Identification of Immunodominant B- and T-Cell Combined Epitopes in Outer Membrane Lipoproteins LipL32 and LipL21 of *Leptospira interrogans*

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Leptospirosis is a serious infectious disease caused by pathogenic *Leptospira*. B- and T-cell-mediated immune responses contribute to the mechanisms of *Leptospira interrogans* infection and immune intervention. LipL32 and LipL21 are the conserved outer membrane lipoproteins of *L. interrogans* and are considered vaccine candidates. In this study, we identified B- and T-cell combined epitopes within LipL32 and LipL21 to further develop a novel vaccine. By using a computer prediction algorithm, two B- and T-cell combined epitopes of LipL21 and four of LipL32 were predicted. All of the predicted epitopes were expressed in a phage display system. Four epitopes, LipL21 residues 97 to 112 and 176 to 184 (LipL2197–112 and LipL21176–184, respectively) and LipL32233–247 of LipL32 were selected as antigens by Western blotting and enzyme-linked immunosorbent assay. These selected epitopes were also recognized by CD4+ T lymphocytes derived from LipL21- or LipL32-immunized BALB/c (H-2d) mice and mainly polarized the immune response toward a Th1 phenotype. The identification of epitopes that have both B- and T-cell immune reactivities is of value for studying the immune mechanisms in response to leptospirosis and for designing an effective vaccine for leptospirosis.

Leptospirosis is a widespread zoonotic disease caused by pathogenic *Leptospira* spp. (27, 36). Patients suffering from leptospirosis have a diverse array of clinical symptoms, such as meningitis (10), pneumonia (12), hepatitis (1), nephritis (31), and pancreatitis (35), and death may result (5). It is known that zoonotic infections of humans with leptospires are a significant public health problem in developing countries (24).

Currently, most vaccines against leptospirosis in animal models have been studied using heat-killed or formalin-killed leptospires, outer membrane proteins, and native or recombinant proteins from leptospires (13, 21, 23). In general, currently available vaccines made from either inactivated leptospires or their membrane components may elicit immunity but with the disadvantages of incomplete, short-term, limited serovar-specific effects and poor immunological memory (39). In addition, the increasing number of serovars also provides a challenge to developing an ideal vaccine with full protection against pathogenic leptospires.

In response to leptosporal infection, the host launches vigorous humoral and cellular immune responses. Evidence indicates that leptospires can activate the immune system, but their immunological effects are still unclear. T lymphocytes play an important role in the recognition and subsequent elimination of tumors and intracellular pathogens. Recent studies have shown that heat-killed *Leptospira interrogans* induces CD4+ T-cell and γ/δ T-cell responses and stimulates type I cytokine production (9, 26). These data suggest that the type I or cell-mediated immune response is involved in the protective effect against leptospires.

LipL32 and LipL21 are outer membrane proteins of leptospires. They play important roles in infection by acting as adhesins, targets of specific antibodies, porins, and receptors for soluble molecules and complement proteins (7, 15). A recombinant vaccine based on LipL32 and LipL21 provides partial immunoprotection in a hamster model (17, 22). Since the immune responses evoked by whole-cell vaccine or subunit vaccine are often not optimal, epitope-based vaccines provide an alternative strategy. The potential advantages of the epitope-based approach include an excellent immune response, increased safety, the opportunity to rationally engineer epitopes for increased potency and breadth, and the ability to focus immune responses on conserved epitopes (30). To develop an epitope-based vaccine, the identification of potential effective immunodominant epitopes is an initial and critical step. In this study, we characterized B- and T-cell combined epitopes of the outer membrane proteins LipL32 and LipL21; the discovery of these epitopes is important for understanding immune responses against leptosporal infection and for highlighting a new therapeutic strategy against leptospirosis.

MATERIALS AND METHODS

**Materials.** *Escherichia coli* host strain DH10B was maintained in the lab. Phage vector M13KE and host bacterium ER2738 were from New England Biolabs. The 20-bp DNA ladder marker was from TaKaRa Bio (Dalian) Co., Ltd.
Endonucleases, pGEM-T easy vector, reagents, and chemicals used throughout this work were from Promega, unless otherwise stated. The secondary antibodies, horseradish peroxidase (HRP)-conjugated goat anti-human IgG and goat anti-rabbit IgG-HRP, were from Jackson Immunoresearch and Santa Cruz, respectively. The protein molecular weight marker (P0062), mouse lymphocyte separation medium, mitomycin, and cell proliferation CCK-8 kit were from Beyotime Institute of Biotechnology. Specific enzyme-linkedin immunosorbent assay (ELISA) kits, used to detect gamma interferon (IFN-γ) and interleukin-4 (IL-4), were from R&D Systems. Sera from rabbits immunized with \textit{L. interrogans} strain 56601 and recombinant proteins LipL32 and LipL21 (rLipL32 and rLipL21, respectively) were maintained in our lab (11, 40). Sera from patients infected with leptospires were from hospitals in Guangdong, Sichuan, and Zhejiang provinces and maintained in our lab. Female BALB/c mice aged 6 to 8 weeks were provided by the Experimental Animal Center of Zhejiang University and bred under specific-pathogen-free conditions in the same facility. The animal experiments were approved by our Institutional Review Board.

### Prediction of T- and B-cell epitopes
The amino acid sequences of LipL32 and LipL21 were from the NCBI protein database (GenBank accession numbers AAT45811 and AAT48493, respectively), based on the Chinese reference strain Lai. The SignalP 3.0 Server (http://www.cbs.dtu.dk/services/SignalP/) was used to ensure that the epitope was not located in the signal peptide region. The B-cell epitopes were predicted by the ANTIGENIC program in EMBOSS (http://biotools.biocuckoo.org/EMBOSS/). Potential Th epitopes were predicted by the ANTIGENIC program in EMBOSS (http://emboss.biou.edu/EMBOSS/). Potential Tb epitopes were predicted by the ProPred HLA-DR binding peptide prediction program (http://www.imtech.res.in/emb/linhpred/). (34).

### Preparation of genomic DNAs
Extraction of genomic DNA of \textit{L. interrogans} strain Lai was described previously (19). Briefly, genomic DNA of cultured leptospires was extracted by protease K treatment and phenol-chloroform extraction. After precipitation, the genomic DNA was suspended in sterilized water and stored at −20°C.

### Expression of peptides using phage display
A total of six peptides were expressed, and of these four epitopes were from LipL32, and the other two were from LipL21. The primers used to amplify genes encoding selected epitopes based on LipL32 and LipL21 are shown in Table 1. An EcoR52I site and a 14-bp sequence encoding the M13KE leader peptide were included in each reverse primer. The individual amplified fragment was first cloned into a pGEM-T easy vector for sequencing before it was subcloned into the phage vector M13KE between the EcoR52I and KpnI sites. Positive clones were determined by colony PCR and sequencing before it was subcloned into the phage vector M13KE between the EcoR52I and KpnI sites. Positive clones were determined by colony PCR and further confirmed by sequencing.

The recombinant phage was proliferated following the manufacturer’s instructions. Briefly, \textit{E. coli} ER2738 was inoculated in 30 ml of LB culture medium and incubated with shaking at 37°C for 2 h. Recombinant phage containing each epitope was used to infect strain ER2738, and the culture was incubated with vigorous aeration at 28°C for 4 h. After centrifugation at 10,000 rpm for 10 min at 4°C, the medium supernatant was transferred to a clean tube. By adding one-sixth volume of 20% polyethylene glycol 8000 (PEG 8000)-2.5 M NaCl, the phage was pelleted by centrifugation at 11,000 rpm for 15 min at 4°C and resuspended in 1 ml of TBS (50 mM Tris-HCl, pH 7.5, 150 mM NaCl). The phage was reprecipitated by adding one-sixth volume of 20% PEG 8000-2.5 M NaCl and allowed to precipitate on ice for 1 h. Finally, the phage was centrifuged at 11,000 rpm for 15 min at 4°C and resuspended in TBS; the optical density (OD) values at 269 nm and 320 nm were determined to calculate the number of phage particles according to the method of Day (8).

### Detection of B-cell epitopes by Western blotting and ELISA
To detect the B-cell epitope that may react with antibodies in the serum, a Western blot assay was performed using the purified phage particles and antiserum raised in rabbit against \textit{L. interrogans} rLipL32 or rLipL21. Recombinant purified phage particles (3 × 10^{14}) were mixed with loading buffer and separated by electrophoresis in an 8% SDS-PAGE gel; then the proteins were transferred to a polyvinylidene difluoride membrane (PVDF; Millipore) according to the Bio-Rad protocols. Membranes were blocked in 6% nonnovine serum-TBST (Tris-buffered saline, 0.1% Tween 20, pH 7.2) for 1 h, followed by overnight incubation at 4°C with serum against leptospirosis (dilution, 1:200; microscopic agglutination test, >1:400); rLipL32 or rLipL21 (dilution, 1:300). After the membrane was washed with TBST, it was incubated for 1 h with HRP-conjugated secondary antibodies (dilution, 1:5,000) at 37°C. Chemiluminescent detection was performed using Lumigen-PS according to the manufacturer’s protocol (GE), and blots were exposed to X-ray film for equivalent times. Wild-type M13KE particles were used as controls.

ELISA was carried out using a 96-well ELISA plate. The wells were coated with 3 × 10^{4} phage particles and incubated overnight at 4°C. After the plate was washed three times with PBST (phosphate-buffered saline [PBS] containing 0.05% Tween 20), blocking was performed at 37°C for about 1 h with 200 μl of 1% bovine serum albumin (BSA) in PBS, and the plate was then washed with PBST. Five antileptospire IgM- and IgG-positive serum samples from leptospirosis-infected humans were pooled and used as a primary antibody (1:50 dilution); the sera were added to the plate at 100 μl per well and incubated at 37°C for about 1.5 h. After the plates were washed, 100 μl of goat anti-human IgG-HRP (1:5,000 dilution) was used as secondary antibody, and the mixture was incubated at 37°C for about 1.5 h. After a washing step, 100 μl of 3,3′,5,5′-tetramethylbenzidine substrate was allowed to react with the HRP for 15 min at 37°C in the dark. Finally, the color development reaction was terminated by 100 μl of 2 M H₂SO₄, and absorbance was read at 450 nm.

### Mice and immunization
Six- to 8-week-old female BALB/c mice were purchased from the Experimental Animal Center of Zhejiang University and immunized with 100 μg of rLipL32 or rLipL21 protein premixed with complete Freund’s adjuvant (CFA; Sigma), administered subcutaneously in both hind limbs. After 2 weeks, the same protein, embedded in incomplete Freund’s adjuvant (IFA; Sigma), was injected to strengthen the immunization. After 7 to 10 days, the mice were used in further experiments. Mice that were immunized using the same procedure with PBS instead of recombinant proteins were used as controls.

### Table 1. Primers for amplifying segments of LipL32 and LipL21 epitope sequences

| Protein | Epitope location | Primer | Sequence (5'→3') |
|---------|-----------------|--------|------------------|
| LipL32  | 46–66           | L32-F46| CCGGTAACCTTTCATTTCTACCTGGTAAAGAAGCTTACCTCCTTA |
|         | 133–160         | L32-R46| TGCCTGGGCAACGGCCTCCGTCATCGTACCGGTA |
|         | 201–218         | L32-F201| CCGGTAACCTTTCATTTCTACCTGGTAAAGAAGCTTACCTCCTTA |
|         | 221–247         | L32-R201| TGCCTGGGCAACGGCCTCCGTCATCGTACCGGTA |
| LipL21  | 97–122          | L21-F97| CCGGTAACCTTTCATTTCTACCTGGTAAAGAAGCTTACCTCCTTA |
|         | 176–184         | L21-R176| TGCCTGGGCAACGGCCTCCGTCATCGTACCGGTA |
|          |                 |        |                  |
| PIIV    | M13PF           |        | GAGATTTCACCACTGAAAGAATTATTT |
|         | M13PR           |        | TGAATTTTCTGTATGAGGAGTTCGTA |

*a Amino acid residues.

*b EcoR52I and KpnI sites are underlined. Sequence encoding the M13KE leader peptide is in boldface. Primers from gene coding phage PIIV protein were designed to determine insertion of the epitope fragment.
TABLE 2. Prediction of T- and B-cell combined epitopes in LipL32 and LipL21

| Protein  | Epitope location | Amino acid sequence (N → C) |
|----------|------------------|-----------------------------|
| LipL32   | 46–66            | KTLLPYGSVNYGYVKPG            |
|          | 133–160          | HIRVERLSAM/PDQIAAKAKPYQKL    |
|          | 201–218          | KLKLLQGLYRISFTYYK            |
|          | 221–247          | FGKSFVASYG/LQFPGGPGVSPHLIS   |
| LipL21   | 97–112           | ASDV/KMWGVGETVEA             |
|          | 176–184          | DALVAKAOEVS                  |

a Amino acid residues.

b Italics indicate residues for potential anchoring of major histocompatibility complex class II; underlining indicates residues for potential binding to B lymphocytes.

Isolation of splenocytes and CD4+ T-lymphocyte proliferative response. After collection of sera from immunized BALB/c mice, splenocytes were aseptically removed and homogenized with a 3-mL syringe plunger, and then splenic T lymphocytes were isolated by lymphocyte separation medium (mouse) according to the manufacturer’s instructions. The cells were resuspended in complete RPMI 1640 medium (RPMI 1640 medium, 10% fetal bovine serum, 2 mM glutamine, 50 U penicillin/ml, 50 μg streptomycin/ml, 50 μg M-2-mercaptoethanol, and 25 mM HEPES) unless otherwise specified.

A total of 100 μl of isolated T cells (5 × 10^4 cells per well) and mitomycin-C inactivated allogeneic splenocytes cells (10^5 cells per well) were seeded into 96-well flat-bottom culture plates. Concanavalin A (ConA; 5 μg/ml) and 5 × 10^-4 recombinant phage particles were added to 200 μl of RPMI 1640 complete culture medium. Cells were incubated at 37°C with 5% CO₂ for 72 h. Cell proliferation was measured using a CCK-8 assay kit according to the manufacturer’s instructions. Briefly, 30 μl of the Cell Counting Kit solution was added to the culture medium and incubated for an additional 3 h. The absorbance was determined at 450 nm with a reference wavelength of 630 nm. Cultures without the culture medium and incubated for an additional 3 h. The absorbance was determined at 450 nm with a reference wavelength of 630 nm. Cultures without any antigen were used as negative controls, and ConA was used as a positive control. Tests were repeated at least three times independently.

In addition to testing synthesized epitope peptides individually, we also pooled the peptides to stimulate the T cells for the purpose of detecting possible antagonistic or synergistic interaction among them.

Effect of each epitope peptide on Th1 and Th2 cytokine secretion. To determine the cytokine secretion pattern and further understand the orientation of Th cell polarization, sandwich ELISA kits specific to IFN-γ and IL-4 were used to evaluate cytokine levels in the culture supernatant (100 μl each) of T lymphocytes. All assays were performed as suggested by the manufacturer. Production of each cytokine was calculated from the titration of the calibrated cytokine standard curve. The molecular mass of M13KE was about 63 kDa, and that of each recombinant phage was a little higher, 65 to 66 kDa (Fig. 1). These results showed that the inserted epitope fragments, encoding about 2- to 3-kDa heterologous peptides, were expressed correctly.

Detection of B-cell epitopes. B-cell epitopes were confirmed by Western blot assay. When 3 × 10^14 phage particles of each epitope were used in SDS-PAGE separation and transferred to the PVDF membrane for Western blot assay, there was a single strip near 65 kDa according to the protein ladder (Fig. 1A). Western blotting results showed that all recombinant phages containing epitopes from LipL32 or LipL21 reacted significantly with not only antileptospire (L. interrogans strain 56001) serum but also homologous anti-LipL32 or -LipL21 serum (Fig. 1A and C). Epitopes from LipL21 showed reactivity similar to the antisera of leptospires and rLipL21. However, epitopes from LipL32 presented different reactivities between antileptospire and anti-rLipL32 sera. The band representing LipL32 residues 133 to 160 (LipL32 133–160) showed most significant reactivity with antileptospire serum among the four epitopes, and LipL32 46–66 was more reactive than LipL32 221–247 and LipL32 201–218 (Fig. 1B). When the phage particles reacted with anti-rLipL32 serum, there were no significant differences among these epitopes; all of them readily recognized the antibodies (Fig. 1C).

We also tested the reactivity of these peptides against a pool of human sera from leptospirosis patients. Five sera, with positive antileptospire IgM and IgG antibodies (19), were pooled for screening the B-cell epitope peptides. Serum antibodies captured by the epitope peptide were detected using goat antihuman IgG-HRP. For comparison, wild-type phage and negative serum were used in the ELISA as controls. The results are shown in Fig. 2. The epitope of LipL32 133–160 presented the strongest specific cross-reactivity with antibodies in patient serum, which was consistent with the Western blotting results. The epitopes of LipL32 46–66 and LipL32 201–218 presented stronger cross-reactivity with patient antisera than LipL32 221–247, which was opposite from the Western blotting experiments.
results. The epitopes from LipL21 also showed significant re-
action with patient antiserum, but LipL21 176–184 appeared to be more reactive than LipL21 97–112.

CD4+ T-cell proliferation responses to each peptide. Groups of mice (n = 3) immunized with recombinant phage were sacrificed, and lymphocytes were collected. The data represent two independent experiments, and the experiments were performed in triplicate. Splenocytes derived from LipL32- or LipL21-immunized mice proliferated vigorously upon stimulation with recombinant phage containing epitopes from the corresponding LipL32 or LipL21 protein (Fig. 3). Compared with splenocytes from native mice stimulated with PBS, this proliferative response was significant.

Cytokine profile of lymphocytes induced by LipL21- and LipL32-derived peptides. To characterize the in vitro polarization of T-helper cells, the production levels of IL-4 and IFN-γ were measured in lymphocyte cultures from immunized mice (Fig. 4). Lymphocytes from LipL32-immunized mice released large amounts of IFN-γ in response to stimulation with epitope peptides from LipL32. Similar results were obtained when LipL21-immunized splenocytes were stimulated with epitope peptides of LipL21. The LipL32,133–160 epitope showed the strongest activity in stimulation of IFN-γ production. The other three epitopes of LipL32 showed a similar ability in the production of IFN-γ. When cells were stimulated with epitope pools, the mixture of the four LipL32 epitopes exhibited synergistic enhancement of stimulation activity, while single or mixed epitopes from LipL21 had a similar ability to stimulate IFN-γ production since there was no significant difference between single or mixed epitopes.

DISCUSSION

Leptospirosis is recognized as a globally reemerging public health problem because it has spread from its habitual rural base to become the cause of urban epidemics in poor communities of industrialized and developing nations (6). The search for new tools for diagnosis and treatment of leptospirosis, especially an effective vaccine, would improve patients’ quality of life. In the last decade, new concepts of specific immunotherapy have evolved based on recombinant expression of antigens, especially epitope antigens. Antigenic epitopes are the parts (contact points) of an antigen involved in specific interaction with the antigen-binding site (the paratope) of an antibody or a T-cell receptor, which is composed of several or scores of amino acid residues (38). The epitopes can be divided into two groups: T-cell epitopes which induce cellular immunity and B-cell epitopes which induce humoral immunity (20). Detailed analysis of epitopes is important both for the under-

FIG. 2. Direct binding assay of epitopes with antibodies in human serum. Histograms show the binding of the indicated epitopes in sera from patients with (+) or without (−) leptospiral infection. Compared with the control (M13KE), each epitope of LipL32 (residues 46 to 66, 133 to 160, 201 to 218, 221 to 247) and LipL21 (97 to 112 and 176 to 184) recognized the antibody in the patient sera. The data represent the average of three separate experiments.

FIG. 3. Cell proliferation assay of splenocytes from mice immunized with LipL32 or LipL21 and stimulated with the different epitopes. Lymphocytes (5 × 10⁴) and mitomycin-inactivated allogeneic splenocytes (10⁵) were mixed and stimulated with phage particles containing epitopes from LipL32 (A) or LipL21 (B) for a proliferation assay. The response to each antigen is presented as the mean of three independent experiments. Lymphocytes isolated from mice treated with PBS served as controls to determine if the responses were LipL32 or LipL21 specific. Cells stimulated with ConA or wild-type phage were used as controls.
standing of immunological events and for the development of more effective vaccines and diagnostic tools for various diseases.

B-cell epitopes can be exploited in the development of epitope-based marker vaccines and diagnostic tools (28). A number of such vaccines have been designed to act against viruses (37), bacteria (18), and cancer (41). In our experiments on the identification of B-cell epitopes, we used phage display and Western blotting, which are efficient methods for such studies (29). The results showed that the selected epitopes were specifically recognized by antibodies in serum from rabbits infected by *L. interrogans* or recombinant LipL32 or LipL21. The epitopes from LipL21 showed similar reactive abilities to antileptospire and anti-LipL21 sera. Although all the epitopes from LipL32 recognized the corresponding sera, they had different reactivities to the sera from leptospire- or LipL32-immunized rabbits. All of the epitopes showed more remarkable reactions with antibody in serum immunized by rLipL32 than that infected by *L. interrogans*. This may be because only specific antibodies are induced by recombinant protein antigen while infection with whole bacterial components provides many antigens (e.g., outer membrane proteins, LigA, LigB, and other stimulatory factors); the antibodies that emerge in whole bacterial immunity are therefore more complicated, which may affect the reaction of antigen and antibody. Briefly, the reactive abilities of LipL32246–266 and LipL32201–218 were similar, and they were lower than the reactive ability of LipL32133–160 but higher than that of LipL32221–247, thus representing an important target of the humoral response to leptospiral infection.

CD4+ T-lymphocytes can differentiate into a variety of effector subsets, including classical Th1 and Th2 cells, which play a critical role in the immune reaction of host against bacterial infection (4, 16). The differentiation decision is governed predominantly by cytokines (3). Th1 cells are characterized by their production of IFN-γ and are involved in cellular immunity (32, 33). Previous research reported that a typical Th1 response occurs during the vaccination process (2) while other researchers suggest that the Th2 response is also important in the clearing of pathogen and provides helper activity for antibody production (25). In this experiment, the splenocytes from LipL32- or LipL21-immunized BALB/c mice were stimulated with the corresponding epitope; the results showed that the secretion of IFN-γ was more distinctive than that of IL-4. The results showed that the selected epitopes were BALB/c-specific Th1-type epitopes, which implied that these selected epitopes are promising as vaccine candidates. Study of these epitopes may help to explain the role of Th1 and Th2 responses in the pathogenesis and immunity of *L. interrogans* infection, which also may contribute to the development of an effective multiple-epitope vaccine.

During attack by a pathogenic microorganism, the protection against infection depends mainly on the stimulation of an appropriate antibody; highly potent neutralizing antibodies can intercept a pathogen before it attaches to its target cell. This ability is based on the antibodies' specific recognition of epitopes, the sites on the antigen (14). However, cellular immunity plays a crucial role in the production of high titers of antibody. Thus, it is essential to study T- and B-cell combined epitopes for the development of novel vaccines. In this study, T- and B-cell combined epitopes in the outer membrane proteins LipL32 and LipL21 from *L. interrogans*, which can induce the immune response associated with leptospiral infection, were identified and characterized, and these may be used to develop vaccines or therapeutic strategies.

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