Leptospirosis Vaccines: Past, Present, and Future

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ABSTRACT

It is well known that Leptospira vaccine prevents the disease. However specificity for serovars limits the efficacy of killed whole cell vaccines. Leptospiral antigens that induce cross-protective immunity to the various serovars are sought as new vaccine candidates. In this paper, we have summarized both past and current findings about leptospiral antigens that are conserved among pathogenic leptospires and that induce protective immunity in animal models. The full-length genome sequences of two Leptospira strains have been published and reverse vaccinology has been used to identify leptospiral vaccine candidates. Although humoral immunity is thought to be dominant in protection from leptospiral infection, a role for cell-mediated immunity is now being explored.

KEY WORDS: *Leptospira*, Leptospirosis, Vaccine, Killed whole cell vaccine, Serovar, LPS, Protein antigen, Genome, Cellular immunity

Leptospirosis is an important zoonotic disease that is distributed worldwide.[1-3] In mammals, leptospirosis is transmitted either by direct contact with infected animals or by exposure to water or soil contaminated by the urine of infected animals. The efficacy of vaccine for preventing leptospirosis was shown soon after *Leptospira* was proven to be a causative agent of Weil's disease in Japan. The heat-killed whole cell vaccine made from leptospiral cultures dramatically protected coal miners from Weil's disease in the Kyushu area where Weil's disease was endemic.[4] The development of killed whole cell vaccine against leptospirosis in the middle of the last century has been reviewed,[5] and subsequently results of vaccine trials were published.[1,6-8]

Killed whole cell vaccine

Killed whole cell leptospiral vaccines for humans are available in some countries, including Japan. The Japanese leptospiral vaccine consists of formalin-killed leptospires. The concentrations and serovars are 250 million/ml each of Australis, Autumnalis, and Hebdomadis and 500 million/ml of Copenhageni. The leptospires are grown in media containing rabbit serum and/or bovine serum albumin, inactivated with formalin, and washed by centrifugation. The standard procedures for production and verification of the vaccine were established in 1952.[9] The vaccine is administered initially using two subcutaneous injections of 1.0 ml given at a 7-day interval. The booster injection is 1.0 ml of vaccine injected subcutaneously and given within 5 years after the second initial dose. The antibody titres (MAT titres) after vaccination were significantly lower than those developed after natural infection and seroconversion occurred with low frequency (about 20-60%).[10-13] However, protection was reported to be high in such populations and efficacy rates of whole cell vaccines were about 60-100%.[6-8,14] The effectiveness of the killed vaccine is serovar-specific. The studies done in the Izena-jima Island in Okinawa prefecture showed that people who were inoculated with a serovar Pyrogenes monovalent vaccine were protected from the infection with serovar Pyrogenes, but not from infection with serovars Autumnalis and Hebdomadis. However, polyvalent vaccine, consisting of serovars Autumnalis, Hebdomadis and Pyrogenes, prevented infection with the three leptospiral serovars strains for at least for 7 years after immunization.[14] The serovar-specific protection of killed leptospiral vaccine has also been documented in other studies.[6,15] There are more than 230 serovars among the pathogenic leptospires. The local variability in serovars of endemic leptospiral strains complicates the development of a vaccine that can be used worldwide.[1,16] The study done in the Izenajma Island also demonstrated that the killed vaccine induced long-term immunity, although the prevalence of leptospirosis among the unvaccinated population after the introduction of the vaccine dramatically decreased from those rates previously documented. However, one study has reported that the duration of immunity induced by killed vaccine ranges between 6 months to a 1 year at the longest and a second study reported a duration of at least 3 years.[5,6] Persistence of immunity in dogs is also controversial.[17,18] The side effects of the whole cell vaccines were reported, which included both systemic and local reactions at a various frequency. To reduce side effects ascribable to serum in cultures, a vaccine that consisted of leptospires grown in the protein-free medium was developed.[11,19,20] A Japanese research group, however, reported that there was no difference in the frequency of side-effects be-

tween the vaccines derived from either cultures in Korthof's or protein-free medium.[21] In addition to side-effects, the whole cell vaccine may induce autoimmune diseases, such as uveitis.[22]

There is a report on the outer envelope vaccine for leptospirosis which was studied in China.[23] The results of the study showed the good protection with less side effects and higher agglutinating titre than those in a whole cell vaccine. Vaccination using the outer envelope vaccine also reduced the number of the patients with vaccine-unrelated serogroup strains.

Lipopolysaccharides

The variations in the carbohydrate composition of lipopolysaccharide (LPS) reflect the antigenic diversity among pathogenic leptospires. The protective immunity conferred by leptospiral LPS as an immunogen is generally serovar specific.[16] Sera from patients with leptospirosis cross-reacted with antigens from non-pathogenic (saprophytic) *L. biflexa* serovar Patoc (strain Patoc I).[24,25] The cross-reactivity is the basis for the development of LEPTO dipstick assay for the diagnosis of leptospirosis and uses the heat stable antigen (LPS) from *L. biflexa* Patoc I.[26,27] Matsuo et al extracted the antigenic components from *L. biflexa* Patoc I by the hot phenol–water method and obtained three immunoreactive fractions.[28] The fractions strongly reacted with not only anti-*L. biflexa* serum, but also with various antisera against pathogenic leptospires in ELISA assays, regardless of their serovars or serogroups. The ELISA reaction was specifically inhibited only by β -(1→4)-mannobiose and not by any other monosaccharides or oligosaccharides that were tested. NMR studies demonstrated that the main structural part of the antigenic fractions had a repeating disaccharide of '→)- β -D-Man*p*-(1→4)- β -D-Man*p*-(1→. A direct binding between antisera and disaccharides of →3)- β -D-Man*p*-(1→4)- β -D-Man*p*-(1→ was not shown. However, β -(1→4)-mannobiose inhibited the reaction and after deacylation of one of the antigenic fractions with some of the fatty acyl groups immunoreactivity was the same as the intact preparation, suggesting that polysaccharides of →3)- β -D-Man*p*-(1→4)- β -D-Man*p*-(1→ are antigenic components of LPS. Furthermore, not only antisera against various pathogenic leptospires, but also sera from patients with leptospirosis, reacted with exocelluar mannans from *Rhodotorula glutinis* that have the same structural backbone with →3)- β -D-Man*p*-(1→4)- β -D-Man*p*-(1→.[29,30] Administration of *L. biflexa* LPS preparation in hamsters was protective against a challenge with virulent *L. interrogans* serovar Manilae without any side effects.[31] Immunization protected hamsters from bacteremia and prevented a renal carrier state. Since protein-conjugated polysaccharide vaccines against *Haemophilus influenzae* type b, pneumococci and meningococci have been successful, similar conjugated polysaccharide vaccines for leptospirosis could be developed.

Protein antigens

The immunogenic proteins, especially the outer membrane surface proteins, of pathogenic *Leptospira*, may be effective as vaccinogens. The identification of proteins, which are conserved among pathogenic leptospires and can generate cross-protection against various serovars, has become a major focus of leptospirosis research.[32-54] Subunit vaccines may also have fewer side-effects than killed whole cell vaccine. Sera from patients with leptospirosis have antibodies against several protein antigens.[39] Protein extracts prepared from a pathogenic *Leptospira* can induce protective immunity against challenge with a heterologous serovar strain in an experimental animal model.[55] These data point to the potential use of leptospiral protein(s) as candidates for a new vaccine that could induce good protection against diverse serovars.

Some leptospiral protein antigens have been shown to elicit protective immunity in animal models. These are described as follows:

1. OmpL1 and LipL41

OmpL1 is a transmembrane protein,[33,56] and LipL41 is an outer membrane lipoprotein.[34] 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.[37] Although the mechanism of this synergistic protection remains to be solved, a steric hindrance of OmpL1 by LipL41 has been proposed.[52] The results from this same study also indicated that immunization using the membrane-associated OmpL1/LipL41 (also referred to as lipidated LipL41) was critical for inducing immunoprotection. The authors suggested that conformational integrity of OmpL1/LipL41 epitopes for antibody binding might require the association with membrane and/or lipid attached to LipL41 for immunogenicity. A similar condition for immunogenicity exists with *Borrelia burgdorferi* lipoprotein, OspA, in which immunogenicity and immunoprotective capacity were enhanced by lipidation of the protein.[57, 58] Southern and western blot analyses revealed that *ompL1* gene product and LipL41 protein were present in various pathogenic leptospiral serovars, but not in non-pathogenic leptospires.[33,34] Patients with leptospirosis have antibodies against these proteins in their sera.[38,59] Whether or not these proteins will confer cross-protection against a heterologous challenge remains to be determined.

2. LipL32/Hap-1

LipL32 is one of the most abundant proteins in *Leptospira*.[38] 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. A recombinant LipL32 antigen has good sensitivity and specificity when used in an ELISA for human leptospirosis IgG.[59] LipL32 is also called haemolysis associated protein-1 (Hap-1) because *E. coli* harbouring the plasmid encoding this gene showed some haemolytic activity on sheep, but not on human erythrocytes.[60] 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.[61] Vaccination using an adenovirus vector encoding the *lipL32/hap-1*

gene induced cross-protection in the gerbil model of leptospirosis.[40] 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. In addition, OmpL1, either alone or in combination with LipL32/Hap-1, had no protective activity. This result is in contrast to the synergistic activity observed with OmpL1 and LipL41.

3. Leptospiral immunoglobulin-like proteins

Leptospiral immunoglobulin-like (Lig) proteins are surface-exposed outer membrane proteins punctuated by tandem repeats of about 90 amino acids of bacterial immunoglobulin (Ig) like domains.[45,49,51,62] LigA consists only of Ig-like domains, whereas LigB has an additional unique domain at the C terminus. The bacterial immunoglobulin-like domains are found in various adhesion proteins of pathogenic bacteria; for example, the intimin of *E. coli*,[63, 64] and the invasin of *Y. pseudotuberculosis*,[65] and may function as adhesion molecules. Both LigA and LigB contain an N-terminal lipobox and LigA was lipidated when expressed in *E. coli*.[51] Expression of Lig protein and *lig* mRNA was lost after attenuation by culture of *L. kirshneri* and *L. interrogans*, suggesting that Lig proteins are associated with virulence. Lig proteins were not detected in some virulent strains during *in vitro* culture, although specific mRNAs were transcribed.[45,62] We also failed to detect expression of LigA protein in fresh human isolates of serogroup Autumnalis and Hebdomadis.[66] Recent studies show that expression of Lig proteins, surface exposure of LigB and extracellular release of LigA are all enhanced by physiological osmolarity.[67] Sera from either rats immunized with extracts of *in vitro* cultured low-passage strain or sera from vaccinated dogs failed to react with Lig proteins in Western blot analysis, while sera from captured rat reservoirs or dogs infected with *Leptospira* gave a positive response.[49, 62] Therefore expression of Lig proteins is upregulated during infection of a mammalian host. The *lig* genes are present among various pathogenic, but not non-pathogenic, leptospires.[49,51,62] Sera from human patients infected with different leptospiral serogroup strains reacted with Lig proteins.[51] Furthermore, in a mouse model of leptospirosis, the Lig proteins elicited protective immunity against challenges, not only with the homologous serovar Manilae infection,[51] but also the heterologous serovar Icterohaemorrhagiae.[66] These data suggest that Lig proteins could induce protective immunity against the challenge with strains of various leptospiral serovars.

Exploration of the full-length leptospiral genome to detect genes encoding candidate vaccine proteins

The ability to rapidly determine full-length genome sequences has opened a new approach to vaccine design that may be relevant for the treatment of bacterial infections.[68] The strategy of 'reverse vaccinology', in which the full-length genome is "mined" by various computer algorithms, for genes that encode proteins with desired characteristics, has been applied to some bacteria and novel vaccine candidate sequences have been identified.[69,70] The full-length genome sequences of two strains of *L. interrogans*, serovar Lai 56601 and serovar Copenhageni Fiocruz L1-130 are published.[71-73] A full-length genome analysis of the strain Fiocruz L1-130 has been used to identify candidate antigens for leptospiral vaccine.[74] Genes that contained exportation signal peptides, transmembrane domains, lipoprotein signatures and homologies to known surface proteins were selected by computer analysis. A total of 206 genes had been predicted and 150 of them were expressed in *E. coli*, purified, and used for immunoblotting with leptospirosis patient sera. A total of 16 proteins reacted with convalescent patient sera in immunoblotting. The 16 proteins identified in the study contained some previously identified antigens like LipL32 and Loa22, but LipL41 and OmpL1 were not detected, which points out a weakness of the "reverse vaccinology" for analysis of some proteins. There is ample room for improving the accuracy of screening. Four of the 10 proteins tested were highly conserved among different pathogenic leptospires. However, the protective activity of these proteins remains to be determined.

Cell-mediated immunity

Although immunity against leptospiral infection was thought to be primarily humoral,[16] recent studies point to a role for cell-mediated immunity in protection from leptospirosis. Peripheral blood mononuclear cells (PBMCs) from cattle immunized with a killed *L. borgpetersenii* serovar Hardjo vaccine proliferated and produced IFN-γ after *in vitro* stimulation with leptospiral antigens.[75] $CD4^+$ cells were the main source of IFN, but $CD8^+$ and gd T cells also produced it.[76] The PBMCs from Hardjo-vaccine immunized cattle also responded to stimulation with serovar Grippotyphosa antigen preparation.[76] PBMCs from nonvaccinated cattle also responded to leptospiral antigens, but the responses were lower than those of vaccinated cattle. The low cell-mediated immune response in unvaccinated cattle may be associated with lack of protection from chronic infection.[77] Thus, protective immunity against serovar Hardjo infection in cattle correlates with establishment of Th1 immunity. An uncharacterized leptospiral glycolipoprotein elicited *in vitro* production of TNF-α and IL-10 and upregulated the expression of CD69 and HLA-DR on human PBMCs.[78] The heat-killed leptospires also induced the production of IFN-γ, IL-12 and TNF-α in human whole blood *in vitro*.[79] Naïve human PBMCs from healthy individuals proliferated and produced IFN-γ, IL-12, and TNF-α *in vitro* after stimulation with *L. interrogans*.[80] High numbers of leptospires caused the expansion of gd T cells, while low numbers of leptospires induced ab T cells. In patients with acute leptospirosis the number of peripheral blood gd T cells increased significantly. The *in vivo* role of gd T cells in protection from leptospiral disease remains to be elucidated.

Concluding remarks

Both the mechanisms of pathogenesis of *Leptospira* and nature of protective immunity against leptospiral infection are poorly understood. The availability of full-length genome sequences of two *Leptospira* strains will be helpful for future studies. The development of the genetic analysis tools for pathogenic leptospires, which are currently available only for

non-pathogenic leptospires,[81-83] will accelerate both the identification of virulence factors, as well as our understanding of the pathogenesis of leptospires. As new information evolves, insights will be gained into ways to improve existing, and develop effective new, vaccines for leptospirosis.

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