Elsevier Ltd. All rights reserved.

1. Introduction

Leptospirosis is an emerging global zoonosis, resulting in a systemic infection with high mortality rates in tropical and developing countries. *Leptospira* is a member of the Spirochaetacae family, a group of helical, gram-negative organisms. Over 230 serovars of pathogenic *Leptospira* have currently been identified. The lipopolysaccharide (LPS) of *Leptospira* is the principle antigen to which agglutinating, opsonic antibodies bind [1]. Antigenically related serovars of *Leptospira* are grouped into serogroups depending on the similarity of the carbohydrate component of the LPS of each strain [2]. Due to the specificity of the immune response generated towards LPS and the diversity in leptospiral LPS carbohydrate structure, current commercial vaccines stimulate protection only against homologous or closely related serovars [3,4]. Vaccines that confer heterologous protection would enhance protection in vaccinated animals and reduce transmission to humans. However, this goal has so far eluded researchers.

Several potential vaccine candidates have been identified. Many of these target antigens lie in the leptospiral outer membrane (OM). The OM of pathogenic *Leptospira* spp. contains a number of components including LPS, lipoproteins (including LipL32, LipL21 and LipL41 [5]), the leptospiral immunoglobulin-like proteins LigA and B, porins such as OmpL1 [6], and phospholipids. The OM proteins are highly conserved across the pathogenic species [7]. Therefore, these proteins have been the focus for development of novel vaccines for heterologous protection.

LipL32 is the major OM protein of pathogenic *Leptospira* spp. [8]. It is expressed *in vivo* and is highly immunogenic; over 95% of patients with leptospirosis produced antibodies against LipL32 [9]. Due to its antigenic properties, LipL32 has been a major candidate antigen in a number of vaccine trials and has been shown to confer partial protection against infection in some animal models [7,10], while other trials have shown no protection (reviewed in [11]). LipL32 binds to extracellular matrix (ECM) proteins [12–14]. However, recent studies have shown that a *LipL32* mutant retains ECM-binding capabilities *in vitro* and virulence in hamsters [15], suggesting redundancy in ECM-binding capability. The antibody dominant epitopes of LipL32 have been identified [13,16] and

* Corresponding author at: Department of Microbiology, Monash University, VIC 3800, Australia. Tel.: +61 3 9902 9177; fax: +61 3 9902 9222.
E-mail address: Ben.Adler@monash.edu (B. Adler).
[1] These authors contributed equally to this work.

0264-410X/$ – see front matter © 2011 Elsevier Ltd. All rights reserved.
doi:10.1016/j.vaccine.2011.02.084

Table 1
Primer sequences used in this study.

Primer name	Sequence 5′–3′	Serovar
LipL32 5′ for gateway plasmid	GGGG ACA AGT TTG AAA AAA GCA GGC TCT GGT GCT TTC GGT GGT CTG CCA AGC	Manilae
LipL32 3′ for gateway plasmid	GGG GAC CGC TTT GTA CAA GAA AGC TGG GTT CTT AGT CGC GTC AGA AGC AGC TTT	Manilae
Inverse PCR primers for LipL32Δ155–200 Primer 1	GTA AGA GGT CTT TAC AGA	Hardjo
Inverse PCR primers for LipL32Δ155–200 Primer 2	TTT GGC GAT TTG GTC AGG	Hardjo
Inverse PCR primers for LipL32Δ155–200 Internal Primer	GTA AGA GGT CTT TAC AGA ATT TCT	Hardjo
5′ LigANI for gateway plasmid	GGGG ACA AGT TTG AAA AAA GCA GGC TCT AAT ACG AAT ATT CTT AGC GTT TCC	Manilae
3′ LigANI for gateway plasmid	GGG GAC CGC TTT GTA CAA GAA AGC TGG GTT TGG CTC CGT TTT AAT AAGA GGC TAA	Manilae
5′LigANI EcoRI for LipL32Δ155–200–LigANI fusion protein	GCA GAA ATT CTC TAA TAC GAA TAT TCT TAG CGT TTC CAA C	Manilae
3′LipL32EcoRI for LipL32Δ155–200–LigANI fusion protein	TGC GAA TTC CTT AGT CGC GTC AGA AGC AGC TTT	Hardjo

have been shown to comprise two regions spanning amino acids 155–177 and 181–204 [16]. Hauk et al. [13] constructed three truncations of mature LipL32: N-terminal truncation (amino acids 21–92), an intermediate protein fragment (amino acids 93–184) and a C-terminal truncation (amino acids 185–272). The recombinant proteins were screened using serum samples from patients with confirmed leptospirosis. Both the intermediate and C-terminal truncations were recognised by the serum [13]. An additional study identified the specific amino acids in the immunodominant region through the screening of a synthetic peptide library against serum samples from leptospirosis patients [16]. Therefore, in the present study a truncated form of LipL32 (LipL32 Δ155–200) was included to determine the immunogenicity of LipL32 when the immunodominant epitopes are absent.

An alternative protein vaccine candidate is the leptospiral immunoglobulin-like protein A (LigA). LigA is a 130 kDa surface exposed lipoprotein expressed during infection and is highly immunogenic [17]. LigA is not expressed under normal *in vitro* growth conditions and requires the medium to be adjusted to physiological osmolarity to induce expression [17,18]. LigA binds ECM proteins and fibrinogen [19], and therefore may be involved in cellular adhesion and colonization. The six carboxy-terminal unique Ig-like repeat domains of the LigA (LigANI) may contribute to tissue specificity during infection [20]. Vaccination with LigANI was shown to protect hamsters from leptospirosis [21].

To assess the protective capacity of LigANI and LipL32 in the hamster model, we cloned and expressed variations of recombinant LigANI and LipL32 in *Escherichia coli*. These recombinant proteins included PBS-soluble mature LipL32 (minus the signal peptide), truncated LipL32 (LipL32 Δ155–200), the six C-terminal repeats of LigA (LigANI) based on the findings of Silva et al. [21] and a hybrid protein comprising truncated LipL32 (LipL32 Δ155–200) fused with LigANI. Immunity to leptospirosis is predominantly antibody mediated in a number of species, including humans and hamsters [11], therefore Alhydrogel was selected as the adjuvant for this study, as it stimulates a strong antibody response [22]. These proteins were then assessed for their capacity to protect hamsters from infection with *Leptospira*.

2. Methods

2.1. Bacterial strains and growth conditions

Leptospira interrogans serovar Manilae (L495), *L. interrogans* serovar Copenhageni (Fio Cruz L1-130), and *Leptospira borgpetersenii* serovar Hardjo subtype Hardjobovis (L550) were grown in EMJH medium [23] at 30 °C to a density of 4×10^8/mL. To induce LigA expression in serovar Copenhageni, NaCl was added to a final concentration of 0.15 M then all cultures were incubated at 30 °C for an additional 20 h before harvest. *E. coli* BL-21Codon Plus (Stratagene) was grown at 37 °C in Luria–Bertani medium supplemented with 100 μg/μL ampicillin (LB Amp^{100}).

2.2. Expression and purification of recombinant proteins

The target genes were amplified from leptospiral genomic DNA by PCR using Taq DNA polymerase. The PCR products were then cloned into the gateway cloning vector pDonrZeo (Invitrogen). PCR products for LipL32 and LigA were amplified from *L. interrogans* serovar Manilae (L495). The sequence identiy of *L. interrogans* serovar Manilae (L495) and *L. interrogans* serovar Copenhageni (Fio Cruz L1-130) LigANI is 97%. The PCR product for LipL32 Δ155–200 was amplified from *L. borgpetersenii*. The sequence identity between *L. interrogans* and *L. borgpetersenii* LipL32 is 98%. Primer sequences are shown in Table 1. All plasmids were sequenced to confirm orientation and absence of PCR-derived infidelities.

Plasmids were introduced by transformation using the heat shock method into *E. coli* BL21-CodonPlus cells made competent with calcium chloride [24]. To induce expression of recombinant proteins, cells were grown overnight in LB Amp^{100} at 37 °C. For large scale production of protein, 500 mL fresh LB Amp^{100} were inoculated with 10 mL of overnight culture and incubated for 90 min at 37 °C with shaking at 150 rpm. 1 mM IPTG (final concentration) was then added and the cultures were incubated for a further 4 h (at 37 °C with shaking at 150 rpm). Cultures were harvested by centrifugation 10,000 × *g* for 15 min and the pellets stored at −20 °C.

The *E. coli* cell pellets were resuspended in 10 mL of lysis buffer (100 mM NaH_2PO_4, 10 mM Tris–HCl, 8 M urea, pH 8) and sonicated on ice four times for 30 s. The resultant whole cell lysate (WCL) was centrifuged at 10,000 × *g* for 15 min at room temperature and the supernatant added to 2 mL of the Ni-NTA Superflow™ resin slurry (1 mL bed volume) (Qiagen); samples were agitated at room temperature for 1 h. After incubation, the contents were transferred to a 10 mL column, allowing the resin to settle prior to collection. The resin was washed three times with wash buffer (100 mM NaH_2PO_4, 10 mM Tris–HCl, 8 M urea, pH 6.3); proteins were then eluted using elution buffer (100 mM NaH_2PO_4, 10 mM Tris–HCl, 8 M urea, pH 4.5).

2.3. PBS dialysis of recombinant LipL32 for solubility

Purified recombinant LipL32 was dialysed against PBS (2 mL sample to 4L PBS) at room temperature for 12 h, after which the buffer was refreshed and samples were left stirring at room temperature for an additional 4 h. The sample was then removed from the dialysis tubing and centrifuged for 15 min at 14,000 × *g* at room temperature. Protein concentration was measured using a Nanodrop ND-1000 UV-Vis spectrophotometer (Nanodrop Technologies, DE, USA). Proteins or WCL were analyzed by SDS–PAGE using a 12.4% Tris-glycine gel (200 V, 55 min) and visualised by Coomassie Blue (Biorad) staining.

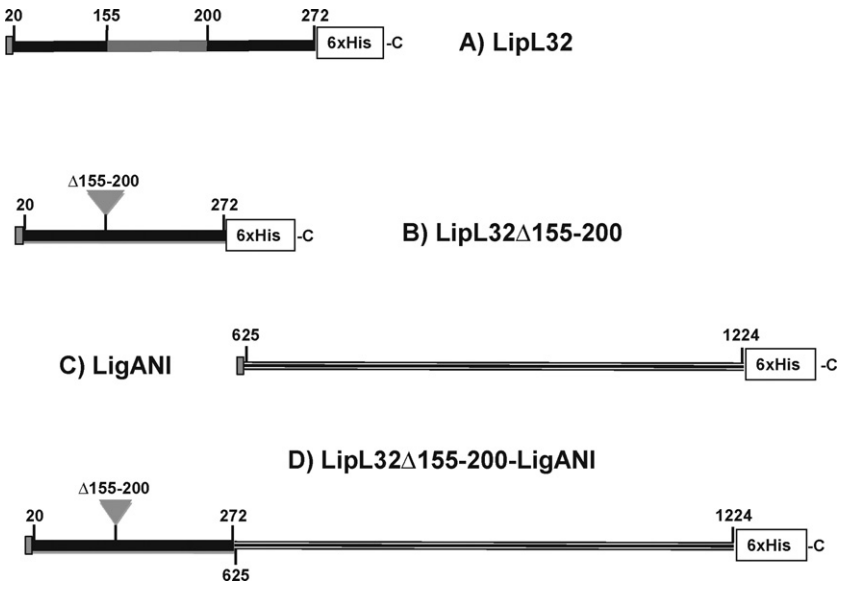

Fig. 1. Schematic representation of proteins used in this study. (A) Mature length LipL32 (amino acids 20–272); the immunodominant region is denoted by the grey bar (amino acids 155–200). (B) Truncated LipL32 (LipL32 Δ155–200). (C) Truncated LigA (LigANI) (amino acids 625–1224). (D) LipL32 Δ155–200–LigANI fusion protein. The C-terminal hexahistidine tag (6× His) is indicated.

2.4. Immunisation of hamsters with recombinant proteins

Groups of 10 hamsters aged 4 weeks were vaccinated with either PBS, 8 M urea, formalin-killed whole serovar Manilae (L495), soluble LipL32, LipL32 Δ155–200, LigANI or LipL32Δ155–200–LigANI. Samples for vaccination were prepared by mixing 30 μL Alhydrogel, with 120 μL of PBS, 8 M urea, WCL or recombinant protein (at a concentration of 30 μg/120 μL). A booster vaccination was administered after 2 weeks; hamsters were then challenged 2 weeks later via intraperitoneal injection with 2×10^3 virulent *L. interrogans* serovar Manilae and monitored for two weeks. The lethal dose for strain L495 is approximately 10 leptospires [25]. 100 μL of blood were collected from each animal before immunisation and challenge. Animals were euthanised if they appeared moribund, kidneys were harvested upon death and inoculated into EMJH to check for colonisation. Animal experiments were approved by the Animal Ethics Committee, Khon Kaen University, Thailand.

2.5. Immunoblotting

Proteins or WCL were separated by SDS–PAGE then transferred onto Immobilon-PVDF membranes (Millipore). Membranes were blocked overnight at 4 °C in 5% skim milk–TBS-0.05% Tween 20 buffer. The membranes were incubated with the appropriate pooled sera from vaccinated hamsters. Sera were diluted 1/250 in skim milk–TBS-0.05% Tween 20 buffer. Bound antibodies were detected using horseradish peroxidase-conjugated goat anti-hamster IgG (ab7146, Abcam), diluted 1/2500 in skim milk–TBS-0.05% Tween 20 buffer.

Immunoblots were developed using the ECL Western blot detection system (GE healthcare) and visualised with a Fuji LAS 3000 digital imager.

2.6. Statistical analysis

The Fisher exact test was used to evaluate significant differences between hamster groups immunised with the various recombinant proteins in this study. All *P* values were two-sided and a *P* value of <0.05 was considered statistically significant.

3. Results

3.1. Expression, purification and immunogenicity of recombinant proteins

All recombinant proteins (Fig. 1) were highly expressed in *E. coli* and purified by metal affinity chromatography to >95% purity (Fig. 2).

Hamsters were immunised with formalin-killed L495 (control), PBS (control), 8 M urea (control), soluble LipL32, LipL32 Δ155–200, LigANI and LipL32 Δ155–200–LigANI, 30 μg in Alhydrogel on day 0 and day 14. Serum was collected from all hamsters on day 28 before they were challenged with 2×10^3 cells of serovar Manilae (L495). Sera from hamsters vaccinated with either PBS or 8 M urea alone did not recognise any of the recombinant proteins (data not shown). Sera from hamsters immunised with the individual recombinant proteins were able to recognise their corresponding purified proteins (Fig. 3). Importantly, sera from hamsters immunised with either soluble LipL32 or LipL32 Δ155–200 recognised LipL32 in leptospiral WCL (Fig. 4, lanes 1 and 2). Sera from hamsters immunised

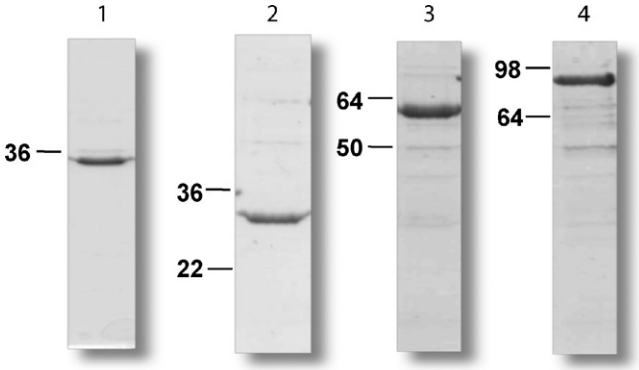

Fig. 2. Coomassie Blue staining of purified recombinant proteins. Lane 1: soluble LipL32, lane 2: LipL32 Δ155–200, lane 3: LigAN1, and lane 4: LipL32 Δ155–200–LigAN1. The positions of standard molecular mass markers (kDa) are shown on the left of each lane.

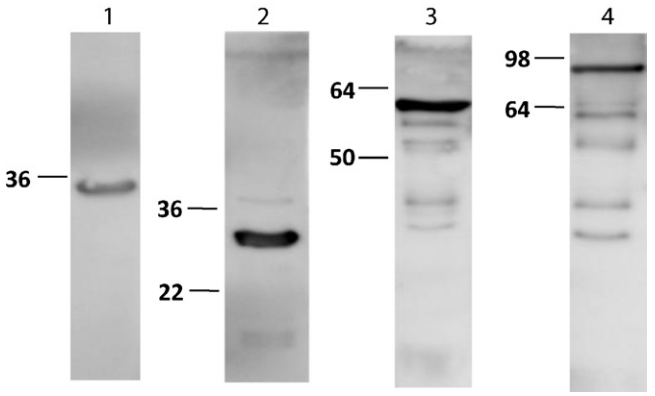

Fig. 3. Detection of recombinant proteins with sera from hamsters immunised with the corresponding proteins. Lane 1: LipL32 detected with sera from hamsters vaccinated with soluble LipL32, lane 2: LipL32 Δ155–200 detected with sera from hamsters vaccinated with LipL32 Δ155–200, lane 3: LigANI detected with sera from hamsters vaccinated with LigANI, and lane 4: LipL32 Δ155–200–LigANI detected with sera from hamsters vaccinated with LipL32 Δ155–200–LigANI. The positions of standard molecular mass markers (kDa) are shown on the left of each lane.

with LigANI reacted with intact LigA (Fig. 4, lane 3), while sera from hamsters immunised with the LipL32 Δ155–200–LigANI fusion protein recognised both LigA and LipL32 present in leptospiral WCL (Fig. 4, lane 4).

3.2. Vaccinated hamsters are not protected against leptospiral infection

Hamsters were challenged on day 28 with virulent serovar Manilae (L495), and monitored for signs of illness over 12 days. Hamsters vaccinated with formalin-killed L495 were completely protected from acute infection compared with those receiving PBS ($P = 0.0007$) or 8 M urea ($P = 5.4 \times 10^{-6}$) (Table 2). In contrast, none of the hamsters immunised with any of the recombinant protein constructs were protected ($P > 0.47$) (Table 2). After 12 days, the surviving hamsters were killed and one kidney from each animal was cultured in EMJH medium. Leptospires were cultured from all

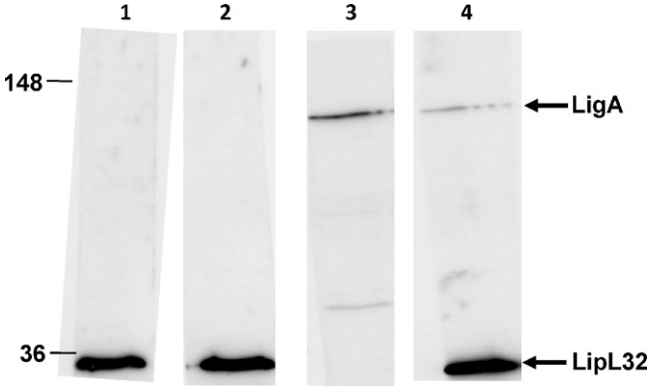

Fig. 4. Western blot of *L. interrogans* serovar Copenhageni (Fio Cruz L1-130) WCL (salt induced for LigA expression) detected with sera from hamsters vaccinated with soluble LipL32 (lane 1), LipL32 Δ155–200 (lane 2), LigANI (lane 3) etc. Lane 1: sera from hamsters vaccinated with soluble LipL32, lane 2: sera from hamsters vaccinated with LipL32 Δ155–200, lane 3: sera from hamsters vaccinated with LigANI, and lane 4: sera from hamsters vaccinated with LipL32 Δ155–200–LigANI. The positions of standard molecular mass markers (kDa) are shown on the left. Sera from hamsters vaccinated with either PBS or 8 M urea did not recognise any proteins in leptospiral WCL (data not shown). Bands corresponding to leptospiral LigA and LipL32 are indicated on the right.

Table 2
Survival of hamsters following immunisation with recombinant proteins or control preparations and challenge with 2×10^3 serovar Manilae.

Antigen	Survivors	*P* value*
PBS	2/10	–
8 M urea	0/10	0.474
Formalin-killed serovar Manilae	10/10	0.0007
Soluble LipL32	0/10	0.474
LipL32 Δ155–200	0/10	0.474
LigANI	0/10	0.474
LipL32 Δ155–200–LigANI	2/10	1.0

* Compared with PBS control.

survivors except for the 10 control hamsters vaccinated with killed leptospires.

4. Discussion

Each of the recombinant antigens elicited an antibody response against the homologous recombinant protein (Fig. 3) and against the corresponding protein in leptospiral WCL (Fig. 4). However, none of the immunised hamsters was protected when challenged with virulent *L. interrogans*, whereas the killed bactrin elicited 100% protection. Hamsters were challenged via the recommended intraperitoneal route of infection [26]. Serovar Manilae was selected for challenge as it causes systemic, lethal infection in hamsters. Notably, a recent study demonstrated that hamsters vaccinated with a live attenuated derivative of serovar Manilae (L495) produced antibodies against both LipL32 and LigA, indicating that LigA is expressed during *in vivo* growth of Manilae in hamsters [27]. The dose of 30 μg protein per vaccination was selected based on previous studies demonstrating protective immunity using similar amounts of protein and similar vaccination strategies [21,28].

These results highlight a recurring issue with LipL32 as a potential protective antigen. Despite being a highly immunogenic protein, LipL32 appears to stimulate protective immunity against leptospires only under very specific conditions. Hamsters vaccinated with recombinant LipL32 expressed in the live attenuated *Mycobacterium bovis* strain BCG were protected from leptospirosis [10]. However, further analysis of the data indicated that statistical significance was achieved in only one out of three experiments [11]. BCG induces a strong Th1 immune response and has been used as an effective vaccine to protect against tuberculosis in humans. Therefore, the adjuvant used for formulation may play a critical role in stimulating the appropriate immune response for protective immunity. A study investigating the ability of recombinant adenovirus expressing LipL32 to protect gerbils obtained statistically significant protection [7]. However, 50% of the control animals also survived after challenge. When gerbils were immunised with a *LipL32* DNA vaccine there was 66% protection [29] but since 33% of unvaccinated animals also survived, claims on partial protection are compromised. A recent study using cholera toxin B subunit (CTB) LipL32 fusion protein observed a significant seroconversion of anti-CTB–LipL32 IgG antibodies in mice immunised with the CTB–LipL32 fusion protein in comparison to mice immunised separately with CTB and LipL32 and unimmunised control mice [30]. However, since the mice were not challenged in this study it is therefore not possible to draw any conclusions about whether this construct would confer protective immunity [30]. No study has demonstrated 100% protection with LipL32 in the face of appropriate challenge. Notably, in our study we observed no protective immunity in any hamsters immunised with LipL32, despite eliciting an antibody response to both the mature length LipL32 and the truncated form of the protein.

LigA has also been successfully used as a protective antigen in previous experiments. An immunisation study using the C-terminal portion of LigA (LigANI) was shown to protect hamsters [21]. In that study, hamsters were immunised subcutaneously with LigA in Freund's complete adjuvant on day 0 and a second immunisation of antigen in Freund's incomplete adjuvant on day 14. Immunisation was performed with a range of recombinant protein doses that included 80/40 μg (first/second immunisation); 60/30 μg, 40/20 μg; and 20/10 μg. Protection varied from 63% to 100% depending on the vaccine dose [21].

A study using hamsters vaccinated with LigA combined with Alhydrogel [31] claimed to have demonstrated the immunoprotectiveness of LigA in hamsters, as the vaccinated group was protected 100% against challenge (8/8 survivors). However, 80% of unvaccinated control animals (6/8) also survived challenge, yielding an insignificant *P* value of 0.467 (Fisher's exact test). Although this study found that no leptospiral associated histopathological lesions were found in the LigA-immunised hamsters in contrast to the control animals, claims of protection are compromised by the survival data.

Another study using C3H/HeJ mice also showed greater than 90% protection with LigA [20]. Freund's complete adjuvant was used for the primary vaccinations and the mice were boosted without adjuvant 2 and 4 weeks later. However, mice are not an accepted animal model for acute leptospirosis and a higher inoculum ($>10^6$ bacteria) is required to produce lethal infection [32]. These data again highlight the importance of adjuvant used in formulation and the difficulty involved in formulating a vaccine with an acceptable adjuvant. Although Freund's complete adjuvant has been used in formulations with LigANI that successfully protected hamsters against leptospirosis, it is not licenced for commercial use as it is highly toxic [22]. In our study Alhydrogel was selected as it is regularly used in commercial animal vaccines and is approved for use in human vaccines [22]. Strict guidelines must also be adhered to when claiming statistical significance in studies.

In our study, hamsters were immunised and boosted with LigANI in Alhydrogel. However, this formulation was unable to stimulate protective immunity, despite eliciting an antibody response. Hamsters immunised with the LipL32 Δ155–200–LigA fusion protein also produced an antibody response towards LigA. However the response to LigA was weaker compared to hamsters immunised with LigANI alone. These data suggest that the antibody response appeared to be directed primarily against LipL32 Δ155–200 and the absence of the immunodominant epitopes in the LipL32 truncation did not appear to affect the humoral response generated against it.

The first published data demonstrating partial protective immunity against homologous challenge was reported by Haake et al. [28]. Hamsters vaccinated with *E. coli* membrane fractions containing a combination of OmpL1 and LipL41 induced significant, but not complete, protection against homologous challenge with *Leptospira kirschneri* serovar Grippotyphosa. Neither protein was able to protect when administered alone, nor were the purified non-membrane associated proteins able to protect, suggesting that the way in which these proteins associate with the membrane is important in inducing a protective immune response and may be due to the epitope conformation required for antibody binding [28]. These results have not been reproduced. No other individual proteins have been reported as eliciting protective immunity against infection with *Leptospira* in any animal model. Further research to identify additional protein components of pathogenic *Leptospira* involved in immunity to infection is thus clearly warranted.

Acknowledgements

We gratefully acknowledge the excellent technical assistance of Vicki Vallance, Kate Rainczuk and Chen Ai Khoo. This work was supported by grants from the National Health and Medical Research Council, and the Australian Research Council, Canberra, Australia.

References

[1] Farrelly HE, Adler B, Faine S. Opsonic monoclonal antibodies against lipopolysaccharide antigens of *Leptospira interrogans* serovar Hardjo. J Med Microbiol 1987;23:1–7.
[2] Levett PN. Leptospirosis. Clin Microbiol Rev 2001;14:296–326.
[3] Brown RA, Blumerman S, Gay C, Bolin C, Duby R, Baldwin CL. Comparison of three different leptospiral vaccines for induction of a type 1 immune response to *Leptospira borgpetersenii* serovar Hardjo. Vaccine 2003;21:4448–58.
[4] Faine S, Adler B, Bolin C, Perolat P. *Leptospira* and leptospirosis. 2nd ed. Australia: MediSci; 1999.
[5] Cullen PA, Xu XY, Matsunaga J, Sanchez Y, Ko AI, Haake DA, et al. Surfaceome of *Leptospira* spp. Infect Immun 2005;73:4853–63.
[6] Shang ES, Exner MM, Summers TA, Martinich C, Champion CI, Hancock RE, et al. The rare outer membrane protein OmpL1, of pathogenic *Leptospira* species is a heat-modifiable porin. Infect Immun 1995;63:3174–81.
[7] Branger C, Sonrier C, Chatrenet B, Klonjkowski B, Ruvoen-Clouet N, Aubert A, et al. Identification of the hemolysis-associated protein 1 as a cross-protective immunogen of *Leptospira interrogans* by adenovirus-mediated vaccination. Infect Immun 2001;69:6831–8.
[8] Haake DA, Chao G, Zuerner RL, Barnett JK, Barnett D, Mazel M, et al. The leptospiral major outer membrane protein LipL32 is a lipoprotein expressed during mammalian infection. Infect Immun 2000;68:2276–85.
[9] Guerreiro H, Croda J, Flannery B, Mazel M, Matsunaga J, Galvao Reis M, et al. Leptospiral proteins recognized during the humoral immune response to leptospirosis in humans. Infect Immun 2001;69:4958–68.
[10] Seixas FK, da Silva EF, Hartwig DD, Cerqueira GM, Amaral M, Fagundes MQ, et al. Recombinant *Mycobacterium bovis* BCG expressing the LipL32 antigen of *Leptospira interrogans* protects hamsters from challenge. Vaccine 2007;26:88–95.
[11] Adler B, de la Peña Moctezuma A. *Leptospira* and leptospirosis. Vet Microbiol 2010;140:287–96.
[12] Hoke DE, Egan S, Cullen PA, Adler B. LipL32 is an extracellular matrix-interacting protein of *Leptospira* spp. and *Pseudoalteromonas tunicata*. Infect Immun 2008;76:2063–9.
[13] Hauk P, Macedo F, Romero EC, Vasconcellos SA, de Morais ZM, Barbosa AS, et al. In LipL32, the major leptospiral lipoprotein, the C terminus is the primary immunogenic domain and mediates interaction with collagen IV and plasma fibronectin. Infect Immun 2008;76:2642–50.
[14] Tung JY, Yang CW, Chou SW, Lin CC, Sun YJ. Calcium binds to LipL32, a lipoprotein from pathogenic *Leptospira*, and modulates fibronectin binding. J Biol Chem 2010;285:3245–52.
[15] Murray GL, Srikram A, Hoke DE, Wunder Jr EA, Henry R, Lo M, et al. Major surface protein LipL32 is not required for either acute or chronic infection with *Leptospira interrogans*. Infect Immun 2009;77:952–8.
[16] Lottersberger J, Guerrero SA, Tonarelli GG, Frank R, Tarabla H, Vanasco NB. Epitope mapping of pathogenic *Leptospira* LipL32. Lett Appl Microbiol 2009;49:641–5.
[17] Matsunaga J, Barocchi MA, Croda J, Young TA, Sanchez Y, Siqueira I, et al. Pathogenic *Leptospira* species express surface-exposed proteins belonging to the bacterial immunoglobulin superfamily. Mol Microbiol 2003;49:929–45.
[18] Matsunaga J, Sanchez Y, Xu X, Haake DA. Osmolarity, a key environmental signal controlling expression of leptospiral proteins LigA and LigB and the extracellular release of LigA. Infect Immun 2005;73:70–8.
[19] Choy HA, Kelley MM, Chen TL, Moller AK, Matsunaga J, Haake DA. Physiological osmotic induction of *Leptospira interrogans* adhesion: LigA and LigB bind extracellular matrix proteins and fibrinogen. Infect Immun 2007;75:2441–50.
[20] Koizumi N, Watanabe H. Leptospiral immunoglobulin-like proteins elicit protective immunity. Vaccine 2004;22:1545–52.
[21] Silva EF, Medeiros MA, McBride AJ, Matsunaga J, Esteves GS, Ramos JG, et al. The terminal portion of leptospiral immunoglobulin-like protein LigA confers protective immunity against lethal infection in the hamster model of leptospirosis. Vaccine 2007;25:6277–86.
[22] Petrovsky N, Aguilar JC. Vaccine adjuvants: current state and future trends. Immunol Cell Biol 2004;82:488–96.
[23] Johnson RC, Walby J, Henry RA, Auran NE. Cultivation of parasitic leptospires: effect of pyruvate. Appl Microbiol 1973;26:118–9.
[24] Sambrook J, Fritsch EF, Maniatis T. Molecular cloning: a laboratory manual. 2nd ed. New York: Cold Spring Harbour Laboratory Press; 1989.
[25] Murray GL, Morel V, Cerqueira GM, Croda J, Srikram A, Henry R, et al. Genome-wide transposon mutagenesis in pathogenic *Leptospira* species. Infect Immun 2009;77:810–6.
[26] Haake DA. Hamster model of leptospirosis. Curr Protoc Microbiol 2006 [Chapter 12:Unit 12E 2].

[27] Srikram A, Zhang K, Bartpho T, Lo M, Hoke DE, Sermswan RW, et al. Cross-protective immunity against leptospirosis elicited by a live, attenuated lipopolysaccharide mutant. J Infect Dis 2011.
[28] Haake DA, Mazel MK, McCoy AM, Milward F, Chao G, Matsunaga J, et al. Leptospiral outer membrane proteins OmpL1 and LipL41 exhibit synergistic immunoprotection. Infect Immun 1999;67:6572–82.
[29] Branger C, Chatrenet B, Gauvrit A, Aviat F, Aubert A, Bach JM, et al. Protection against *Leptospira interrogans* sensu lato challenge by DNA immunization with the gene encoding hemolysin-associated protein 1. Infect Immun 2005;73:4062–9.
[30] Habarta A, Abreu PA, Olivera N, Hauk P, Cedola MT, Ferrer MF, et al. Increased immunogenicity to LipL32 of *Leptospira interrogans* when expressed as a fusion protein with the Cholera toxin B subunit. Curr Microbiol 2010.
[31] Palaniappan RU, McDonough SP, Divers TJ, Chen CS, Pan MJ, Matsumoto M, et al. Immunoprotection of recombinant leptospiral immunoglobulin-like protein A against *Leptospira interrogans* serovar Pomona infection. Infect Immun 2006;74:1745–50.
[32] Adler B, Faine S. Host immunological mechanisms in resistance of mice to leptospiral infections. Infect Immun 1977;17:67–72.