

Leptospiral Proteins Recognized during the Humoral Immune Response to Leptospirosis in Humans

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Leptospirosis is an emerging zoonosis caused by pathogenic spirochetes belonging to the genus *Leptospira*. An understanding of leptospiral protein expression regulation is needed to develop new immunoprotective and serodiagnostic strategies. We used the humoral immune response during human leptospirosis as a reporter of protein antigens expressed during infection. Qualitative and quantitative immunoblot analysis was performed using sera from 105 patients from Brazil and Barbados. Sera from patients with other diseases and healthy individuals were evaluated as controls. Seven proteins, p76, p62, p48, p45, p41, p37, and p32, were identified as targets of the humoral response during natural infection. In both acute and convalescent phases of illness, antibodies to lipopolysaccharide were predominantly immunoglobulin M (IgM) while antibodies to proteins were exclusively IgG. Anti-p32 reactivity had the greatest sensitivity and specificity: positive reactions were observed in 37 and 84% of acute- and convalescent-phase sera, respectively, while only 5% of community control individuals demonstrated positive reactions. Six immunodominant antigens were expressed by all pathogenic leptospiral strains tested; only p37 was inconsistently expressed. Two-dimensional immunoblots identified four of the seven infection-associated antigens as being previously characterized proteins: LipL32 (the major outer membrane lipoprotein), LipL41 (a surface-exposed outer membrane lipoprotein), and heat shock proteins GroEL and DnaK. Fractionation studies demonstrated LipL32 and LipL41 reactivity in the outer membrane fraction and GroEL and DnaK in the cytoplasmic fraction, while p37 appeared to be a soluble periplasmic protein. Most of the other immunodominant proteins, including p48 and p45, were localized to the inner membrane. These findings indicate that leptospiral proteins recognized during natural infection are potentially useful for serodiagnosis and may serve as targets for vaccine design.

Infection by pathogenic *Leptospira* species is an important and frequently life-threatening cause of human disease characterized by hematogenous dissemination to multiple organs including the brain, aqueous humor, liver, lungs, and kidneys. Leptospirosis occurs in a variety of urban and rural settings, and is considered to be the most widespread zoonosis in the world (10, 22, 43, 46). The wide distribution of *Leptospira* species results from their ability to colonize the renal tubules of a diverse group of wild and domestic animals. After urinary shedding, *Leptospira* species are transmitted directly to a new host or indirectly through contact with organisms contaminating moist environments. The ability to survive as free-living organisms is unique among the invasive spirochetes and presumably reflects differential expression of proteins involved in adaptation to the environment outside the mammalian host. Based upon these biological considerations, it is anticipated that certain leptospiral proteins expressed in cultivated organisms may or may not be expressed during infection (5). Proteins expressed during infection may serve as determinants in

leptospiral pathogenesis and as targets for the host immune response. To develop a more comprehensive understanding of leptospiral protein expression, we have used the humoral immune response during human leptospirosis as a reporter of protein antigens expressed during infection.

The identification of leptospiral antigens expressed during infection has potentially important implications for the development of new serodiagnostic and immunoprotective strategies. Most research on leptospiral antigens has been focused on lipopolysaccharide (LPS). Variations in the carbohydrate side chains of LPS are responsible for the antigenic diversity observed among leptospiral serovars, of which over 250 have been identified (10). As a result of the immunodominance of LPS, leptospiral vaccines consisting of inactivated whole-cell immunogens, termed bacterins, are based largely on inducing antibodies against carbohydrate epitopes within this moiety. For this reason, currently used vaccines often do not provide cross-protection against serovars not contained in vaccine preparations. In contrast, leptospiral protein extracts can induce protection against challenge with heterologous serovars in experimental animal models (39).

The antigenic variability of leptospiral LPS is also a limitation for serodiagnosis. The microscopic agglutination test (MAT) has been the “gold standard” confirmatory test for the

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past 70 years and is most likely based on seroreactivity with the LPS antigens. The need to assess agglutination by dark-field microscopy and maintain a large battery of live leptospiral antigens in culture restricts the use of the MAT to a few reference laboratories worldwide. More widely accessible serologic approaches have been developed, approaches which take advantage of cross-reactive antigens in crude extracts which are shared among diverse leptospiral serovars. These cross-reactive antigens could include proteins and/or components of leptospiral LPS (30). Currently available serologic assays include the macroscopic agglutination (31), indirect hemagglutination (28), and microcapsule agglutination (2) tests, all of which are less sensitive than the MAT and identify less than 50% of patients presenting with early-phase leptospirosis. Assays that detect immunoglobulin M (IgM) and are based upon crude antigen (1, 14, 27, 38, 44) appear to be more sensitive for serodiagnosis but may be subject to variations in specificity.

The need to develop better serodiagnostic strategies has become even more critical now that leptospirosis has been recognized as an emerging cause of epidemics such as the 1995 outbreak of severe pulmonary hemorrhage syndrome in Nicaragua (42). In the rest of Latin America, large epidemics occur annually among impoverished populations in major urban centers and are associated with case fatality rates of over 15% (22, 29). In order to respond to this emerging public health problem, case identification needs to be performed promptly so that rapid outbreak investigations and timely administration of antibiotic therapy can be implemented. However, the broad spectrum of clinical presentations associated with leptospirosis hampers case identification. In several outbreak situations, leptospirosis was initially confused with dengue (22, 26, 35, 42). Therefore, early diagnosis must rely on an efficient laboratory test that can be easily implemented in the field without dependence on reference laboratory settings.

For the purpose of developing a diagnostic test that can be applied to the variety of epidemiological situations associated with human and veterinary leptospirosis, ideally an antigen which is highly conserved among diverse pathogenic leptospiral strains should be selected. The amino acid sequences of leptospiral proteins, such as the major outer membrane protein, LipL32, appear to be highly conserved across leptospiral species (16). To identify candidate protein antigens for serodiagnosis, we characterized the humoral immune response in leptospirosis by studying the immunoblot reactivity of a large number of patients and by characterizing the recognized protein antigens. Earlier one-dimensional immunoblot studies used clinical sera to identify the relative mobility of several immunogenic proteins but were unable to further characterize these antigens (7, 8). Recent molecular characterization of leptospiral proteins such as GroEL (4, 33), DnaK (3), the OmpL1 porin (15, 36), and the lipoproteins LipL41 (37) and LipL32/MOMP (16) has provided the antibody reagents needed to definitively identify many of the major protein antigens. Sera from leptospirosis patients from Barbados and Brazil were used to perform one- and two-dimensional immunoblot analyses of leptospiral proteins. The major outer membrane protein, LipL32, and heat shock proteins GroEL and DnaK were found to be the dominant immunoreactive protein antigens. The human immune response also identified the sur-

face-exposed lipoprotein, LipL41, the outer membrane porin, OmpL1, and a series of other less well characterized membrane-associated proteins. On the other hand, sera from patients did not recognize the previously described protein, LipL36, which is a prominent component of the leptospiral outer membrane in organisms cultured in vitro. We believe that these data provide useful insights into the pathogenesis of leptospirosis and the identification of candidate protein antigens for serodiagnosis and immunoprotection.

MATERIALS AND METHODS

Bacterial strains and media. *Leptospira kirschneri* strain RM52 (41) and other leptospiral strains were obtained from the National Leptospirosis Reference Center (National Animal Disease Center, Agricultural Research Service, U.S. Department of Agriculture, Ames, Iowa). Most of the leptospiral strains used in this publication are described in a recent DNA relatedness study (6). Leptospiral strains analyzed by 1D and 2D electrophoresis were clinical isolates from Salvador, Brazil (*Leptospira interrogans* serogroup Icterohaemorrhagiae serovar copenhageni) (22) and Barbados (*L. kirschneri* serogroup Autumnalis serovar bim) (21). Leptospires were cultivated in Johnson-Harris Bovine Serum Albumin Tween 80 medium (Bovuminar PLM-5 Microbiological Media; Intergen) (20). *Escherichia coli* BLR(DE3)pLysS [F^- ompT hsdS_B (r_B^- m_B⁻) gal dcm Δ(*srl-recA*)306::Tn10(TcR) (DE3) pLysS(CmR)] (Novagen) was used as the host strain for the pRSET expression vector (Invitrogen). *E. coli* cells were routinely grown in Luria-Bertani broth or on Luria-Bertani agar (34).

Patients and control individuals. During active hospital-based surveillance for epidemic leptospirosis in the city of Salvador, Brazil, consecutive patients were identified between March 1996 and February 1998 according to a clinical definition based on the presence of characteristic severe manifestations (jaundice and acute renal failure) without laboratory or radiological evidence for a disease other than leptospirosis (22). According to the surveillance routine, a first, acute-phase serum sample was collected at the time of hospital admission. A second, convalescent-phase serum sample was collected 14 or more days after the collection of the acute-phase sample, typically during outpatient evaluation after hospital discharge. Informed consent was obtained from patients or their guardians, and the guidelines of the Brazilian Ministry of Health, Barbados Ministry of Health, the New York Presbyterian Hospital, and the U.S. Department of Health and Human Services were followed in the conduct of the clinical research.

Among more than 700 cases with clinically suspected leptospirosis identified in 1996 and 1997, 100 were selected randomly from three groups of patients from whom paired serum samples were obtained. Cases in this sample reported a mean of 7.4 days (standard deviation, ± 3.9 days) of illness prior to hospitalization. The mean interval between collections of paired serum samples was 23.7 days (± 9.7 days). Leptospirosis Group 1 ($n = 73$) consisted of patients with laboratory-confirmed leptospirosis defined by a fourfold rise in the titer of the MAT between paired serum samples, a reciprocal MAT titer greater than 800 in one or more serum samples, or leptospires identified in cultures of blood or urine by dark-field microscopy (22). Leptospirosis Group 2 ($n = 11$) consisted of patients defined as probable cases based on a reciprocal MAT titer of more than 100 in one or more serum samples. Leptospirosis Group 3 ($n = 16$) consisted of patients without MAT evidence for a confirmed or probable diagnosis. Sera were also obtained from five patients with culture-documented leptospirosis acquired in Barbados. One hundred thirty-five serum samples were selected from existing collections obtained from five control groups of individuals from Salvador, Brazil, without known leptospirosis. Control Group 1 ($n = 60$) members were randomly selected from healthy participants of a city-wide population-based seroprevalence survey. Control Group 2 ($n = 30$) consisted of blood bank donors. Control Group 3 ($n = 15$) comprised individuals with positive serum Venereal Disease Research Laboratory (VDRL) test results. Control Group 4 ($n = 15$) consisted of patients with laboratory-confirmed acute hepatitis A or B virus infection. Control Group 5 ($n = 15$) consisted of patients with serologically confirmed dengue.

Rabbit antisera. Leptospiral GroEL serum was a generous gift of B. Adler (Monash University, Clayton, Victoria, Australia). Leptospiral DnaK serum was a generous gift of J. Timoney (Gluck Equine Institute, Lexington, Ky.). Antisera to OmpL1 (15), LipL32 (16), LipL36 (17), LipL41 (37), and LipL45/31 (J. Matsunaga, M. Mazel, T. Young, and D. A. Haake, unpublished data) were prepared by immunizing New Zealand White rabbits with purified His6 fusion proteins.

Gel electrophoresis and immunoblotting. For one-dimensional sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), samples were solubilized in a final sample buffer composed of 62.5 mM Tris hydrochloride (pH 6.8), 10% glycerol, 5% 2-mercaptoethanol, and 2% SDS and were separated on a discontinuous buffer system (23). Two sets of molecular mass standards were used in SDS-PAGE: for quantitative immunoblot analyses with individual patients' sera, prestained high-range protein standards (Gibco BRL) which contained rabbit skeletal muscle myosin H-chain (200 kDa), rabbit muscle phosphorylase B (97.4 kDa), bovine serum albumin (68 kDa), hen egg white ovalbumin (43 kDa), bovine carbonic anhydrase (29 kDa), beta lactoglobulin (18.4 kDa), and hen egg white lysozyme (14.3 kDa) were used; for qualitative analyses with pooled human sera and two-dimensional electrophoresis, protein standards (Bio-Rad) which contained rabbit skeletal muscle myosin (200 kDa), *E. coli* beta-galactosidase (116 kDa), rabbit muscle phosphorylase B (97 kDa), bovine serum albumin (66.2 kDa), hen egg white ovalbumin (45 kDa), bovine carbonic anhydrase (31 kDa), soybean trypsin inhibitor (21.5 kDa), and hen egg white lysozyme (14.4 kDa) were used. Two-dimensional gel electrophoresis was performed according to the method of O'Farrell (32) modified by Görg et al. (11, 12). Samples for 2D gel electrophoresis were solubilized in a rehydration solution composed of 8 M urea, 2% Triton X-100, 20 mM dithiothreitol, and 2% carrier ampholyte mixture (IPG Buffer; Pharmacia). Immobiline DryStrips (Pharmacia) were rehydrated overnight in rehydration solution containing leptospiral material. Isoelectric focusing was performed using a Pharmacia Multiphor II system. After isoelectric focusing, SDS-PAGE was performed as described above. Gels were stained with Coomassie brilliant blue or were transferred to 0.45- μ m Immobilon-P membranes (Millipore) for immunoblotting.

Paired serum samples from patients with leptospirosis and single samples from control subjects were evaluated in one-dimensional immunoblot analyses. Immunoblots of whole-cell leptospiral extract separated in 10% polyacrylamide gels were blocked with 5% nonfat dry milk TBS (0.05 M Tris buffered saline, pH 7.4)-0.05% Tween 20 (TBS-T) and probed with individual serum samples diluted 1:100 in TBS-T, and after being washed they were probed with anti-human IgG or IgM goat antibodies conjugated to alkaline phosphatase (Sigma Chemical Co.), diluted 1:1,000 in TBS-T. Individual immunoblots were then developed in NBT/BCIP solution (Bio-Rad) and scored when dry. For analyses with pooled human sera, immunoblots of 12% polyacrylamide gels were treated with sodium periodate in order to reduce background reactivity with carbohydrate antigens and enhance visualization (45). Membranes were blocked with 5% nonfat dry milk in 0.1 M phosphate-buffered saline (PBS) (pH 7.4)-0.1% Tween 20 (PBS-T) and probed with pooled human sera diluted 1:1,000 in PBS-T. Two separate pools of human sera were utilized, consisting of convalescent-phase sera with high MAT titers from Leptospirosis Group 1 patients ($n = 20$) identified in Salvador, Brazil, or from patients who acquired leptospirosis in Barbados ($n = 5$). As an additional method to enhance visualization, pooled sera were incubated with Immobilon-P membrane coated with His6-LipL32 fusion protein (16) to remove antibodies that recognize a predominant immunoreactive leptospiral antigen. After incubation with pooled sera, immunoblots were probed with anti-human immunoglobulin mouse antibodies conjugated to horseradish peroxidase (Amersham) diluted 1:1,000. Antigen-antibody binding was detected using the enhanced chemiluminescence system (ECL; Amersham). Blots were incubated in ECL reagents for 1 min and then exposed to Hyperfilm (Amersham).

Scoring of immunoblots and statistical analysis. A pilot study was performed to identify the spectrum of antigen bands recognized by 30 convalescent-phase sera from Leptospirosis Group 1 patients. Relative mobility (M_r) was estimated for identified antigens based on comparisons with prestained high-range protein molecular mass standards (Gibco BRL). Two serum samples which in combination recognized all identified antigen bands were chosen and used in subsequent analyses as quality control standards to identify the positions of antigen bands in each immunoblot. For the purpose of determining the proportion of sera that react to individual antigen bands, two investigators used a scale based on visual intensity (1 [barely visible] to 4 [intense staining]) to score immunoblots. After performing independent observations, the investigators jointly reviewed discordant results and assigned final values after arriving at an agreement. Positive reactions to a particular antigen band were defined by scores of ≥ 2 . Data were entered into EpiInfo (version 6.04, Centers for Disease Control and Prevention) and analyzed using the SAS system (version 6.11, SAS Institute). The frequencies of band recognition of sera from leptospirosis cases were compared with those of sera from healthy community controls using the chi-square test with Yates' correction. Logistic regression analysis was used to assign an order to the antigens and sequential combinations were graphed in a receiver-operator characteristic curve.

Cell fractionation studies. (i) Soluble and total membrane fractions. A leptospiral culture containing 4×10^{10} *L. kirschneri* isolates was washed twice in 5 mM $MgCl_2$ -PBS at 4°C and resuspended in 6 ml of lysis buffer (20 mM Tris [pH 8]-150 mM NaCl-2 mM EDTA-2 mg of lysozyme per ml). The bacterial suspension was subjected to three cycles of freezing, thawing, and tip sonication, followed by centrifugation at $100,000 \times g$ for 30 min to separate the soluble supernatant fraction from the membrane pellet fraction. The supernatant was precipitated with acetone.

(ii) Triton X-114 fractions. *L. kirschneri* organisms were also fractionated by solubilization with 1% Triton X-114 by a modification of the method described previously (16). In brief, a leptospiral culture containing 4×10^{10} *L. kirschneri* isolates was washed twice in 5 mM $MgCl_2$ -PBS and extracted in the presence of 1% protein grade Triton X-114 (Calbiochem), 150 mM NaCl, 20 mM Tris (pH 8), and 2 mM EDTA at 4°C. The insoluble material was removed by centrifugation at $17,000 \times g$ for 10 min. After centrifugation, 20 mM $CaCl_2$ was added to the supernatant. Phase separation was performed by warming the supernatant to 37°C and subjecting it to centrifugation for 10 min at $1,000 \times g$. The detergent and aqueous phases were separated and precipitated with acetone.

RESULTS

Quantitative analysis of humoral immune response to leptospirosis. In initial one-dimensional SDS-PAGE and Western blot analyses, sera from patients with laboratory-confirmed leptospirosis (Group 1) recognized up to 25 distinct leptospiral antigen bands with M_r greater than 20 kDa from *L. interrogans* serovar copenhageni, the etiologic agent of urban epidemics in Salvador, Brazil (22). The predominant humoral response against these antigens during infection was IgG antibodies, regardless of whether samples were analyzed from the acute or convalescent phase of illness (mean interval and standard deviation between onset of illness and sample collection, 8.4 ± 4.2 and 32.1 ± 10.6 days, respectively) (Fig. 1). Although an IgM antibody response was consistently detected against low M_r species corresponding to leptospiral LPS, little or no detectable IgM response to higher M_r antigens was identified during the acute or convalescent phase of illness (Fig. 1). Two exceptions were observed: IgM reactivity to p37 and a doublet of antigen bands that has M_r values (35 to 36 kDa) consistent with those for leptospiral flagellar proteins (Fig. 1, lanes 2, 4, and 6).

The positions of the 13 most frequently recognized leptospiral antigens are shown in Fig. 1. In subsequent immunoblot analyses, the frequencies of IgG antibody reactivity to these protein antigens were quantified for acute- and convalescent-phase sera from leptospirosis patients and compared to those for sera from groups of control individuals (Table 1). During leptospiral infection, patients generated markedly heterogeneous IgG responses with respect to the number and distribution of antigens that were recognized (Fig. 1). However, an IgG response was frequently observed against leptospiral antigens p32 and p62. In Group 1 (patients with laboratory-confirmed leptospirosis), 37% (27/73) and 45% (33/73) had IgG antibodies to p32 and p62, respectively, during the acute phase of illness. This proportion increased to 84% (61/73) and 59% (43/73) for p32 and p62, respectively, during the convalescent phase. Between the acute and convalescent phases of illness, 50% of Group 1 patients had seroconversion of the immunoblot reactivity to p32 in contrast to 16% with seroconversion responses to p62. The anti-p32 IgG response appeared to be highly specific for leptospirosis, with 0 to 5% reactivity in all control groups except hepatitis patients. In contrast, the anti-p62 response was less specific, with up to 23% reactivity in

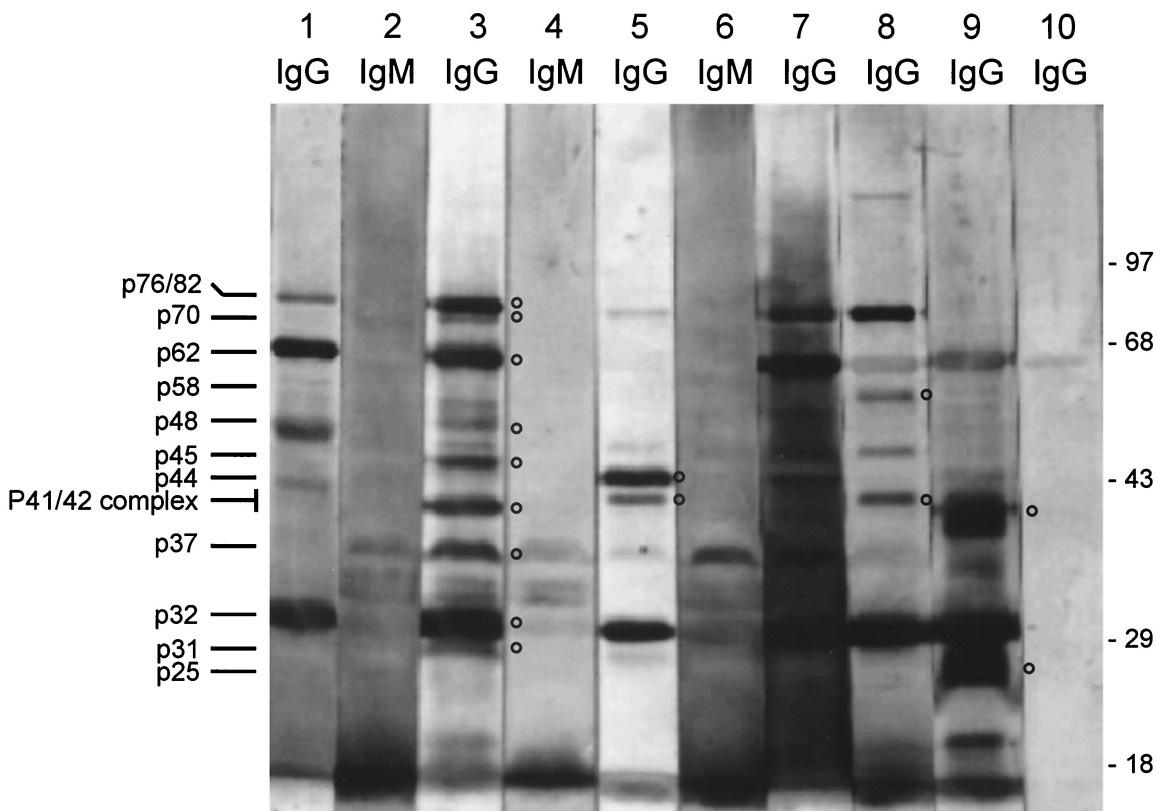


FIG. 1. Representative immunoblots of sera from patients with leptospirosis during urban epidemics in the city of Salvador, Brazil. SDS-PAGE (10% polyacrylamide) was used to separate antigen extracts of a clinical isolate of *L. interrogans* serovar copenhageni. Immunoblots were incubated with serum samples of individual patients (patients 235 [lane 1], 66 [lanes 2 and 3], 7 [lanes 4 and 5], 134 [lanes 6 and 7], 205 [lane 8], and 66 [lane 9]) and probed with either anti-IgM (IgM) or anti-IgG (IgG) secondary antibody conjugate. Lane 10 was incubated with serum from a healthy control individual. The locations of molecular mass markers (in kilodaltons) and selected leptospiral proteins are shown on the right and left, respectively. In addition, the positions of leptospiral proteins are marked (○) within the immunoblot and correspond to the following, in descending order: p76/82, p70, p62, p48, p45, the p41/42 complex, p37, p32, and p31 in lane 3; p44 and the p41/42 complex in lane 5; p58 and the p41/42 complex in lane 8; and the p41/42 complex and p25 in lane 9.

blood bank donors. Significant, although less uniform, immunoblot reactivity (16 to 22% in acute-phase sera and 25 to 59% in convalescent-phase sera from Group 1) was observed against a second group of antigens that included p37, the p41/42 complex, p45, p48, p76, and p82 (Table 1). The p41/42 complex comprised at least two distinct antigen bands with M_r of 41 to 42 kDa that were not consistently discriminated in immunoblot analyses. In community control groups, reactivity against each of the six antigens was less than or equal to 10%.

Samples from patients with leptospirosis that was clinically suspected but unconfirmed by MAT had higher frequencies of immunoblot reactivity to leptospiral antigen bands than those from healthy individuals and patient control groups selected from populations at risk for epidemic leptospirosis in Salvador, Brazil. As shown in Table 1, anti-p32 IgG antibodies were detected during the acute and convalescent phases of illness in 36 and 73% of the probable cases (Group 2), respectively, and 6 and 25% of unconfirmed cases (Group 3), respectively. Immunoblot reactivity appeared to be associated with illness rather than prior exposure in many cases unconfirmed by MAT. Among Group 2 and 3 patients, 64% (7/11) and 38% (6/16), respectively, demonstrated seroconversion with respect to their immunoblot reactivity to one or more antigens. p32

was the predominant antigen recognized in seroconversion responses: 45% (5/11) of Group 2 patients and 25% (4/16) of Group 3 patients had positive reactions to this antigen in convalescent-phase and not acute-phase serum samples.

Because of the heterogeneity in patients' IgG response to leptospiral antigen bands, combinations of results for individual antigens were evaluated to determine whether the sensitivity of the immunoblot reaction could be augmented. The best combinations were identified in logistic regression models and are presented in Table 2 and Fig. 2. For acute-phase samples, inclusion of the results for the immunoblot reactivity for p62 and p76 with those for p32 increased sensitivity 1.7-fold, from 37 to 63%. However, the false-positive rate increased threefold, from 5 to 15%, because of the reduced specificity of the p62 response. For convalescent-phase samples, no combination significantly improved the sensitivity and specificity of the immunoblot reactivity against a single antigen band (p32).

One limitation of these analyses was that separation of proteins with similar migration patterns was limited in one-dimensional mini-gels. Although positive-control sera were used on every immunoblot to identify major proteins, differentiation between two proteins of similar size was difficult, and frequen-

TABLE 1. Proportion of sera from confirmed and unconfirmed cases of leptospirosis and groups of control individuals which recognize leptospiral proteins by immunoblotting^a

Protein	% Sera with IgG reactivity in:													
	Leptospirosis cases ^b									Control groups ^c				
	Confirmed (n = 73)			Probable (n = 11)			Unconfirmed (n = 16)			Healthy community individuals (n = 60)	Blood bank donors (n = 30)	VDRL positive individuals (n = 15)	Cases of hepatitis (n = 15)	Cases of dengue (n = 15)
	Acute phase	Convalescent phase	% Sero-conversion ^d	Acute phase	Convalescent phase	% Sero-conversion	Acute phase	Convalescent phase	% Sero-conversion					
p82	17	34 ^f	25	18	27	9	13	19	6	5	3	7	7	
p76	18 ^f	49 ^f	36	18	27	9		13	13	2	7			7
p70	10	10	6				6	19	13	7	3		20	
p62	45 ^f	59 ^f	16	18	18		19	19		10	23	7		
p58	5	8	4	9						3	7			7
p48	16 ^f	25 ^f	12	9	18	9		6	6	3	10			
p45	22	59 ^f	41	9	18	18	19	31	13	10	3		7	
p44	5	10	7							5	3			7
p41/42 ^e	21 ^f	36 ^f	18	9	9	9	19	31	13	7	10	20		7
p37	21 ^f	29 ^f	13	9	9		6	13	13	5		7	13	
p32	37 ^f	84 ^f	50	36	73	45	6	25	25	5			13	
p31	3	17	16		9	9								
p25	4	4	1							2				

^a The chi-square test was used to compare the frequencies of band recognition of sera from leptospirosis cases with those of sera from healthy community individuals (n = 60). Values of zero are not shown to improve clarity.

^b Paired acute- and convalescent-phase samples were tested for all leptospirosis cases. A confirmed leptospirosis case was defined as having a fourfold rise in the microagglutination test titer between paired serum samples or a titer of greater than 1:800 in one or more samples. Probable leptospirosis was defined as having a titer of $\geq 1:200$ in a single sample.

^c Control groups were chosen among residents of the city of Salvador, Brazil. Healthy community individuals were selected randomly from participants of a city-wide population-based seroprevalence survey. VDRL, Venereal Disease Research Laboratory test.

^d Seroconversion was defined for patients whose samples were shown by immunoblotting to be nonreactive during the acute phase of illness and reactive during the convalescent phase. Proportions are calculated from the total numbers of sample pairs evaluated in the immunoblot analysis.

^e The p41/42 complex (p41/42) includes at least two antigen moieties that could not be consistently distinguished in these SDS-PAGE and immunoblot analyses. Proportions were calculated based on a reaction to any of the moieties in this complex.

^f The difference (by chi-square test) between frequencies of band recognition of sera from leptospirosis cases and those of sera from healthy community individuals (n = 60) is statistically significant (P < 0.05).

cies may represent reactivity to more than one protein. The diversity of immunoreactive proteins may be greater than suggested by the analysis presented in Table 1.

Conservation of protein antigens among leptospiral strains. Immunoblot analysis in Fig. 3 shows that pooled convalescent-

TABLE 2. Proportion of sera from confirmed and unconfirmed cases of leptospirosis and control individuals which recognize one or more moieties within specific combinations of leptospiral proteins by immunoblot

Combinations of protein bands	% Sera with IgG reactivity to protein bands alone or in combination ^a in:			
	Leptospirosis cases			Community controls (n = 60)
	Confirmed (n = 73)	Probable (n = 11)	Unconfirmed (n = 16)	
Acute phase				
p32	37	36	6	5
p32 + p62	58	45	25	15
p32 + p62 + p76	63	45	25	15
Convalescent phase				
p32	84	73	25	5
p32 + p76	89	73	25	8
p32 + p45 + p76	91	73	38	17
p32 + p41/42 ^b + p45 + p76	94	82	50	20

^a Combined sensitivity and false positives were defined as positive reactions with any single protein of the combination.

^b p41/42c, p41/42 complex.

phase sera from leptospirosis patients from Salvador, Brazil, recognized a consistent pattern of immunodominant antigens in leptospiral strains other than *L. interrogans* serovar copenhageni. Sera from patients who acquired leptospirosis in Barbados produced similar immunoblot reactivity (data not shown). Up to 14 distinct bands were detected in a single leptospiral strain. Among different serovars, small polymorphisms were observed in the M_r of particular immunodominant antigens such as the p37, p45, p48, and p58 proteins. The patterns of reactivity to these antigens fell into four classes. The p62 and p76 proteins were Class I antigens, detected in all organisms within the genus *Leptospira*, including pathogenic and nonpathogenic strains. There were at least five Class II antigens, p31, p32, p41/42, p45, and p82, which were consistently identified among all pathogenic strains. Class III antigens, which were found in most pathogens, included the p48 and p58 proteins and the high-molecular-weight p160 protein. Class IV antigens, such as p37 and p25, were expressed in one or only a few strains. On the basis of these immunoblot results, *Leptospira inadai* and *Leptospira weilii* appear to share few protein antigens (only the p32, p62, and p76 proteins) with other pathogenic *Leptospira* species. The expression of the immunodominant proteins by leptospiral strains is summarized in Table 3.

Identification of protein antigens by 2-D gel electrophoresis.

A comparison of silver-stained 2-D gels prepared using pH 3 to 10 versus pH 4 to 7 Immobiline DryStrips showed that virtually

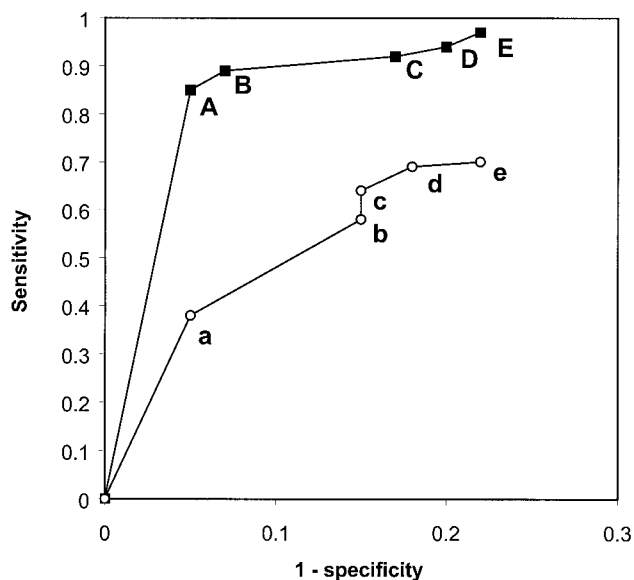


FIG. 2. Receiver-operator characteristic curve showing the sensitivity and false-positive rate for immunoblot seroreactivity to combinations of leptospiral proteins. Sensitivity and 1-specificity were defined as the proportion of sera from confirmed cases and community control individuals, respectively, which recognized at least one leptospiral antigen in a combination. The best order of antigen combinations for acute-phase sera was as follows: p32 alone (a); p32 or p62 (b); p32, p62, or p76 (c); p32, p62, p76, or p41 (d); p32, p62, p76, p41, or p45 (e). For convalescent-phase sera the best order was as follows: p32 (A); p32 or p76 (B); p32, p76 or p41 (C); p32, p76, p41, or p45 (D); p32, p76, p41, p45, or p62 (E).

all leptospiral proteins had isoelectric points within the pH 4 to 7 range (data not shown). In addition, there was better protein separation with 2-D gels prepared using pH 4 to 7 Immobiline DryStrips. On the basis of these preliminary experiments, a decision was made to use pH 4 to 7 Immobiline DryStrips in the 2-D immunoblot studies. Specific antisera were used to identify the location, in 2-D immunoblots, of DnaK, GroEL, OmpL1, LipL32, LipL36, LipL41, and LipL45/31. 2-D immunoblots of leptospiral strain *L. interrogans* serovar copenhageni from and endemic to Brazil or *L. kirschneri* serovar bim from and endemic to Barbados were probed with pooled convalescent-phase sera from leptospirosis patients from the same region. Significant differences were not observed with respect to the antigen patterns recognized by sera from patients of the two epidemiologically distinct regions. As shown in immunoblot analyses of serovar copenhageni antigens (Fig. 4 and Table 3), the electrophoretic mobilities of p76, p62, one antigen band within the p41/42 complex, and p32 allowed identification of these proteins as DnaK, GroEL, LipL41, and LipL32, respectively. Probing of 2-D immunoblots with pooled patient sera also demonstrated reactivity with the 33-kDa form of OmpL1 (Fig. 4). Reactivity of pooled patient sera to other previously characterized leptospiral proteins was either weak (LipL45/31) or not identified (LipL36). In addition, pooled patient sera reacted with several uncharacterized proteins, including the p45, p25, and p22 proteins. The latter two proteins were not well visualized in 1-D immunoblots (Fig. 1 and 3), probably because they were obscured by reactivity to leptospiral LPS antigens migrating in the lower-molecular-mass re-

gion of immunoblots. This demonstrates that in addition to providing definitive identification of protein antigens, a second advantage of the isoelectric focusing step of 2D immunoblots is the resultant separation of leptospiral protein antigens, such as the 25- and 22-kDa proteins, from LPS.

Localization of protein antigens by leptospiral fractionation. We analyzed the behavior of protein antigens in two complementary leptospiral fractionation procedures. The first technique separated organisms into total membrane (cytoplasmic membrane and outer membrane) and soluble (cytoplasm and periplasm) fractions. The second technique involved separation of organisms into Triton X-114 soluble and insoluble fractions, followed by phase partitioning of the Triton X-114 soluble fraction into detergent (hydrophobic) and aqueous (hydrophilic) phases. Previous leptospiral fractionation studies have demonstrated that the Triton X-114 insoluble material consists of the protoplasmic cylinder, including the cytoplasm, cytoplasmic membrane, and peptidoglycan cell wall, including penicillin-binding and flagellar proteins (19). The Triton X-114 detergent phase has been shown to contain outer membrane components, including leptospiral LPS, OmpL1 (an outer membrane porin), and several lipoproteins, including LipL32 (the major outer membrane protein), LipL36, and LipL41, while Triton X-114 aqueous phase would be expected to contain soluble periplasmic proteins (16, 17, 37).

Immunoblotting of these fractions with pooled convalescent-phase sera from leptospirosis patients revealed that most protein antigens were found in the cytoplasmic membrane, as indicated by the similarity of the total membrane (Fig. 5, lane MP) and Triton X-114-insoluble (Fig. 5, lane TP) fractions. Notable exceptions to this pattern are GroEL, LipL32, LipL41, p31, p37, and p25. GroEL is one of only two protein antigens that appear prominently in the soluble fraction (Fig. 5, lane MS). Another indication that its primary location is within the cytoplasm is the inability of Triton X-114 to release GroEL from the protoplasmic cylinder, consistent with the findings of a previous study (17). LipL32, LipL41, and p31 (the 31-kDa form of LipL45) were found in the total membrane and Triton X-114 detergent phase fractions (Fig. 5, lanes MP and TD, respectively), indicative of their outer membrane location (18, 37). LipL41 is one of at least two components in the p41/42 complex; a second component is an inner membrane antigen with slightly higher M_r that partitioned into the total membrane and Triton X-114-insoluble fractions (Fig. 5, lanes MP and TP). The 37- and 25-kDa antigens were predominant bands in immunoblots of the Triton X-114 aqueous phase (Fig. 5, lane TA), indicating that these antigens differ in fundamental ways from most of the other proteins recognized by the humoral immune response. Although similar in size and location to the flagellar proteins, the 37-kDa band is distinguishable from those proteins on the basis of its appearance as a single band, its solubilization by Triton X-114 (Fig. 5, lane TA), and its inconsistent expression in pathogenic leptospiral strains (Fig. 3). The flagellar proteins appear as a 35- and 36-kDa doublet (Fig. 1) and are not solubilized by Triton X-114. In addition, expression of flagellar proteins is conserved among all leptospiral species and serovars (data not shown). Subcellular locations of the immunodominant leptospiral proteins are summarized in Table 3.

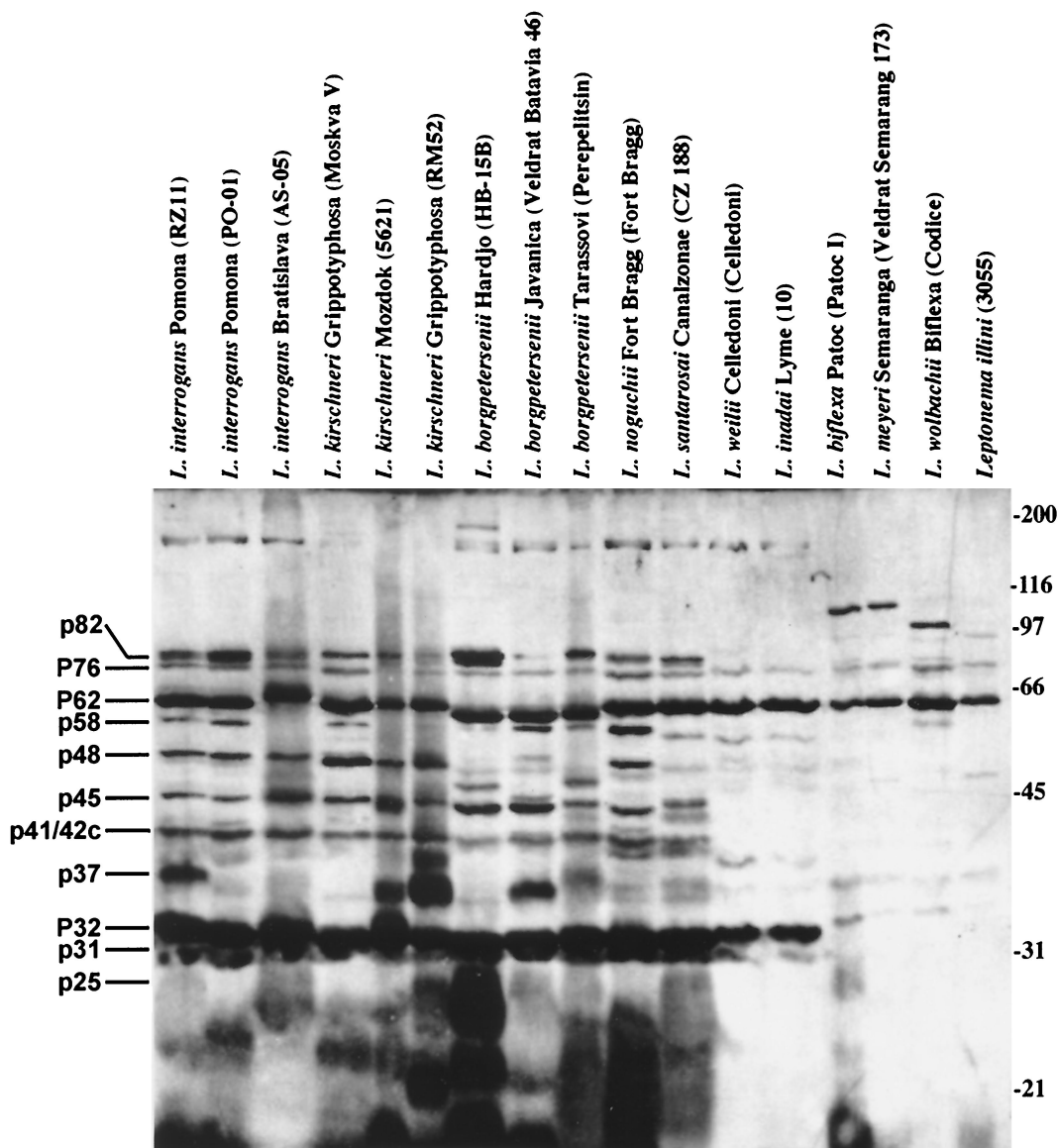


FIG. 3. Immunoblot panel of *Leptospira* species probed with pooled convalescent-phase sera from leptospirosis patients in Salvador, Brazil. The locations of molecular mass markers (in kilodaltons) and selected leptospiral proteins are indicated to the right and left, respectively. Most proteins were conserved among pathogenic *Leptospira* species *L. interrogans*, *L. kirschneri*, *L. borgpetersenii*, *L. noguchii*, *L. santarosai*, *L. weilii*, and *L. inadai*. Two proteins, p62 and p76, which were identified as heat shock proteins GroEL and DnaK, respectively, were exceptions to this pattern. Their expression is conserved in all *Leptospira* species including the nonpathogens *L. biflexa*, *L. meyeri*, and *L. wolbachii* and the related organism *Leptonema illini*.

DISCUSSION

Pathogenic *Leptospira* species possess a number of protein antigens that are expressed during infection of mammalian hosts and become targets for the host immune response. The goals of this study were to perform quantitative and qualitative analyses of the protein antigens recognized by antibodies induced during human leptospirosis. The work presented here builds upon earlier immunoblot studies using leptospirosis patient sera from Australia and New Zealand (7) and from Barbados (8). Our efforts were facilitated by several important technical developments. First, significant improvements in the sensitivity and specificity of immunoblotting techniques have become available (24). Secondly, the formation of the Salvador

Leptospirosis Study Group made it possible to obtain large numbers of well-characterized patient sera (22). Thirdly, many of the major leptospiral protein antigens have now been characterized on a molecular basis, and monospecific antibody reagents for these protein antigens have become available to assist in the interpretation of immunoblot studies (15–17, 37).

Our findings indicate that p32 is the immunodominant protein antigen recognized by the humoral response during natural infection. This conclusion is consistent with those from earlier immunoblot studies of sera from leptospirosis patients in Barbados, which identified a 32-kDa outer membrane protein as a major immunoreactive antigen (8). Our results extend those findings both in terms of frequency analysis based upon

TABLE 3. Immunoreactive leptospiral proteins

Molecular mass (kDa)	Designation	% Seroreactivity ^a	Leptospiral expression	Subcellular location
82		34	All pathogens	Inner membrane
76	DnaK	49	Genus wide	Cytoplasm ^b
62	GroEL	59	Genus wide	Cytoplasm ^b
58		8	Most pathogens	Inner membrane
48		25	Most pathogens	Inner membrane
45		59	All pathogens	Cytoplasm
44		10	ND ^d	Inner membrane
41/42 complex ^c	Includes LipL41	36	All pathogens	p42, inner membrane; LipL41, outer membrane
37		29	Some pathogens	Periplasm
32	LipL32	84	All pathogens	Outer membrane
31	LipL45/31	17	All pathogens	Outer membrane
25		4	Some pathogens	Periplasm

^a Percent seroreactivity of convalescent-phase sera from patients with confirmed leptospirosis.

^b The primary cellular location is indicated. However, these antigens appear to be distributed in additional cellular compartments.

^c This complex includes at least two antigen moieties, p42 and LipL41, which are expressed in all leptospiral pathogens. The percent seroreactivity is represented as the proportion of sera that react to any of the moieties to the complex because they could not be consistently discriminated in one-dimensional SDS-PAGE and immunoblot analyses.

^d ND, not determined.

much larger numbers of sera and by the identification of p32 as the major outer membrane lipoprotein, LipL32. Convalescent-phase sera from Brazilian patients with confirmed leptospirosis reacted with LipL32 more frequently (84%) than with any other antigen (Table 1). Reactivity to p32 also demonstrated high specificity: 0 to 5% background reactivity was observed in all but one of the control groups, including those from regions with both high and low prevalence of leptospirosis (Table 1). The p32 antigen was consistently observed in all leptospiral pathogens tested (Fig. 3), and its identity as LipL32 was confirmed by 2D electrophoresis (Fig. 4).

These results are consistent with the recent characterization of LipL32 as an outer membrane lipoprotein, which is expressed at high levels by pathogenic *Leptospira* species (16). A comparison of LipL32 amino acid sequences from six leptospiral serovars, representing five leptospiral species, found a 97.8% average amino acid sequence identity. The high degree of LipL32 sequence identity indicates that serodiagnostic strategies based upon this antigen would be effective regardless of the infecting serovar. The specificity of this protein antigen for leptospiral infection is supported by BLAST searches of the GenBank database in which no significant homologues of the LipL32 sequence were identified. The antigenicity of LipL32 is presumably enhanced by its high level of expression in leptospiral pathogens and by lipid modification of its amino terminus, a property known to enhance the antigenicity of other spirochetal lipoproteins, such as OspA of *Borrelia burgdorferi* (9).

Two other prominent leptospiral antigens, p62 and p76, were identified in this study to be molecular chaperones GroEL and DnaK, respectively. Expression of bacterial heat shock proteins, including leptospiral GroEL and DnaK, is up-regulated at the elevated temperatures encountered within the mammalian host (3, 4, 40). Both GroEL and DnaK are recognized by significant numbers of acute- and convalescent-phase sera from patients with confirmed leptospirosis (Table 1). In immunoblots with acute-phase sera, GroEL (45%) was the only antigen recognized more frequently than LipL32 (37%). However, only 16% of confirmed cases demonstrated seroconversion to GroEL between the acute and convalescent phases

of illness (opposed to 50% for LipL32), suggesting that the immunoreactivity observed during acute-phase illness may have been due to preexisting, possibly cross-reactive, antibodies or a vigorous memory response. Furthermore, significant seroreactivity was observed among control sera to GroEL and, to a lesser degree, DnaK, probably reflecting the ubiquitous expression of these proteins in eubacteria (13) and the fact that many different types of infections are associated with an immune response to heat shock proteins (47). A recent study found that the dominant antigenic determinant in leptospiral GroEL is a 20-amino-acid region that is highly conserved among prokaryotes (33). This finding indicates that cross-reactivity with GroEL proteins from other bacteria could limit the feasibility of using leptospiral GroEL as a specific marker for leptospiral seroreactivity.

LipL41 was the fourth previously characterized antigen that

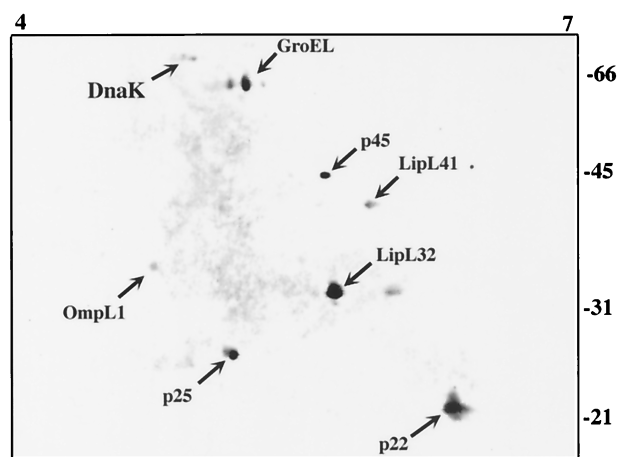


FIG. 4. A two-dimensional immunoblot of *L. interrogans* serovar *copenhageni* proteins probed with pooled convalescent-phase sera from leptospirosis patients in Salvador, Brazil. The locations of DnaK, GroEL, LipL41, OmpL1, LipL32, and three as yet unidentified antigens (p45, p25, and p22) are indicated. The locations of molecular mass markers (in kilodaltons) and reference isoelectric points (pI) are indicated on the right and top of the figure, respectively.

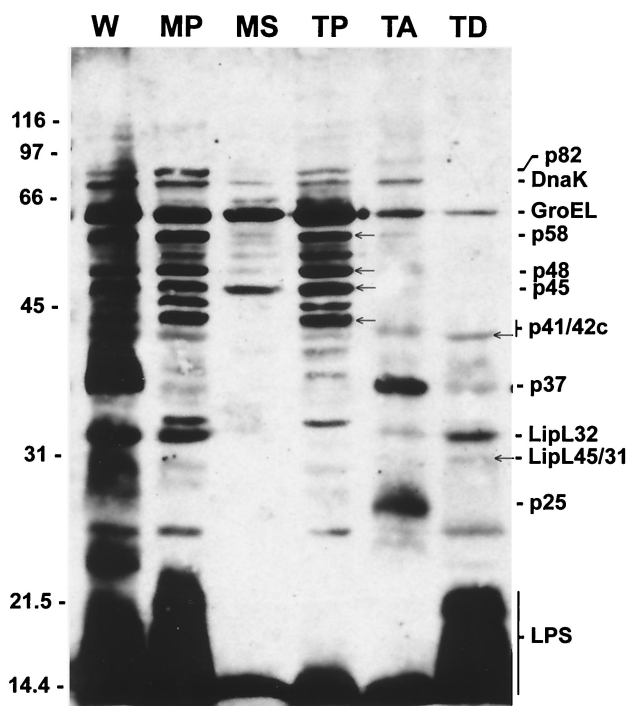


FIG. 5. Localization of leptospiral protein antigens by fractionation of cellular components. Extracts of whole organisms (W) were used as starting material. The total membrane fraction (MP) contains both the cytoplasmic membrane and outer membrane, while the soluble fraction (MS) contains cytoplasmic and periplasmic material. The detergent Triton X-114 selectively releases the outer membrane, leaving the cytoplasm and cytoplasmic membrane in the Triton X-114 insoluble pellet (TP). Phase partitioning of the Triton X-114 extract separates periplasmic material in the aqueous phase (TA) from outer membrane components in the detergent phase (TD). The locations of molecular mass markers (in kilodaltons) and leptospiral protein antigens are shown on the left and right, respectively. In addition, the positions of leptospiral proteins are marked by arrows (\leftarrow) within the immunoblot and correspond to, in descending order, p58, p48, p45, and p42 in the p41/42 complex in lane TP and LipL41, part of the p41/42 complex, and LipL45/31 in lane TD.

we identified to be a target of the humoral immune response during leptospiral infection. Like LipL32, LipL41 is lipidated at its amino terminus and is located in the leptospiral outer membrane (37). A significant fraction of LipL41 appears to be exposed on the leptospiral surface, making it a potential target of a protective antibody response. When used in combination with OmpL1, immunization with recombinant lipidated LipL41 protects hamsters from challenge with virulent *L. kirschneri* (18). Our results indicate that in contrast to LipL32 and LipL41, other leptospiral lipoproteins would have limited usefulness in the serodiagnosis of human leptospirosis. For example, LipL36 is expressed by most leptospiral pathogens grown in culture, including the strains isolated from Salvador, Brazil, and Barbados; however, it was not detected by sera from patients acquiring leptospirosis in those locations in one- or two-dimensional immunoblot analyses (Fig. 4).

OmpL1 is a transmembrane outer membrane protein with porin activity which has been shown to be a protective immunogen (15, 18, 36). Although OmpL1 is expressed during mammalian infection (5), immunoblot reactivity using clinical

leptospirosis sera could be demonstrated only by 2-D immunoblotting (Fig. 4). The difficulty in demonstrating OmpL1 reactivity could be due, in part, to this protein's unusual electrophoretic mobility pattern. In its undenatured form, OmpL1 migrates in SDS-PAGE with an apparent molecular mass of 25 kDa. In its denatured form OmpL1 migrates closer to its true molecular mass of 33 kDa (36). Neither the denatured nor undenatured form of native OmpL1 was detectable by one-dimensional immunoblotting with sera from leptospirosis patients (data not shown). At least two explanations could account for this result. First, reactivity with the 25- and 33-kDa forms of OmpL1 is likely to be obscured on immunoblots by reactivity with LPS and LipL32, respectively. Secondly, OmpL1 is expressed at low levels by *Leptospira* species, so there would be relatively less OmpL1 on immunoblots using native proteins.

An important advantage of the present study is that it uses sera from patients with naturally occurring leptospiral infections that probably result from relatively small infectious doses. For this reason, the immune response would be expected to exclusively target antigens expressed by leptospiral organisms within the mammalian host, not antigens expressed exclusively on environmental organisms at the time of inoculation. Therefore, recognition of lipoproteins LipL32 and LipL41 and heat shock proteins GroEL and DnaK by the humoral immune response to leptospirosis is a strong indication that these proteins are expressed during infection. These results confirm earlier immunoblotting and immunohistochemistry studies involving the hamster model of leptospirosis, which found that LipL32 and LipL41 are expressed by organisms within the proximal renal tubule, while LipL36 expression is down regulated during infection (5, 16). In those studies, efforts were made to avoid exposure to environmental organisms by inoculating hamsters with *L. kirschneri* obtained directly from infected hamster tissues. Immunoblots using sera from hamsters challenged with host-derived organisms recognized OmpL1, LipL41, and moieties that appear to be the p22, p37, and p45 antigens identified in this study (5). Interestingly, LipL32, GroEL, and DnaK were not well recognized by the hamster sera, suggesting that the results were biased by the artificial nature of experimental infection.

A second important advantage of studying sera from patients with clinical leptospirosis is the robust immune response, which allows identification of a much larger number of protein antigens than could be identified using infection-derived hamster sera (5). The diversity of recognized leptospiral protein antigens and the heterogeneous patterns of the antibody response observed among infected individuals are evident in Fig. 1 and 3. Furthermore, immunoreactive proteins were found to be shared among groups of genetically diverse leptospiral strains. The immunoblot pattern in Fig. 3 shows that most strains can be categorized as either pathogens or nonpathogens based upon the immunoreactive proteins which they express. Previous phylogenetic studies have indicated that *L. inadai* is an intermediate between leptospiral pathogens and nonpathogens (25). This observation was confirmed in the present study: among pathogen-specific protein antigens, only LipL32 was detectable in *L. inadai*.

There is an urgent need to address emerging epidemics of leptospirosis, particularly in medically underserved popula-

tions in developing countries, but surveillance and diagnosis have been hampered by the lack of an effective, widely available laboratory tool for case confirmation. The results of the present study serve as a guide to develop new strategies for serodiagnosis. The anti-LipL32 response was identified as the most important serologic marker of infection in immunoblot analyses. Seroreactivity against other leptospiral protein antigens did not significantly enhance the diagnostic sensitivity and specificity observed for the anti-LipL32 response alone (Table 2 and Fig. 2). LipL32 seroreactivity had sensitivity levels of 37 and 84% in detecting leptospiral infection during the acute and convalescent phases, respectively, of illness. In addition, an anti-LipL32 response was detected in 73 and 25% of convalescent-phase sera from probable (according to the MAT) and unconfirmed cases, respectively, of suspected leptospirosis, suggesting that LipL32 seroreactivity may be capable of capturing cases not identified by the standard laboratory confirmation method. The low frequency of reactivity in healthy individuals and patients with syphilis, hepatitis, and dengue from regions where leptospirosis is endemic (Table 1) indicates that the anti-LipL32 response is highly specific and therefore useful in differentiating leptospirosis from other causes of acute febrile illness. Application of more sensitive and rapid detection formats such as recombinant protein-based immunoassays will be the next step in evaluating the usefulness of this marker of infection for laboratory case confirmation in the field.

In addition to their use in serodiagnosis, leptospiral proteins expressed during mammalian infection may also have immunoprotective potential. The present study identified more than 20 immunoreactive proteins, several of which appear to be surface exposed and therefore serve as targets of a protective immune response. It has been demonstrated recently that immunization with whole leptospiral protein preparations confers protection in experimental animal models (39). In contrast to anti-LPS responses, those against leptospiral proteins were found to protect against challenge with heterologous, as well as homologous, leptospiral serovars. Furthermore, leptospiral outer membrane proteins OmpL1 and LipL41 have been shown to induce synergistic immunoprotection when expressed as membrane-associated recombinant antigens in *E. coli* (18). It is anticipated that additional leptospiral proteins identified in this study will be evaluated as immunoprotective antigens leading to the development of improved vaccines for the prevention of leptospirosis.

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