Monoclonal Antibodies Against LipL32, the Major Outer Membrane Protein of Pathogenic *Leptospira*: Production, Characterization, and Testing in Diagnostic Applications

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**ABSTRACT**

Pathogenic serovars of *Leptospira* have a wide antigenic diversity attributed mainly to the lipopolysacharide present in the outer membrane. In contrast, antigens conserved among pathogenic serovars are mainly represented by outer membrane proteins. Surface exposure of a major and highly conserved outer membrane lipoprotein (LipL32) was recently demonstrated on pathogenic *Leptospira*. LipL32 in its recombinant form (rLipL32) was used to immunize BALB/c mice to develop murine monoclonal antibodies (MAbs). Three MAbs against rLipL32 were produced, isotyped, and evaluated for further use in diagnostic tests of leptospirosis using different approaches. MAbs were conjugated to peroxidase and evaluated in a native protein enzyme-linked immunosorbent assay (ELISA) with intact and heat-treated leptospiral cells, conjugated to fluorescein isothiocyanate (FITC) for indirect immunofluorescence with intact and methanol fixed cells and were used for LipL32 immunoprecipitation from leptospiral cells. rLipL32 MAbs conjugated to peroxidase or used as primary antibody bound to intact and heat-treated cells in ELISA, proving that they could be used in enzyme immunosassays for detection of the native protein. In immunofluorescence assay, MAbs labeled bacterial cells either intact or methanol fixed. Two MAbs were able to immunoprecipitate the native protein from live and motile leptospiral cells and, adsorbed onto magnetic beads, captured intact bacteria from artificially contaminated human sera for detection by polymerase chain reaction (PCR) amplification. Results of this study suggest that the MAbs produced can be useful for the development of diagnostic tests based on detection of LipL32 leptospiral antigen in biological fluids.

**INTRODUCTION**

*Leptospirosis*, a zoonotic disease widespread in the world, is caused by pathogenic bacteria of the genus *Leptospira*.1 There are over 230 pathogenic leptospiral serovars identified and this antigenic diversity is mainly attributed to the lipopolysaccharide (LPS) covering bacterial surface.2 Diagnostic approaches based on detection of antibodies generated during infection or on detection of antigens using antibodies generated against *Leptospira* strains have poor sensitivity because LPS is the dominant antigen and antibody reaction is serovar specific. Because of the LPS diversity, recent research focused on the use of outer membrane proteins (OMPs) conserved among pathogenic *Leptospira* species to increase sensitivity of diagnostic tests.

A small number of leptospiral outer membrane lipoproteins are exposed on the cell surface, among which are LipL32, LipL21, and LipL41.3 Expression of the major OMP LipL32 has been demonstrated both in culture and in host infections,4 and its surface exposure on the bacterial membrane has recently been proven.3 Sera from patients with leptospirosis react strongly with the recombinant form of LipL325 and an enzyme assay using this antigen was able to detect human6 and animal cases of leptospirosis.7,8

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In this study we report on the production and characterization of three monoclonal antibodies (MAbs) against recombinant LipL32 (rLipL32) and their use in different approaches to demonstrate their potential for diagnosis of leptospirosis. The MAbs recognized the native protein on the surface of pathogenic Leptospira cells and did not react with saprophytic strains or other microorganisms, suggesting they could be a useful diagnostic tool for leptospirosis.

**MATERIALS AND METHODS**

**Leptospira strains and culture conditions**

*L. interrogans* serovar Copenhageni strain FIOCRUZ L1 130 used in this study was provided by A.I. Ko (Centro de Pesquisa Gonçalo Moniz, FIOCRUZ, Salvador, BA, Brazil). Other *Leptospira* serovars used were obtained from the Center for Zoonosis Control (Universidade Federal de Pelotas, Brazil). Leptospires were grown at 30°C in Ellinghausen-McCullough-Johnson-Harris (EMJH) medium (Becton Dickinson Co., MD), supplemented with 8% of bovine serum albumin.

**Antigen preparation**

The lipL32 gene, obtained by polymerase chain reaction (PCR) using the DNA from *L. interrogans* L1 130 as template, was cloned into the pAE expression vector that allows fusion of the protein with a 6×His tag. This plasmid was used to transform *Escherichia coli* BL21 (DE3). Purification of the protein was accomplished by affinity chromatography with Ni-NTA resin using the QIA EXPRESSIONIST Kit (Qiagen Corporation, Valencia, CA) following the manufacturer’s instructions. The eluate was then dialyzed against phosphate-buffered saline (PBS) and glycine 0.1%, pH 8.0, for approximately 16 hours at 4°C. Protein in the final preparation was quantified by the Bradford method.

**Generation and purification of MAbs**

Two 6-week-old BALB/c female mice were immunized intraperitoneally on day 1 with 100 µg of the recombinant protein LipL32 (rLipL32) mixed with Freund’s complete adjuvant (Sigma Aldrich Co., St. Louis, MO). This was followed by three intraperitoneal injections of rLipL32 mixed with Freund’s incomplete adjuvant (Sigma Aldrich) on days 14, 21, and 28. Three days before fusion the mouse with the highest titer of serum antibodies against rLipL32 in an indirect ELISA was cloned twice by limiting dilution. A secondary screening was performed by immunoblotting and indirect ELISA using supernatant from cloned cells and heat-treated *Leptospira* from different serogroups as antigen. Specific hybrid cell lines identified in this second screening were expanded and stored in liquid nitrogen. For ascites production the hybridomas were removed from liquid nitrogen, cultivated on DMEM with 10% FCS, collected by centrifugation, washed five times in DMEM without FCS, and injected into pristane-primed BALB/c mice. MAbs were purified from ascitic fluid by affinity chromatography on a protein A-Sepharose CL-4B column (GE Healthcare Company, Piscataway, NJ) according to the manufacturer’s instructions. Purification efficacy was evaluated by SDS-PAGE and final concentration was measured by spectrophotometry at 280 nm. Purified MAbs were stored at −20°C. The MAbs were isotyped by ELISA with a mouse subsotyping kit following manufacturer instructions (Sigma Aldrich).

**Gel electrophoresis and immunoblotting**

Proteins immunoprecipitated from outer membrane or heat-treated leptospiral cells were solubilized in final sample buffer 50 mM Tris-HCl (pH 6.8), 100 mM dithiothreitol, 0.1% bromophenol blue, 2% SDS, and 20% glycerol and separated in a 12% polyacrylamide gel using a discontinuous buffer system. After electrophoresis, the gels were stained with 0.2% Coomassie brilliant blue R-250 (in 10% acetic acid—45% methanol) or transferred electrophoretically to a polyvinylidene difluoride (PVDF) membrane (GE Healthcare) at 25 volts for 60 minutes. The membranes were blocked with 0.1 M PBS containing 0.1% Tween 20 (PBS-T, pH 7.4) and 5% skim milk for 30 minutes, washed twice in PBS-T and incubated for 1 hour with the MAbs diluted 1/1000 in PBS-T. After washing the membrane three times with PBS-T, a rabbit anti-mouse immunoglobulin (Ig) horseradish peroxidase conjugate (Sigma Aldrich) diluted 1:2000 in PBS-T or a MAb anti-LipL32 peroxidase conjugate was added for 1 hour and the membrane was again washed three times with PBS-T. The MAb-peroxidase conjugate was prepared according to established protocol. Bands were visualized after the addition of the substrate/chromogen solution (H2O2/4-chloro-1-naphthol).

**ELISA with intact and heat-treated leptospiral cells**

Seven-day cultures of *L. interrogans* serovar Copenhageni strain Fiocruz L1 130, and of saprophytic *L. biflexa* serovar patoc (strain Patoc I) were harvested by centrifugation (15,000g, for 30 minutes) at 4°C and washed once in PBS (0.01 M, pH 7.2). The cells were resuspended in PBS, counted in a Petroff-Hauser chamber and the concentration was adjusted to approximately 10⁸ cells per milliliter. For ELISA with intact leptospira cells, microtiter plates (Nunc Polysorp, Nalge Nunc International, Rochester, NY) were first coated overnight at 4°C with 100 µL of a 10 times diluted 0.1% poly l-lysine solution and then with 100 µL of the bacterial suspension in PBS for 2 hours at 30°C. Wells were washed 3 times with leptospira culture medium (LCM) and 100 µL of twofold dilutions of each ascites MAb in LCM was added to the wells for 2 hours at 30°C. Washing was repeated and 100 µL of rabbit anti-mouse Ig-POase conjugated was added for 2 hours at 30°C. After 2
washes with LCM and 3 washes with PBS, 100 μL of enzyme substrate/chromogen solution ([H2O2/orthophenylenediamine) in citrate-phosphate buffer, pH 5.0, was added and the reaction was allowed to take place in the dark for 10 minutes. Optical density was read at 450 nm in an ELISA reader (Multiskan MCC/340, Titerktek Instruments, Huntsville, AL). ELISA with heat-treated leptospiral cells was performed in the same way except that leptospiral cultures were first inactivated overnight at 56°C and stored at −20°C until use. As control of cell integrity, rat serum against cytoplasmatic protein GroEL was included in both tests. To investigate how protein conjugation would affect MAb performance in ELISA, a conjugate of MAB 1D9 and POase was also used in this experiment.

Direct and indirect immunofluorescence

Slide chambers (ICN Biomedicals Inc., Costa Mesa, CA) were coated with a 0.01% Poly l-lysine solution (Sigma Aldrich) and dried for 1 hour at room temperature. A 7-day culture of L. interrogans L1-130 was washed once in PBS, resuspended to a density of 10⁶ cells per milliliter in PBS, and incubated in the slide chamber for 2 hours at 30°C. The slides were washed twice with LCM and coated with MAb ascsites diluted 1:10 in LCM. After incubating for 1 hour at 30°C, the slides were washed again twice with LCM and with a 1:10 dilution of rabbit anti-mouse FITC conjugate was added and incubated for 1 hour in a dark humid chamber at 30°C. After washing with LCM a drop of mounting medium was added and a coverslip was sealed with acrylic. In experiments with permeabilized membrane, slides were incubated in 5 mL of methanol for 10 minutes at 4°C followed by washing twice with LCM. The following controls were used in this experiment: (1) MAb against Salmonella OMP was used as primary antibody, (2) rabbit anti-mouse FITC conjugate was applied to slides without primary antibody, and (3) normal mouse serum was used as primary antibody. For direct immunofluorescence MAB 1D9 FITC conjugate was produced following established procedures (14) and used together with a conjugate of rabbit antibodies against whole Leptospira and FITC (Ames Laboratories, National Veterinary Services Laboratory, Ames, IA) as positive control. Labeling was visualized by fluorescence microscopy (Olympus BX 51) with excitation wavelength of 450 nm.

Immunoprecipitation of native LipL32 and immunomagnetic separation of leptospiral cells

Surface immunoprecipitation of native LipL32 was performed according to Shang et al. (15) with modifications. A 2-mL volume of each heat-inactivated MAB ascites was mixed with 30 mL of a culture of L. interrogans L1-130 containing 3 x 10⁴ actively motile bacteria. After shaking the suspension gently for 1 hour at 30°C the cells were pelleted at 2000g for 15 minutes at 4°C, resuspended in PBS with 5 mM MgCl₂, centrifuged again, and resuspended in 9 mL of 10 mM Tris-HCl (pH 8.8), 2 mM ethylenediaminetetraacetic acid (EDTA), 1 mM phenylmethyl sulfonyl fluoride (PMSF). A volume of 1 mL of 10% protein grade Triton X 100 (Sigma Aldrich) was added and the suspension was agitated for 30 minutes at 4°C. The insoluble material was removed by centrifugation at 16,000g for 20 minutes and 1 mL of 2% deoxycholate, 50 μL of 20% SDS and 500 μL of Sepharose-SPa (Sigma Aldrich) were added to the supernatant. After agitating the mixture gently for 30 minutes at 4°C, the complex of Sepharose SpA-MAB-antigen was washed by centrifugation two times with 0.01% Triton X 100 in 10 mM Tris-HCl (pH 8.8) and resuspended in final sample buffer. The complex was submitted to SDS-PAGE and immunoblotting using MAB 1D9-POase as probe. As negative controls two additional immunoprecipitation experiments were carried out in parallel: in the first one MAB against LipL32 was omitted and in the second one the culture of L. interrogans L1-130 was replaced with L. biflexa serovar Patoc. For the immunomagnetic separation (IMS) procedure, 1D9 MAB was absorbed onto protein A-coated microspheres (Bangs Laboratories Inc., Fishers, IN). Briefly, 100 μL of particles with 1% solids was suspended with 900 μL of 50 mM pH 8.2 borate buffer (BB), washed twice with BB, resuspended in 1 mL of BB containing 1.2 mg of MAB and incubated with gentle agitation at 4°C for 16 hours. The MAB-coated particles were washed twice with BB and resuspended in stock buffer (100 mM borate pH 8.5, 0.1% bovine serum albumin [BSA], 0.05% Tween 20, 10 mM EDTA, 0.1% Na₂SO₄). An immunomagnetic separator (Invitrogen Corporation, Carlsbad, CA) was used in washing steps. Standard IMS was performed with 5 μL of MAB-coated particles per milliliter of different dilutions of a pool of sterile human sera artificially contaminated with pathogenic and saprophytic Leptospira species. The serum samples were gently agitated for 15 minutes at room temperature and then washed twice on a magnetic separator. The particles were then resuspended in 20 μL of lysis buffer (0.02 M Na₂HPO₄, 0.15 M NaCl, pH 7.2), boiled for 10 minutes and stored at −20°C until use in polymerase chain reaction (PCR) as described below.

PCR conditions

PCR primers lipL32 F: 5’ CGC TTG TGG TGC TGT CCG TGG T 3’ and lipL32 R: 5’ CTC ACC GAT TTC GCC TGT TG 3’ were used, resulting in a 264 bp amplicon of the lipL32 coding region. Briefly, 2 μL of DNA-IMS template was added to a tube with 1 U Taq DNA polymerase (Invitrogen) 150 ng of primers, 2.5 μL of 10x reaction buffer containing MgCl₂ and 0.2 mM dNTP. For DNA template optimization, volumes of 10 μL, 5 μL, 3 μL, 2 μL, and 1 μL of DNA-IMS were tested in the same conditions. Amplification was carried out in a Perkin Elmer 2400 thermocycler (PE Biosystems, Foster City, CA) with 1 cycle at 94°C for 5 minutes, 35 cycles at 94°C for 1 minute, 55°C for 1 minute, 72°C for 1 minute, and an extension of 7 minutes at 72°C at the end of the final cycle. Aliquots were analyzed by electrophoresis in 2% agarose gel with ethidium bromide and visualized under UV transillumination.

RESULTS

Generation of MABs

From a total of 54 hybridomas tested in the primary ELISA, 3, named 1D9, 36C4, and 412H4, have shown a specific reaction in the secondary ELISA screening and were selected for further characterization and testing in diagnostic applications.
The immunoglobulin classes of MAbs 1D9, 36C4, and 412H4 were IgG2b, IgG2a, and IgG3, respectively. All MAbs reacted with a band of an estimated molecular mass of 32 kd and neither reacted with *E. coli* as seen by immunoblotting (Table 1).

**ELISA with intact and heat-treated leptospira**

To evaluate MAb reactivity against native LipL32 protein ELISA protocols with intact and heat-treated leptospiral cells were used. A decrease in the absorbance of ELISA with intact cells occurred as the concentration of antibodies decreased, demonstrating the specificity of the reaction (Fig. 1A). In addition, antisera against the cytoplasmatic protein GroEL did not react in this ELISA. A conjugate of MAb 1D9-POase reacted with native LipL32 in both intact and heat-treated cell ELISA, but higher absorbance values were observed with heat-treated bacteria (Fig. 1B). In these ELISA experiments MAbs did not react with saprophytic bacteria and sera against GroEL reacted with heat-treated cells (data not shown).

**Direct and indirect immunofluorescence**

Reaction of MAbs with native LipL32 on the outer membrane of pathogenic *Leptospira* was investigated by indirect immunofluorescence and by an in-house prepared conjugate of MAb 1D9 and FITC. Bacterial cells were applied to slides with and without methanol treatment to evaluate the effect of the fixing method on MAb reactivity. The three MAbs labeled leptospiral cells in both fixing methods as could be seen by the intense fluorescence (Fig. 2). Labeling of leptospiral cells with MAb anti-*Salmonella* or normal mouse serum was not observed (data not shown).

**Immunoprecipitation of native LipL32 and use of a MAb on IMS-PCR**

The MAbs were used in immunoprecipitation assays to investigate their ability in binding the surface of live and motile leptospiral cells in suspension. Two MAbs were able to precipitate LipL32 from the suspension of live pathogenic bacteria (Fig. 3). In addition, MAb 1D9 was adsorbed onto protein A magnetic beads to explore its potential for use in diagnostic assays, which associates IMS with PCR amplification. With the use of IMS for capturing bacterial cells prior to DNA extraction and PCR amplification, it was possible to detect as low as 10 leptospires per milliliter of artificially contaminated human sera (Fig. 4). Sera contaminated with saprophytic *Leptospira* submitted to IMS and PCR did not show any amplification product (data not shown).

### Table 1. Reactions of Anti-LipL32 MAbs with Different Strains of *Leptospira* in Immunoblotting

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MAbs, monoclonal antibodies.
DISCUSSION

Leptospiral outer membrane protein LipL32 is prominent on pathogenic strains and is the major detergent-phase protein extracted by Triton X 114. Human sera from cases of leptospirosis reacted with native LipL32 and its recombinant form expressed in E. coli. After these findings this antigen has been used for developing diagnostic tests that detect antibodies in human and animal leptospirosis. However, diagnostic tests based on antibody detection suffer from lack of sensitivity in the first 10 days of infection. Antigen detection tests should be preferred when early diagnosis is important for initiating treatment, as is the case of leptospirosis. MAbs are important tools for antigen detection tests because of their high specificity and permanent availability. The three MAbs reported in this study reacted with a 32-kd polypeptidic band from dif-

FIG. 1. Enzyme-linked immunosorbent assay (ELISA) reactions of monoclonal antibodies (MAbs) anti-LipL32 using intact and heat-treated L. interrogans L1-130 cells. A: ELISA using 10^9 intact leptospiral cells per milliliter (100 μl per well) and MAbs 1D9 (●), 412H4 (■), 36C4 (▲), and GroEL antiserum (×) diluted 1:4 (1), 1:8 (2), 1:16 (3), 1:32 (4), and 1:64 (5). B: ELISA reactions with intact (□) and heat-treated cells (■) at 10^9 cell per milliliter (1), 4 × 10^8 cell per milliliter (2) and 2 × 10^8 cell per milliliter (3) and 1D9-POase conjugate diluted 1:1000.

FIG. 2. Staining of LipL32 from L. interrogans L1-130 by indirect immunofluorescence. L. interrogans L1-130 was fixed to microscope slides with and without methanol and probed with monoclonal antibodies (MAbs). Test control slides were made by reacting fixed bacteria with normal mouse sera and rabbit anti-mouse fluorescein isothiocyanate (FITC).
ferent leptospiral serovars on immunoblotting, a molecular mass similar to that of LipL32. Moreover, anti-LipL32 MAbs 1D9, 36C4 and 412H4 did not react with the other microorganisms tested. The MAbs were also able to detect native LipL32 antigen when used as primary antibody in indirect ELISA with intact and heat-treated leptospiral cells. Furthermore, MAb 1D9 maintained antibody activity after conjugation with peroxidase suggesting it is suitable for two-site immunoassays such as sandwich ELISA.

Indirect immunofluorescence was used to investigate MAb reaction after fixing leptospiral cells with or without methanol treatment. When anti-LipL32 MAB 1D9, 36C4 and 412H4 did not react with the other microorganisms tested. The MAbs were also able to detect native LipL32 antigen when used as primary antibody in indirect ELISA with intact and heat-treated leptospiral cells. Furthermore, MAb 1D9 maintained antibody activity after conjugation with peroxidase suggesting it is suitable for two-site immunoassays such as sandwich ELISA.

In conclusion, the results reported in this study demonstrated that MAbs generated against the recombinant form of LipL32 were able to recognize the native protein from pathogenic Leptospira. Because LipL32 is a surface protein conserved among pathogenic serovars and is not found on saprophytic strains, the MAbs against this target will be useful in the development of different test formats for diagnosis of human and animal leptospirosis.

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REFERENCES


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