Vaccines Against Leptospirosis

Ben Adler

Abstract Vaccines against leptospirosis followed within a year of the first isolation of *Leptospira*, with the first use of a killed whole cell bacterin vaccine in guinea pigs published in 1916. Since then, bacterin vaccines have been used in humans, cattle, swine, and dogs and remain the only vaccines licensed at the present time. The immunity elicited is restricted to serovars with related lipopolysaccharide (LPS) antigen. Likewise, vaccines based on LPS antigens have clearly demonstrated protection in animal models, which is also at best serogroup specific. The advent of leptospiral genome sequences has allowed a reverse vaccinology approach for vaccine development. However, the use of inadequate challenge doses and inappropriate statistical analysis invalidates many of the claims of protection with recombinant proteins.

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1 Bacterin Vaccines

Pathogenic *Leptospira* was first isolated in Japan in 1914. Within a year, Japanese researchers had successfully immunized guinea pigs (Ido et al. 1916). They showed that injection of leptospires inactivated with phenol elicited protective immunity in guinea pigs and that immunity could be transferred with immune serum administered concurrently with the infecting leptospires, demonstrating for the first time the importance of antibodies in immunity to leptospirosis in an animal model. The first large-scale human use took place in Japan, where Wani vaccinated 10,000 coal miners between 1919 and 1921. He also showed passive protection of guinea pigs with human immune serum (Alston and Broom 1958).

In the ensuing years, a variety of methods was used to kill leptospires for use as vaccines, including heat, formalin, phenol, ethanol, freeze-thawing, and irradiation. However, in the past 100 years very little has changed and at the present time the only licensed vaccines remain whole cell, inactivated bacterins. These have been used widely in cattle, swine, and dogs; specific usage for individual animal species is detailed in chapter by W.A. Ellis, this volume. Because of problems with reactogenicity due to components of the leptospires and constituents of the growth media, bacterin vaccines for humans have not gained acceptance to the same degree as for animals. Attempts to overcome these problems have included the development of protein-free growth media (Christopher et al. 1982; Torten et al. 1973). However, yields were generally much poorer than in conventional media containing serum or BSA. Nevertheless, human bacterin vaccines have been used successfully in several regions, including China, Japan, Cuba, and Europe. The use of currently available human vaccines is described in the chapter by D.A. Haake and P.N. Levett, this volume.

Immunity elicited by bacterin vaccines is restricted to serovars with related agglutinating antigens and is generally humorally mediated, with the exception of Hardjo infection in cattle (Naiman et al. 2001). This restriction, therefore, requires a good knowledge of the regional epidemiology, which can be reliably gained only by culture and identification of locally prevalent serovars. Reliance on serological surveys to predict local serovars is not recommended. A second consequence of the limitation of immunity to serologically-related serovars is that in situations where multiple serovars are circulating, multivalent vaccines are required. Accordingly, bacterin vaccines containing up to four serovars are commonly used in many countries, especially in dogs and pigs (see the chapter by W.A. Ellis, this volume). Claims for protection against additional, closely-related serovars are probably valid. In some countries, locally-produced vaccines, especially for use in cattle, may contain up to eight serovars. Efficacy studies to demonstrate protection against all the included serovars have very rarely, if ever, been performed. Any possible antigenic competition effects between such large numbers of serovars are completely unknown. A final drawback of bacterin vaccines arises from the fact that the immunity elicited is directed mainly against the leptospiral lipopolysaccharide

(LPS), a T-independent antigen, and therefore involves IgM antibodies and lack of a memory response. The duration of immunity is therefore relatively short, with annual vaccination recommended in almost all cases.

2 Live Vaccines

The lack of detailed knowledge about leptospiral pathogenesis (see the chapter by G.L. Murray, this volume) and the unavailability of genetic tools for easy manipulation of pathogenic *Leptospira* spp. (see the chapter by M. Picardeau, this volume) have to date precluded any development of rationally-attenuated, live vaccines. Nevertheless, serovar Pomona attenuated by laboratory passage was used as a live vaccine and shown to elicit protective immunity in hamsters, swine, and cattle (Stalheim 1968). Although demonstrated to be safe and to be effective in stimulating a duration of immunity which lasted at least 14 months (Stalheim 1971), the basis for attenuation was unknown; the vaccine has not been developed further and no live vaccines are currently licensed.

More recently, a defined LPS biosynthetic mutant of serovar Manilae was shown to be attenuated in hamsters, which showed no clinical signs of infection, and did not become renal carriers (Srikram et al. 2010). The mutant was also unable to colonize the kidneys of mice (Marcsisin et al. 2013). Immunization with the mutant elicited protective immunity against homologous challenge and also against heterologous challenge with serovars Pomona (Srikram et al. 2010) or Autumnalis (Unpublished results). Killed bacteria stimulated only homologous protection. Significantly, there was no detectable reactivity against either Pomona or Autumnalis LPS, strongly suggesting that immunity was mediated by protein antigens. The identity of the protective antigens is unknown.

3 Lipopolysaccharide Vaccines

Unlike some of the other major spirochete genera *Treponema* and *Borrelia*, the major surface component of *Leptospira* is LPS. Furthermore, leptospiral LPS is a protective antigen. Monoclonal antibodies against LPS can protect against acute lethal infection in guinea pigs and hamsters (Jost et al. 1986; Schoone et al. 1989) and also protected dogs, based on recovery of leptospires from blood (Schoone et al. 1989). The protection shown in early studies with an "outer sheath" preparation (Auran et al. 1972) was almost certainly mediated by LPS; indeed, LPS was shown by silver staining and western blotting to be a major constituent of this preparation (Adler B; unpublished results). Likewise, it is clear that a reported protective "glycolipid" antigen (Masuzawa et al. 1990) was in fact LPS. Immunization with as little as 2.5 μg of purified LPS, or the polysaccharide (PS) component of LPS, was sufficient to elicit the production of agglutinating, protective antibodies in hamsters

(Jost et al. 1989). The immunogenicity of PS could be enhanced by conjugation with a protein carrier, diphtheria toxoid (Midwinter et al. 1990). An oligosaccharide derived from LPS and conjugated to diphtheria toxoid elicited the production of agglutinating, opsonic antibodies (Midwinter et al. 1994), suggesting that the conjugate would be protective, but protection studies were not undertaken. The structure of leptospiral lipid A has been determined (Que-Gewirth et al. 2004), but the structure of the carbohydrate component remains unknown. However, genome sequences have identified LPS biosynthetic loci with close to 100 genes, suggesting that the LPS structure is very complex (Bulach et al. 2006; Nascimento et al. 2004).

An intriguing possibility was raised by the use of LPS derived from saprophytic *Leptospira biflexa* to immunize hamsters against infection with the pathogenic serovar Manilae (Matsuo et al. 2000). However, the claims of protection must be tempered by the fact that all hamsters, including controls, survived challenge, despite the use of a high $>10^6$ challenge dose. Protection was based on clinical and pathological criteria, but the use of small animal groups precludes the drawing of any statistically meaningful results. It is possible that the effects observed were due to activation of the innate immune response by *L. biflexa* LPS. The work has not been reproduced, but significantly, an earlier study in which children were immunized with an inactivated *L. biflexa* vaccine reported no agglutinating antibodies against pathogenic serovars (Rottini et al. 1972). In addition, another study found no protection against challenge with Canicola in gerbils immunized with *L. biflexa* (Sonrier et al. 2000).

The clear capacity of LPS and LPS-derived components to elicit protective immunity held the possibility of development of immunoconjugate vaccines, similar to those developed against pneumococcal and *Haemophilus influenzae* infections. However, this development has not eventuated, most probably because of the large number of leptospiral serovars, the cost involved, and the unknown but complex structure of leptospiral LPS. There is no prospect of LPS-based vaccines in the near future.

4 Cross-Protective Immunity

It seems apparent that heterologous immunity does not usually follow natural infection, at least in humans. The author has experience of two examples where culture confirmed infection with one serovar (Pomona) was followed less than 3 months later by infection, again culture confirmed, with a different serovar (Hardjo). Nevertheless, the stimulation of cross-protective immunity remains an important focus and goal in leptospirosis vaccine research. Several studies have shown that this is feasible.

Some inkling that cross immunity might exist was noticed as early as 1928 (Fletcher 1928) and perhaps even earlier, although these studies were primitive by today's standards. In work now 50 years old, Kemenes (1964) investigated cross immunity in guinea pigs, which recovered from one infection and were then

infected with a heterologous serovars. He showed a level of cross immunity between serovars Pomona and Canicola and also between Pomona and Icterohaemorrhagiae. Cross immunity did not extend to serovars Sejroe or Hyos (now Tarassovi). Of course, the nature of the antigens involved was and remains unknown, but interestingly, both of these serovars are now classified in the species *Leptospira borgpetersenii*, while Pomona, Canicola, and Icterohaemorrhagiae belong to *Leptospira interrogans*.

Using a similar approach, Plesko and Lataste-Dorolle (1970) showed limited, but incomplete, cross immunity between several serovars, including Bratislava, Icterohaemorrhagiae and Copenhageni, Pomona, Lora (all *L. interrogans*) and Grippotyphosa (*L. kirschneri*). In a subsequent study, significant, but not complete, interserovar immunity was demonstrated in hamsters between four serovars of *L. borgpetersenii*, viz. Tarassovi, Javanica, Poi and Arborea (Plesko and Hlavata 1971). Again, the responsible antigens remain unknown. However, it is notable that all of these studies used live leptospires, highlighting the possibility that cross-protective antigens may be expressed exclusively in vivo, or at best, expressed at low levels in vitro.

There appears to have been no further work in this area for several decades until Sonrier et al. (2000) investigated the cross-protective capacity of LPS and whole cell extracts, obtained from in vitro-grown bacteria, in gerbils. Not surprisingly, LPS elicited only homologous protection. However, whole cell extracts from Icterohaemorrhagiae ($p = 0.003$) but not from Autumnalis ($p = 0.2$) protected against challenge with Canicola. In a modified reverse experiment, a chloroform-methanol-water extract of the phenol phase (designated as protein in this paper) LPS preparation from Canicola protected against challenge with Icterohaemorrhagiae ($p = 0.002$). However, the claim of protein-mediated cross protection must be tempered by two caveats. The phenol phase can also contain leptospiral LPS (Shinagawa and Yanagawa 1972; Vinh et al. 1989) and there is a well-established MAT cross-reactivity between serovars Canicola and Icterohaemorrhagiae. The possibility that the observed protection was actually mediated by LPS cannot be excluded.

A more recent study examined cross immunity between four serovars, using formalin-inactivated vaccines in hamsters (Rosario et al. 2012). Serovars Canicola and Copenhageni belong to *L. interrogans*, serovar Ballum to *L. borgpetersenii*, while serovar Mozdok may be classified as either *L. borgpetersenii* or *L. kirschneri*, depending on the strain (not specified in this paper). All four vaccines elicited 100 % homologous immunity, as well as varying levels of cross immunity. The Mozdok vaccine elicited only homologous immunity. Immunization with Copenhageni stimulated immunity against challenge with Ballum but not the other two heterologous serovars. The claimed protection against Canicola was not significant ($p = 0.087$). However, vaccination with Ballum or Canicola induced solid heterologous immunity except against Mozdok, with 100 % survival except for the Ballum-Copenhageni combination (70 %; $p = 0.003$).

As described above (Sect. 2), a genetically defined LPS biosynthesis mutant of serovar Manilae was able to stimulate 100 % immunity in hamsters against challenge with Autumnalis (Unpublished results) or Pomona (Srikram et al. 2010).

What conclusions can realistically be drawn from these studies? Although the results have been variable, it seems clear that it is possible to stimulate heterologous immunity and that the antigens involved are probably proteins which may well be expressed poorly or not at all under the standard conditions used to culture leptospires in vitro. Although some progress has been made in recent years, the identity of the antigen(s) involved remains poorly understood.

5 Recombinant Proteins as Vaccines

The development of recombinant DNA techniques and the availability of leptospiral whole genome sequences have resulted in a resurgence of activity to identify protective antigens and to develop defined subunit vaccines. However, much of the vaccination work reported in the literature suffers from the use of inadequate challenge doses, lack of reproducibility, and inappropriate statistical analysis. Accordingly, many of the claims about protection from infection, especially those claiming partial protection, cannot be substantiated.

5.1 The Lipoprotein LipL32

The leptospiral outer membrane lipoprotein LipL32 (Haake et al. 2000) would appear to have all the hallmarks of a virulence factor and/or protective antigen. It is the most abundant protein in the leptospiral cell and the outer membrane and is present exclusively in pathogenic *Leptospira* spp., where it is highly conserved. Surprisingly, therefore, a defined *lipL32* mutant retained virulence for hamsters, whether infected parenterally or conjunctivally, and was unaffected in its ability to colonize the renal tubules of rats (Murray et al. 2009). Originally identified as a major component of the leptospiral surface (Cullen et al. 2005), its surface location has recently been re-evaluated (Pinne and Haake 2013).

LipL32 is the most studied leptospiral protein (Murray 2013). Its ability to elicit protective immunity against acute infection with several different serovars has been reported numerous times using a range of antigen delivery methods and animal models (Table 1). However, a rigorous evaluation reveals major deficiencies in many of these reports, with the most common problems being the use of inadequate challenge dose, small groups of animals, and inappropriate statistical analysis. Accordingly, the majority of publications does not present a credible case for protection when survival is used as the criterion (Table 1).

The original work in gerbils delivered LipL32 either as a recombinant protein or via the *lipL32* gene introduced as plasmid DNA or using an adenovirus vector. Claims of protection must be tempered by the high survival rates in control animals resulting in lack of statistically significant protection (Table 1). Similar problems arise when assessing the results of attempts to immunize hamsters with LipL32

Table 1 Protection studies with LipL32

Origin serovar	Challenge serovar	Antigen	Animal	Delivery	Claimed protection[a]	*P* value[b]	Notes	References
Autumnalis	Canicola	*lipL32* adenovirus	Gerbil	Adenovirus 10^9 pfu	86 %	0.054–0.06	50 % survival in control groups	Branger et al. (2001)
Autumnalis or Copenhageni	Canicola	*lipL32* DNA	Gerbil	Plasmid DNA 100 μg	60 %	0.18	35 % survival in control group	Branger et al. (2005)
Autumnalis or Copenhageni	Canicola	LipL32	Gerbil	Protein 50 μg	0–50 %	0.48–0.71	13–60 % survival in control groups	Branger et al. (2005)
Copenhageni	Copenhageni	LipL32	Hamster	BCG 10^6 cfu	16–60 %	0.015–0.72	Highly variable results across three experiments	Seixas et al. (2007)
Icterohaemorrhagiae	Pomona	Anti-LipL32 monoclonal antibody	Hamster	Anti-LipL32 monoclonal antibody	100 %	<0.0001	Low challenge dose 5xLD_{50}. Leptospires isolated from tissues at 21 day necropsy	Maneewatch et al. (2008)
Lai	Lai	LipL32	Guinea pig	Protein 200 μg	50 %	0.01	High challenge dose > 10^8	Luo et al. (2010)
Lai	Ballum	LipL32	Guinea pig	Protein 200 μg	58 %	0.005	High challenge dose > 10^8	Luo et al. (2010)

(continued)

Table 1 (continued)

Origin serovar	Challenge serovar	Antigen	Animal	Delivery	Claimed protection[a]	*P* value[b]	Notes	References
Pomona	Pomona	LipL32	Hamster	Protein 50 μg	Nil	1.0	No protection based on lung histopathology	Cao et al. (2011)
Manilae or Hardjo	Manilae	LipL32 and fragments	Hamster	Protein 50 μg	Nil	1.0	No survival in control groups. Hamster sera reacted with intact LipL32	Deveson Lucas et al. (2011)
Copenhageni	Copenhageni	LipL32-LTB fusion	Hamster	Protein 43–60 μg	40–100 %	0.44	0–60 % survival in control groups	Grassmann et al.(2012)
Copenhageni	Copenhageni	LipL32 +LTB	Hamster	Protein 43–60 μg	60–100 %	0.17–0.44	0–60 % survival in control groups	Grassmann et al.(2012)

[a] Protection based on survival compared with control animals
[b] Fisher's exact test

expressed in *Mycobacterium bovis* BCG. A proper statistical analysis shows that significance was achieved ($p = 0.022$, Fisher's exact test) in only one of three experiments. Claimed protection of hamsters immunized with LipL32 and LTB, either as a mixture or as a fusion protein, likewise do not withstand proper analysis. Importantly, two additional studies in hamsters showed unequivocal lack of protection following immunization with recombinant entire LipL32 or fragments thereof (Table 1; Cao et al. 2011; Deveson Lucas et al. 2011).

In contrast, partial but significant protection was generated in guinea pigs immunized with LipL32 and challenged with either serovar Lai (homologous) or Ballum (Table 1; Luo et al. 2010). However, even here the conclusion must be tempered by the apparently low virulence of the challenge strains, necessitating the use of a very high challenge dose.

A more compelling case for LipL32 as a protective antigen arises from the clear and highly significant protection conferred to hamsters that received mouse monoclonal antibodies against LipL32 (Table 1; Maneewatch et al. 2008). What then, are we to make of these apparently contradictory findings? Given that the most credible protection has been mediated by antibodies generated in mice or guinea pigs, is it possible that these animal species respond with antibody isotypes that are protective, but hamsters do not? Or do these protective antibodies recognize unique LipL32 epitopes? LipL32 in *Leptospira* is glycosylated (Ricaldi et al. 2012), but recombinant proteins used in vaccination studies would almost certainly not be glycosylated. Any role for LipL32-linked glycans in immunity remains completely unknown.

A further possibility is that cell-mediated immunity plays an as yet undefined role in the variable levels of protection observed. Intriguingly, in cattle where immunity, at least to serovar Hardjo, is not antibody dependent but is correlated with IFN-γ release by T-cells, the antigen which is the major stimulator of IFN-γ release is LipL32 (Deveson Lucas et al. 2014). The possibility thus exists that LipL32 is a protective antigen in some animal species but not in others. In the case of humans at least, there is a compelling argument that LipL32 does not mediate protection. LipL32 is expressed during human infection and is immunogenic; indeed it has been used as a serodiagnostic antigen (Flannery et al. 2001). However, in the face of this antibody response, immunity following human infection is restricted to serovars with related LPS. Whether this restriction is true for other animal species remains equivocal. Another possibility might be that antibodies elicited during natural infection are not directed against as yet undefined protective epitopes. The role of LipL32 in immunity thus remains enigmatic and warrants further investigation.

5.2 The Lig Proteins

The Lig proteins were identified as major components of the leptospiral surface which are not expressed under normal in vitro growth conditions (Matsunaga et al.

2003, 2005). The Lig proteins have been investigated as vaccine candidates against challenge infection with three different serovars (Table 2). As is the case with LipL32 studies, many of the claims of protection must be tempered due to the use of inadequate challenge doses, poor reproducibility, and inappropriate statistical analyses. There are no studies which present a credible case for LigB as a protective antigen (Table 2). Koizumi and Watanabe (2004) reported >90 % protection of C3H/HeJ mice immunized with LigA and/or LigB, but with 40 % survival in the GST-immunized control groups, yielding statistical insignificance. A study with LigA in hamsters claimed efficacy (Table 2; Palaniappan et al. 2006), but control animals showed 75 % survival. A subsequent attempt to immunize hamsters with DNA encoding LigA also claimed protection (Faisal et al. 2008); however, a proper statistical analysis of those data does not support the claim of enhanced survival (Table 2). Nevertheless, several other studies have now shown unequivocal protection of hamsters immunized with the C-terminal portion of LigA, with *ligA* DNA, or with specific domains within the LigA C-terminus (Table 2). An alternative approach was used to deliver lipidated LigA expressed in *E. coli* orally to hamsters. Significant protection was elicited in single experiments against a low intradermal challenge dose, but not against a slightly higher intraperitoneal challenge (Table 2; Lourdault et al. 2014). LigA therefore shows significant promise as a protective protein antigen, at least against some serovars. Potential explanations for the conflicting results include the use of different adjuvants, challenge doses, and challenge serovars. Indeed, one study found no homologous protection in hamsters immunized with Canicola LigA (Table 2; N. Bomchil, Personal communication), whereas Copenhageni LigA elicited 100 % protection against homologous challenge. A similar lack of homologous protection was observed with serovar Manilae (Table 2; Deveson Lucas et al. 2011). The possibility therefore remains that the protective capacity of LigA may not necessarily extrapolate to all species or serovars.

5.3 Other Recombinant Proteins

There has been a range of other recombinant proteins tested for protective capacity. Similar problems arise with the majority of claims, which report a single experiment, or lack of reproducibility and therefore do not withstand proper statistical analysis. These are summarized in Table 3 and will not be discussed further here. Notably, Murray et al. (2013) found no protection elicited by any of the 238 recombinant proteins tested from serovar Hardjo, when colonization of hamster kidneys was used as the criterion for protection. The list of proteins tested contained several for which claims of protection had been made previously.

Two studies report protection elicited by the FlaB flagellar subunit protein (Table 3). The first (Dai et al. 1997) was performed in mice, which were challenged with a very high dose of 2.5×10^{10} leptospires; despite this dose, 40 % of controls survived, but the numbers used yielded significant protection (Table 3). In the

Table 2 Protection studies with Lig proteins

Origin serovar	Challenge serovar	Antigen	Animal	Delivery	Claimed protection[a]	*P* value[b]	Notes	References
Manilae	Manilae	LigA or LigB C–terminus. GST fusions	C3H/HEJ mouse	Protein 10 μg	90 %	0.077	40 % survival in GST control group. Protection significant only when compared with PBS group	Koizumi and Watanabe (2004)
Manilae	Manilae	LigA C–terminus plus LigB C–terminus. GST fusion	C3H/HEJ mouse	Protein 10 μg	100 %	0.167	40 % survival in GST control group. Protection significant when compared with PBS group	Koizumi and Watanabe (2004)
Pomona	Pomona	LigA C-terminus plus LigA N-terminus. GST fusions.	Hamster	Protein 50 μg	100 %	0.077–1.0	57–88 % survival in control groups	Palaniappan et al. (2006)
Copenhageni	Copenhageni	LigA C-terminus. His tag	Hamster	Protein 10–80 μg	63–100 %	0.00008–0.026	Better protection with higher protein doses	Silva et al. (2007)
Copenhageni	Copenhageni	LigB C-terminus. His tag	Hamster	Protein 10–80 μg	Nil	1.0	No protection	Silva et al. (2007)

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Table 2 (continued)

Origin serovar	Challenge serovar	Antigen	Animal	Delivery	Claimed protection[a]	*P* value[b]	Notes	References
Pomona	Pomona	*ligA* DNA	Hamster	Plasmid DNA 50 µg	100 %	0.077–0.20	50–75 % survival in control groups	Faisal et al. (2008)
Pomona	Pomona	LigB fragments	Hamster	Protein 50 µg	25–87 %	0.01–1.0	Variability across experiments. Significant protection with C-terminus ($p = 0.04$) or C- plus N-terminal fragments ($p = 0.01$) obtained in 2 of 3 experiments	Yan et al. (2009)
Pomona	Pomona	LigB fragments	Hamster	Protein 50 µg	33–50 %	0.182–0.455	2 or 3 survivors in groups of 6	Cao et al. (2011)
Pomona	Pomona	LigA C-terminus	Hamster	Liposomes or PLGA microspheres 10/20 µg	76–92 %	$<10^{-7}$	LigA with alum adjuvant elicited 50 % protection ($p = 0.002$)	Faisal et al. (2009)
Copenhageni	Copenhageni	LigA C-terminus and sub-fragments. His tag	Hamster	Protein 100 µg	50–100 %	0.01–0.43	Domains 10–13 required for 100 % protection. Use of four animals	Coutinho et al. (2011)

(continued)

Table 2 (continued)

Origin serovar	Challenge serovar	Antigen	Animal	Delivery	Claimed protection[a]	*P* value[b]	Notes	References
							per group limits conclusions	
Manilae	Manilae	LigA C-terminus	Hamster	Protein 30 µg	Nil	0.474	Hamster sera reacted with intact LigA in *Leptospira*	Deveson Lucas et al. (2011)
Canicola	Copenhageni	*ligA* or *ligB* DNA	Hamster	Plasmid DNA 100 µg	62*–100 %	0.03 to $<10^{-6}$	*One LigB group. remainder all 100 % protection	Forster et al. (2013)
Canicola	Canicola	LigA C-terminus	Hamster	Protein 100 µg	Nil	1.0	No protection. 100 % homologous protection with Copenhageni in the same study	Bomchil, N. (Personal communication)
Copenhageni	Copenhageni	LigA lipidated C-terminal domains 7–13	Hamster	*E. coli* total 37–148 mg. repeat oral immunization	38–63 %	0.026–0.2	Protection against intradermal but not intraperitoneal challenge. Single experiments	Lourdault et al. (2014)

[a] Protection based on survival compared with control animals
[b] Fisher's exact test

Table 3 Protection studies with other recombinant proteins

Origin serovar	Challenge serovar	Antigen	Animal	Delivery	Claimed protection[a]	*P* value[b]	Notes	References
Lai	Lai	*flaB* DNA	Mouse	*E. coli* 10^7 cfu plus plasmid DNA	78 %	0.02	Challenge dose 2.5×10^{10} 40 % survival in control group which received only plasmid vector without *E. coli*	Dai et al. (1997)
Grippotyphosa	Grippotyphosa	OmpL1 LipL41 and OmpL1	Hamster	Protein 50 µg, in *E. coli* membrane	100 %	0.009	33 % survival in control group. Protection seen in one of three experiments. No protection with His-tagged protein(s)	Haake et al. (1999)
Lai	Lai	*flaB2* DNA	Guinea pig	Plasmid DNA 100 µg	90 %	0.005	Single experiment reported	Dai et al. (2000)
Copenhageni	Pomona	*ompL1* DNA	Hamster	Plasmid DNA 100 µg	33 %	0.455	2/6 survival compared with 0/6 in control group	Maneewatch et al. 2007)
Pomona	Pomona	Lp1454, Lp1118, MceII. GST fusions.	Hamster	Protein 50 µg	71–100 %	0.07–0.59	50 % survival in control groups. No significant protection when all three combined	Chang et al. (2007)
Lai	Lai	*lipL21* DNA	Guinea pig	Plasmid DNA 100 µg	Nil	1.0	All animals survived. Protection claimed on the basis of changes in weight gain	He et al. (2008)

(continued)

Table 3 (continued)

Origin serovar	Challenge serovar	Antigen	Animal	Delivery	Claimed protection[a]	*P* value[b]	Notes	References
Pomona	Pomona	Lp0607, Lp118, Lp1454 combined	Hamster	Protein trapped in leptospiral or *E. coli* liposomes	75 %	0.0006	No protection with proteins trapped in phosphatidylcholine liposomes ($p = 0.245$). No protection with proteins mixed with any liposomes ($p = 1.0$)	Faisal et al. (2009)
Copenhageni	Copenhageni	Lic12720, Lic10494, Lic12922 His tag	Hamster	Protein 50 μg	30–44 %	0.04*–1.0	*Lic 12720 in one of two experiments	Atzingen et al. (2010)
Pomona	Pomona	Lp0222, Lp3685, Lp4337	Hamster	Protein 50 μg	33–83 %	0.08–1.0	Protection not significant	Yan et al. (2010)
Copenhageni	Copenhageni	Lic10325, Lic13059 His tag	Hamster	Protein 60 μg	33 %	0.455	2/6 survival compared with 0/6 in control group	Felix et al. (2011)
Copenhageni	Copenhageni	Lsa21, Lsa66, Lic11030. His tag	Hamster	Protein 50 μg	20–30 %	0.21–0.48	Claimed protection for any of the proteins was not reproduced in a second experiment	Atzingen et al. (2012)

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Table 3 (continued)

Origin serovar	Challenge serovar	Antigen	Animal	Delivery	Claimed protection[a]	*P* value[b]	Notes	References
Copenhageni	Icterohaemorrhagiae	*lemA* DNA and LemA prime boost	Hamster	Protein 100 µg plasmid DNA 100 µg	87 %	0.002	LemA alone ($p = 0.8$). *lemA* alone ($p = 0.03$). Survivors culture and lesion positive. Results from single experiment	Hartwig et al. (2013)
Copenhageni	Copenhageni	Leptallo1. His tag	Hamster	Protein 50 µg	30 %	0.211	No significant protection	Hashimoto et al. (2013)
Lai	Lai	GroEL His tag	Hamster	Protein 100–200 µg	50–75 %	0.007–0.077	Single experiment reported. Claim that GroEL antiserum agglutinated multiple serovars	Li et al. (2013)
Hardjo	Hardjo	238 recombinant proteins	Hamster	Protein 25 µg in pools of five	Nil	1.0	Kidney colonization used to assess protection	Murray et al. (2013)

[a] Protection based on survival compared with control animals
[b] Fisher's exact test

second study (Dai et al. 2000), guinea pigs were immunized with plasmid DNA encoding FlaB, and again apparently significant protection was obtained, although only a single experiment was performed. It is difficult to know how to interpret these results. Leptospiral flagellar antigens are not surface exposed and indeed, do not react with specific antibodies unless the leptospiral cells are first permeabilized (Zhang et al. 2013). It is the opinion of this author that the protection claims with flagellar antigens are not credible.

An even stranger claim is that of a recent study which reported significant protection of hamsters with recombinant GroEL (Table 3; Li et al. 2013). The chaperone GroEL is a cytoplasmic protein; indeed, it is used routinely as a cytoplasmic marker in cell fractionation experiments. The report that anti-GroEL antiserum agglutinated all eight leptospiral serovars tested thus borders on bizarre. An independent assessment of specific rabbit anti-GroEL antiserum found no evidence whatsoever of agglutination of whole leptospires (B. Adler, unpublished observations).

Despite these mainly negative or unconvincing findings, there are some credible reports of immunoprophylaxis. One of the earliest reports of protective immunity elicited by defined protein antigens (Haake et al. 1999) involved immunization of hamsters with *E. coli* membrane fractions containing a combination of OmpL1 and LipL41. This preparation induced significant protection against homologous challenge with *Leptospira kirschneri* serovar Grippotyphosa, but only in one of three experiments. These studies have not been repeated.

The hypothetical LemA protein induced partial, but highly significant, protection in hamsters when delivered using a prime boost, DNA plus protein strategy. Surviving animals were culture and lesion positive. Interestingly, LemA alone did not elicit significant immunity, whereas *lemA* alone was marginally protective ($p = 0.03$) despite not eliciting a detectable antibody response (Table 3; Hartwig et al. 2013). Unfortunately, these results were based on a single experiment.

Faisal et al. (2009) reported significant protection in hamsters immunized with a combination of three proteins of unknown function, Lp0607, Lp118 and Lp1454, when delivered trapped in liposomes derived from polar lipids of *L. biflexa* (termed leptosomes). Intriguingly, no protection was observed if the proteins were trapped in phosphatidylcholine liposomes, nor if the proteins were delivered mixed with, rather than trapped in, either of the liposome preparations (Table 3). The leptosomes stimulated significantly higher Th1 and Th2 responses, although it is not clear which of these was involved in mediating protection. However, here again, the protection data were based on a single experiment.

6 Conclusions

On the centennial of the discovery of *Leptospira* as the causative agent of Weil's disease the only vaccines licensed for use in animals and humans are inactivated bacterins not very different from those first used 90 years ago. The post-genomic era has seen a flurry of activity to identify protein components of the leptospiral cell that

are able to elicit cross-protective immunity. As outlined in this review, the majority of claims for protection are not credible, based on the data reported. However, this should not be viewed as painting too bleak a picture. It may well be that some of these proteins will be shown to be capable of stimulating immunity when they are tested in repeat experiments, perhaps in combination, and with sufficiently large numbers of animals, proper challenge doses, and with appropriate statistical analysis. This prospect is exemplified by LigA. Despite a number of protection claims which do not withstand proper scrutiny, there is now sufficient evidence that LigA is a protective antigen, at least in some leptospiral species and serovars, and currently represents the most likely candidate vaccine antigen. There is thus a realistic possibility of the development of defined, protein-based vaccines in the next decade.

A final cautionary point should be borne in mind. The experience with Hardjo vaccines in cattle has emphasized the fact that mechanisms of immunity to leptospirosis, and therefore the identity of the antigen(s) mediating that immunity, cannot necessarily be extrapolated from laboratory animals to production or companion animals, to humans, or even among different animal species. The caveat may also hold for different serovars; the assumption should not be made that immunity elicited by an antigen against a particular serovar in a particular animal, can be generalized to other species.

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