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Changes in the Surface of *Leptospira interrogans* Serovar grippotyphosa during In Vitro Cultivation

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Surface components of virulent and attenuated *Leptospira interrogans* serovar grippotyphosa were compared by using Triton X-114 solubilization and phase partitioning, immunoprecipitation of intact organisms, and freeze-fracture electron microscopy. Removal of the leptospiral outer membrane by using 0.1% Triton X-114 was demonstrated by whole-mount electron microscopy and by essentially complete solubilization of a lipopolysaccharidelike substance (LLS) from the outer membrane. Triton X-114 (0.1%) did not solubilize subsurface proteins, such as endoflagellar filaments or penicillin-binding proteins, which are markers for the periplasmic space and inner membrane, respectively. Triton X-114 solubilized material from both the virulent and attenuated strains, which partitioned into the hydrophobic, detergent phase, contained LLS and major proteins of 41 and 44 kDa, which were also immunoprecipitable from intact organisms. The virulent strain contained greater amounts of an LLS component with an apparent molecular mass of 30 kDa (R_f = 0.57), whereas the attenuated strain contained larger amounts of an LLS component with an apparent molecular mass of 20 kDa (R_f = 0.74). Differences in protein components between virulent and attenuated organisms were also detected; whereas the 41- and 44-kDa proteins were immunoprecipitated in equal amounts from both the virulent and attenuated strains, a 33-kDa protein was immunoprecipitated in significantly greater amounts from the attenuated strain. Quantitation of outer membrane particle density by freeze-fracture electron microscopy showed that both strains had a low transmembrane outer membrane protein content compared with that of typical gram-negative bacteria. The virulent and attenuated strains had 443 and 990 particles ($P < 0.000001$) per μm^2, respectively, in the concave outer membrane fracture face. These findings suggest that in vitro cultivation of *L. interrogans* is accompanied by quantitative and qualitative changes in both LLS and outer membrane-associated proteins.

Leptospirosis is a widespread zoonosis caused by *Leptospira interrogans*, a pathogen capable of infecting most mammalian species through either direct contact with an infected animal or indirect contact with contaminated soil or water. In humans, it is a common cause of serious acute febrile illness in tropical areas of the world (17). Leptospirosis is also an important cause of abortion, stillbirth, infertility, decreased milk production, and death in livestock (50). Control efforts have been hampered because virulent leptospires have the capacity for both long-term survival in the environment as well as persistent infection and shedding by wildlife and livestock. Currently available leptospiral vaccines produce short-term immunity and do not provide cross-protection against many of the serovars of *L. interrogans* (50). These vaccines consist of inactivated whole organisms or outer envelope preparations which produce seroreactivity as determined by microscopic agglutination of intact organisms (5). The nature of the protective immunogens in these vaccine preparations has not been conclusively elucidated, although several lines of evidence suggest that lipopolysaccharidelike substance (LLS) antibodies confer a considerable degree of protection (19, 28, 29, 35).

There is little knowledge regarding the molecular basis of leptospiral pathogenicity. The LLS lacks the endotoxic properties of lipopolysaccharide (LPS) derived from gram-negative bacteria. Although some *L. interrogans* strains produce a hemolysin with sphingomyelinase activity (42), other potential virulence factors have not been described. The outer membrane of *Leptospira* spp. is a labile structure easily damaged by mechanical trauma or chemical treatment (23). Techniques used to identify outer membrane proteins in gram-negative bacteria have not adequately addressed the structural fragility of other spirochetes, such as *Treponema pallidum* (15, 38, 39, 41, 47, 58, 59).

In this report we describe ultrastructural and antigenic studies designed to address the identity of surface molecules of virulent and isogenic culture-attenuated *L. interrogans*. Our findings include the observations that the content of transmembrane outer membrane protein of virulent *L. interrogans* was low compared with that of other bacteria and most closely resembled the rare content of transmembrane protein of the *T. pallidum* outer membrane (59) and that this protein content increased in parallel with culture attenuation along with a corresponding increase in amount of a 33-kDa polypeptide surface antigen. Differences in LLS composition and surface exposure were also noted between virulent and attenuated *L. interrogans*.

MATERIALS AND METHODS

Bacteria. *Leptospira interrogans* serovar grippotyphosa (strain RM52) was originally isolated from material submitted to the Veterinary Diagnostic Laboratory at Iowa State University during an outbreak of swine abortion in 1983 (51). Samples of the isolate were either stored in liquid nitrogen

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(3) or passaged weekly or biweekly in liquid EMJH medium (26). Thirty-three-week-old weanling hamsters were used in testing strains for virulence. Groups of three hamsters were inoculated intraperitoneally with serial 10-fold dilutions from a liquid culture. Surviving hamsters were euthanized at 14 days postinoculation, and kidney and liver tissues were collected at necropsy and cultured in semisolid EMJH medium (26). Ultrastructural and biochemical studies of the virulent strain were performed on bacteria that had been passaged fewer than five times from hamster isolation. The attenuated strain has been passaged more than 200 times since 1983.

Antisera. Rabbits were immunized with a 1-ml phosphate-buffered saline (PBS) suspension of 2×10^8 virulent or attenuated whole *L. interrogans* serovar grippotyphosa mixed with 1 ml of Freund's incomplete adjuvant given in equally divided amounts at two intramuscular and two subcutaneous sites. Animals were injected after 1 week with a similar preparation by using the same combined intramuscular-subcutaneous route. Animals were next injected after 4 months with a 1-ml suspension of 4×10^8 *L. interrogans* serovar grippotyphosa given intravenously. Serum samples were collected 2 weeks after the final injection.

SDS-PAGE and immunoblotting. Samples for sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) were boiled for 10 min in final sample buffer (FSB) composed of 62.5 mM Tris hydrochloride (pH 6.8), 10% glycerol, and 2% SDS and then separated by electrophoresis on 2.5% stacking and 10 to 15% linear gradient polyacrylamide gels by using the discontinuous buffer system of Laemmli (32). After electrophoresis, gels either were stained with Coomassie brilliant blue or periodate silver (56) or were transferred to nitrocellulose for immunoblotting (55). For antigenic detection on immunoblots, the nitrocellulose was blocked with 3% nonfat dry milk in Tris-saline-azide (M-TSA), incubated overnight with antiserum diluted 1:15 in M-TSA, and probed with radiolabeled ^{125}I-staphylococcal protein A. Autoradiography was performed with preflashed Kodak XAR-5 film (Eastman Kodak Co., Rochester, N.Y.) and intensifying screens at −70°C.

Triton X-114 extraction and phase partitioning. Extraction and phase partitioning of leptospiral proteins were performed as previously described for *T. pallidum* (15). All manipulations were performed at 4°C except as noted. Leptospires were washed three times in PBS (pH 7.4) containing 5 mM $MgCl_2$. After the third wash, organisms were resuspended in 10 mM Tris hydrochloride–2 mM EDTA (TE; pH 7.4) at a concentration of 5×10^9 organisms per ml. The leptospirae retained motility throughout the three PBS washes. Triton X-114 (TX-114; 10%) was added to bacterial suspensions to yield final concentrations of 0.1 and 1.0%. After incubation for 30 min, samples were centrifuged for 10 min at 17,000 × *g*, and the pellet (protoplasmic cylinders) was resuspended in FSB. Additional 10% TX-114 was next added to the supernatant to give a final concentration of 2%. Phase separation was performed by warming the supernatant to 37°C for 10 min and subjecting it to centrifugation for 10 min at 2,000 × *g* at room temperature. Separated detergent and aqueous phases were then washed four times in the following manner. The aqueous phase was chilled in an ice bath, and 10% TX-114 was added to a final concentration of 2%, which was followed by warming to 37°C and centrifugation as described above. The detergent phase was chilled in an ice bath, diluted with nine parts TE buffer, warmed to 37°C, and centrifuged as described above. After washing, detergent- and aqueous-phase proteins were precipitated with acetone as described previously (15) and resuspended in FSB for analysis by SDS-PAGE and immunoblotting.

Intrinsic radiolabeling of penicillin-binding proteins. *L. interrogans* serovar grippotyphosa penicillin-binding proteins were radiolabeled by a modification of the method of Spratt (46). Briefly, 8.7 nmol of ^{35}S-benzylpenicillin (DuPont Company, New England Nuclear Research Products, Boston, Mass.) was added to 2×10^9 virulent leptospires in 2 ml of culture medium. After incubation at 30°C for 1 h, bacteria were extracted with 0.1% TX-114 and separated into detergent, aqueous, and protoplasmic cylinder fractions as described above. A control experiment was performed in which 1 mM unlabeled benzylpenicillin was added prior to addition of ^{35}S-benzylpenicillin. After SDS-PAGE and Coomassie brilliant blue staining of these fractions, the gel was destained in 5% methanol–7.5% acetic acid, washed three times in distilled water, impregnated with Autofluor (National Diagnostics, Somerville, N.J.), dried, and exposed to preflashed Kodak XAR-5 X-ray film and intensifying screens at −70°C for 21 days.

Electron microscopy. Leptospires were prepared for whole-mount electron microscopy by the single-droplet method as described previously (15), except that organisms were treated with 0.1% TX-114 at 4°C prior to application to Formvar (Ted Pella, Inc., Tustin, Calif.) and carbon-coated copper grids (300 mesh; Ted Pella). The grids were then washed with ice-cold 150 mM NaCl and negatively stained with 1% uranyl acetate (Sigma Chemical Co., St. Louis, Mo.). Micrographs were taken at 80 kV of accelerating voltage on a JEOL 100CXII electron microscope. Freeze-fracture electron microscopy and particle density quantitation were performed as previously described (59).

Surface immunoprecipitation. A modification of the surface immunoprecipitation method of Hansen et al. (21) was used to identify surface-exposed antigens of *L. interrogans* serovar grippotyphosa. Leptospiral culture (60 ml) containing 2.5×10^9 bacteria (>99% actively motile) was mixed with 650 μl of heat-inactivated rabbit antiserum (56°C for 30 min) raised against either the virulent or the attenuated strain of *L. interrogans* serovar grippotyphosa. The suspension was then gently shaken for 2 h at 30°C. Agglutinated leptospires were pelleted at 2,000 × *g* for 15 min, resuspended in 5 mM $MgCl_2$ in PBS, pelleted again at 2,000 × *g* for 15 min, and then resuspended in 900 μl of 10 mM Tris HCl (pH 8.0). To this suspension was added 100 μl of 10% Triton X-100 in 10 mM Tris HCl, pH 8.0, followed by gentle agitation overnight at 4°C. After overnight incubation, the insoluble material was removed by centrifugation at 16,000 × *g* for 15 min. To the supernatant was added 100 μl of 10 mM EDTA, 100 μl of 2% deoxycholate, 100 μl of 1% SDS, and 100 μl of a slurry of staphylococcal protein A-Sepharose CL-4B (Sepharose-SpA) (Sigma). This mixture was gently agitated for 1 h at 4°C. The Sepharose-staphylococcal protein A-antibody-antigen complexes were pelleted at 500 × *g* for 2 min, resuspended in 0.01% Triton X-100 in 10 mM Tris HCl (pH 8.0), and pelleted again at 500 × *g* for 2 min. The pellet was then resuspended in 0.01% Triton X-100 in 10 mM citrate, pH 3, and agitated gently for 30 min at 4°C in order to release antigen-antibody complexes. Following centrifugation at 500 × *g* for 2 min to remove the Sepharose-staphylococcal protein A beads, the supernatant containing the antigen-antibody complexes was concentrated on a SpeedVac concentrator (Savant Instruments, Inc., Farmingdale, N.Y.) and resuspended in FSB. Samples were then processed by SDS-PAGE and immunoblot with antiserum to virulent *L.*

interrogans serovar grippotyphosa. In some experiments, samples were treated with 400 μg of proteinase K per ml (Sigma) at 56°C for 4 h; an additional 400 μg of proteinase K per ml was added after 2 h.

Affinity purification of antibodies to leptospiral polypeptides isolated by surface immunoprecipitation. Antibodies were affinity purified as previously described (52). The material isolated by surface immunoprecipitation was separated by SDS-PAGE and transferred to nitrocellulose, from which were cut 1-cm horizontal strips containing approximately 20 μg of immunoprecipitated protein. Individual immunoprecipitated polypeptides of 33, 41, and 44 kDa were excised and incubated for 1 h with M-TSA before incubation overnight with anti-leptospiral antiserum diluted 1:15 with M-TSA. Following incubation, the strips were washed 10 times with Tris-saline-azide over a 2-h period. To elute antibody, the strips were incubated in 3 ml of 0.1 M glycine hydrochloride buffer (pH 2)–0.5% bovine serum albumin for 30 min, which was followed by the addition of 600 μl of 1 M Tris, pH 9.0. Eluted antibody from the different strips was then diluted 1:2 in M-TSA and used to identify leptospiral polypeptides as described above for immunoblotting.

RESULTS

Virulence testing. The results of virulence testing with the virulent (<5 passages) strain of *L. interrogans* serovar grippotyphosa were as follows (inoculum, number dead/number tested): 10^8, 3/3; 10^7, 3/3; 10^6, 2/3; 10^5, 1/3; and 10^4, 1/3. All survivors of inoculation with the virulent strain were culture positive at necropsy. In contrast, none of the 15 hamsters inoculated with the attenuated (>200 passages) strain died following injection with as many as 10^9 organisms. The culture results following inoculation with the attenuated strain were as follows (inoculum, number culture positive/number tested): 10^9, 1/3; 10^8, 0/3; 10^7, 1/3; 10^6, 0/3; 10^5, 0/3. From these data it was determined that the minimum lethal inoculum was $<10^4$ for the virulent strain and $>10^9$ for the attenuated strain. The minimum infectious inoculum was $<10^4$ for the virulent strain and 10^7 for the attenuated strain.

Effects of TX-114 on leptospiral morphology. Treatment with 0.1% TX-114 for 30 min resulted in a marked decrease in the diameter of *L. interrogans*, as demonstrated by whole-mount electron microscopy (Fig. 1). The diameter of untreated organisms ranged from 0.16 to 0.22 μm (Fig. 1A). After removal of the outer membrane by TX-114, the resulting protoplasmic cylinders had diameters of 0.05 to 0.08 μm (Fig. 1B). This difference represents a 66% decrease in diameter, which is similar to the 58% decrease in diameter observed after treatment of *T. pallidum* with TX-114 (15). No longer constrained by an outer membrane, endoflagella became unwound and in some cases appeared to remain attached by one end to the protoplasmic cylinder (Fig. 1B). Treatment of *L. interrogans* with TX-114 also appeared to result in a slight decrease in length, resulting in an increase in the helical frequency.

Fate of leptospiral proteins and LLS following TX-114 extraction and phase partitioning. As shown by SDS-PAGE, the use of 0.1% TX-114, in contrast to use of buffer alone, solubilized a number of proteins which subsequently partitioned into detergent and aqueous fractions following phase partitioning (Fig. 2). The use of either 0.1 or 1.0% TX-114 showed relatively little difference in the total amounts of solubilized leptospiral proteins detected in both the detergent and aqueous phases (data not shown).

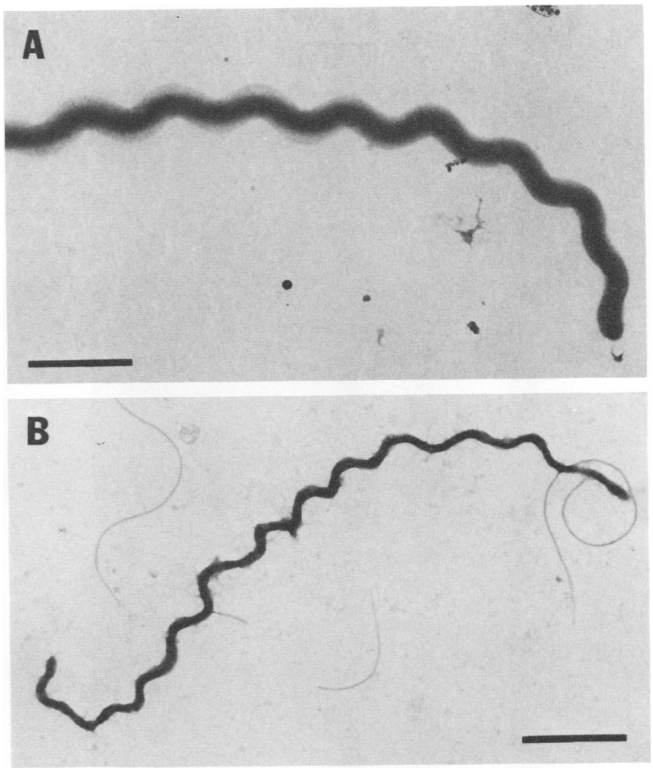

FIG. 1. Effects of TX-114 treatment on *L. interrogans* ultrastructure. Electron micrographs of negatively stained *L. interrogans* serovar grippotyphosa before (A) and after (B) treatment with 0.1% Triton X-114 for 30 min. Bars, 0.77 μm. Note that the filamentous strands which represent endoflagella in some cases appeared to remain attached by one end to the protoplasmic cylinder.

Periodate silver staining of the material solubilized with 0.1% TX-114 detected four separate proteinase K-resistant bands with apparent molecular masses of 24, 20, 16, and less than 14 kDa and R_f values of 0.65, 0.74, 0.88, and 0.97, respectively (Fig. 3). Only a small amount of the carbohydrate-containing material remained in the protoplasmic cylinder fraction, suggesting essentially complete solubilization of the leptospiral outer membrane and its associated LLS. Once solubilized in TX-114, the periodate silver-staining leptospiral LLS partitioned exclusively into the detergent phase, confirming the hydrophobic nature of this glycolipid material. The absence of Coomassie-staining bands with an apparent molecular mass of greater than 14 kDa in a gel run with seven times as much material as shown in Fig. 3 confirms the completeness of the proteinase K treatment used in digesting leptospiral proteins (data not shown).

To determine the selectivity of outer membrane removal by using 0.1% TX-114, endoflagellar protein and penicillin-binding proteins, markers for the periplasmic space and inner membrane, respectively, were identified following TX-114 solubilization and phase partitioning. In both virulent and attenuated strains, the 35- and 36-kDa *Leptospira* endoflagellar proteins (31) were detected by cross-reactive anti-*T. pallidum* endoflagellar antiserum in only the protoplasmic cylinder fractions (Fig. 4A), indicating that these periplasmic constituents were insoluble in 0.1% TX-114 and had not contaminated the outer membrane-solubilized fraction. Similarly, penicillin-binding proteins (82-kDa doublet and 64, 59, and 33 kDa) were only detected in the protoplas-

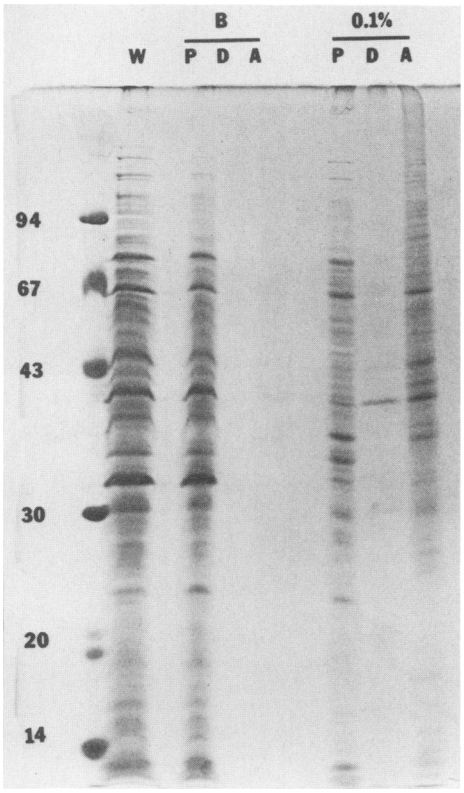

FIG. 2. TX-114 extraction and phase partitioning of virulent *L. interrogans* serovar grippotyphosa. SDS-PAGE analysis of samples representing 2×10^9 virulent serovar grippotyphosa extracted with buffer alone (B) and 0.1% TX-114 (0.1%) and stained with Coomassie brilliant blue. Fractions analyzed were the whole organism (W), protoplasmic cylinders (P), detergent phase (D), and aqueous phase (A). Molecular size standards are in the left lane (in kilodaltons).

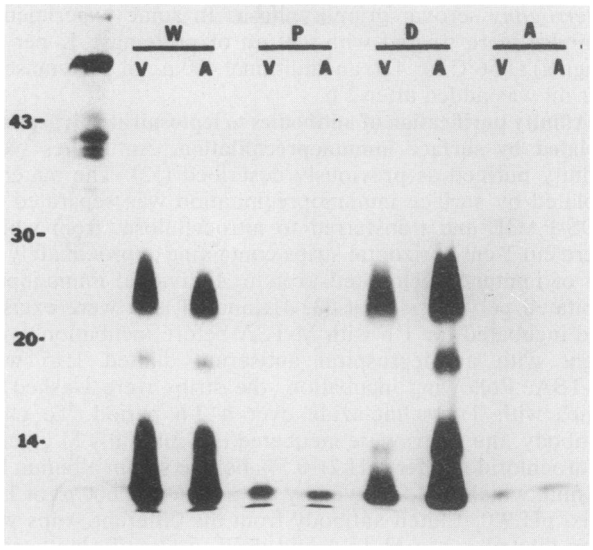

FIG. 3. Fate of *L. interrogans* serovar grippotyphosa LLS after TX-114 extraction and phase partitioning. Periodate silver-stained SDS-polyacrylamide gel of proteinase K-treated material from 7.5×10^7 virulent (V) and attenuated (A) serovar grippotyphosa. Fractions analyzed were the whole organism (W), protoplasmic cylinders (P), detergent phase (D), and aqueous phase (A). Molecular size standards are in the left lane (in kilodaltons).

mic cylinder fraction, indicating the integrity of the inner membrane and associated constituents following 0.1% TX-114 treatment (Fig. 4B). Preaddition of unlabeled benzylpenicillin completely blocked binding of these proteins to ^{35}S-benzylpenicillin (data not shown). The molecular weight profile of the leptospiral penicillin-binding proteins was similar to that of *T. pallidum* (14, 40).

Comparison of LLS extracted from virulent and attenuated *L. interrogans* by using 0.1% TX-114. Figure 5A shows the proteinase K resistance of the periodate silver-staining material present on analysis of the whole organism by SDS-PAGE. Immunoblotting of proteinase K-resistant material demonstrates that leptospiral LLS antigens can be resolved into at least four separate bands: three major bands (apparent molecular masses of 30, 24, and 20 kDa and R_f values of 0.57, 0.65, and 0.74, respectively) and one minor band (apparent molecular mass of 16 kDa, $R_f = 0.88$). Comparison of the LLS immunoblot and periodate silver stain patterns suggests that the antigenicity of LLS material is greater as the molecular weight of leptospiral LLS forms increases. For example, the small LLS band (apparent molecular mass of 30 kDa, $R_f = 0.57$) present at the top of the LLS band with an apparent molecular mass of 24 kDa ($R_f = 0.65$) on the immunoblot of the virulent organism (Fig. 5B, lane WV) is not evident on the periodate silver-stained gel (Fig. 5A, lane WV). Conversely, the LLS material with an apparent molecular mass of less than 14 kDa ($R_f = 0.97$) on the periodate silver-stained gel (Fig. 5A, all four lanes) is not evident on the immunoblot (Fig. 5B, all four lanes). This effect may be due to the limited antigenicity of small LLS precursors that lack terminal sugars. Comparison of the immunoblot patterns of the LLS from the virulent and attenuated strains shows that the LLS antigen with an apparent molecular mass of 30 kDa ($R_f = 0.57$) is present in greater amounts in the virulent strain, whereas the LLS antigen with an apparent molecular mass of 20 kDa ($R_f = 0.74$) is present in greater amounts in the attenuated strain (Fig. 5B).

Comparison of proteins extracted from virulent and attenuated *L. interrogans* by using 0.1% TX-114. Extraction of *L. interrogans* by using 0.1% TX-114 resulted in the solubilization of a large number of proteins varying in molecular mass from greater than 100 to less than 14 kDa (Fig. 6A). Most of the solubilized proteins partitioned into the hydrophilic, aqueous phase, whereas 16 proteins partitioned into the hydrophobic, detergent phase. Both the virulent and attenuated strains possessed several TX-114-solubilized proteins which were unique or present in greater amounts. Detergent phase proteins of 35, 39, and 66 kDa appeared to be unique to the virulent strain. By comparison, detergent phase proteins of 32 and 38 kDa appeared to be unique to the attenuated strain. In addition, the 25- and 33-kDa aqueous-phase proteins and the 27- and 61-kDa detergent-phase proteins appeared to be expressed in greater amounts in the attenuated strain (Fig. 6A). The virulent and attenuated strains contained similar amounts of the major detergent-phase proteins with molecular masses of 41 and 44 kDa. The electrophoretic mobility of the latter in the detergent phase appeared to be distorted by the presence of leptospiral LLS, giving it an apparent molecular mass of 47 to 48 kDa (Fig. 6A). A comparison of the virulent and attenuated *L. interrogans* antigens was performed by immunoblot analysis with antiserum raised against the virulent strain. Aqueous-phase

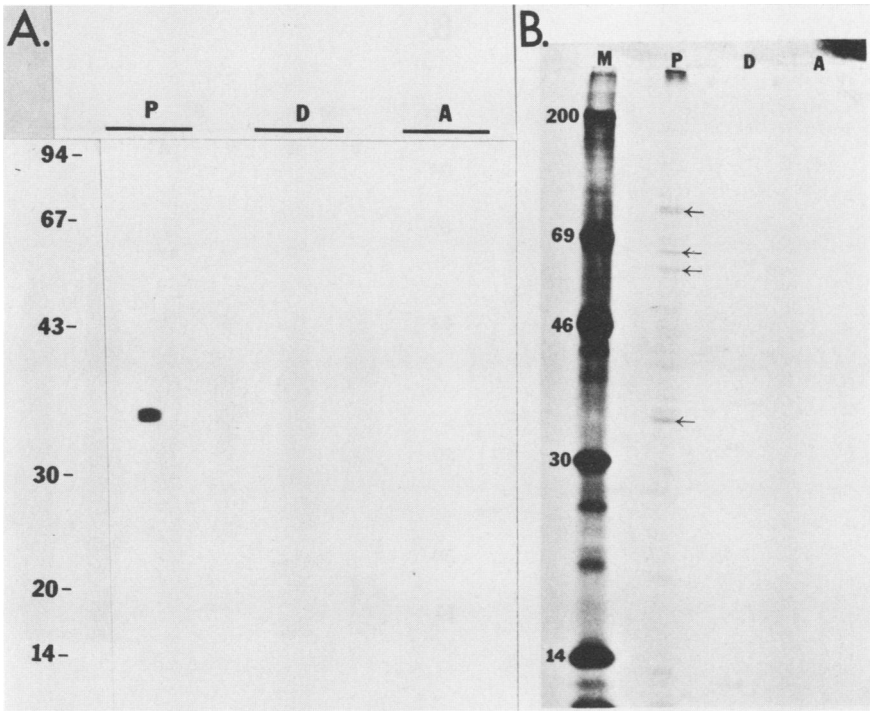

FIG. 4. Fate of *L. interrogans* serovar grippotyphosa endoflagellar proteins and penicillin-binding proteins after TX-114 extraction and phase partitioning. (A) Immunoblot of 5×10^8 attenuated serovar grippotyphosa probed with rabbit antiserum to purified *T. pallidum* endoflagella. Fractions analyzed were the protoplasmic cylinders (P), detergent phase (D), and aqueous phase (A). Molecular size standards are in the left lane (in kilodaltons). (B) Penicillin-binding proteins from 2×10^9 virulent serovar grippotyphosa labeled with ^{35}S-benzylpenicillin were analyzed by SDS-PAGE after 0.1% TX-114 extraction and phase partitioning. Lanes: M, Molecular size standards; P, protoplasmic cylinders; D, detergent phase; A, aqueous phase. Arrows indicate locations of penicillin-binding proteins.

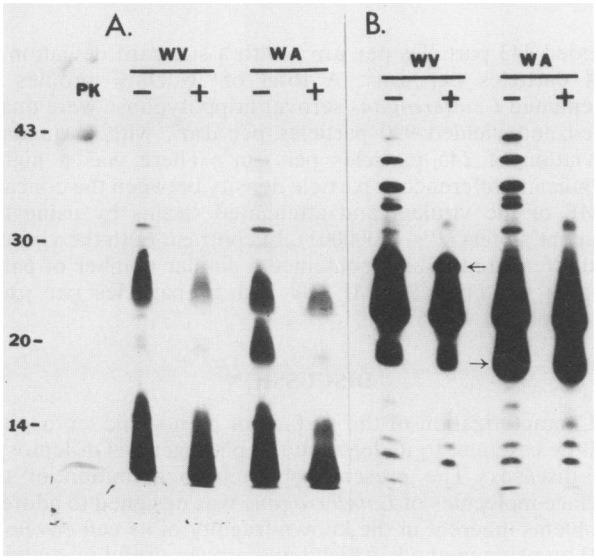

FIG. 5. Periodate silver stain and immunoblot comparison of LLS extracted from virulent and attenuated *L. interrogans* serovar grippotyphosa by using 0.1% TX-114. Periodate silver stain (A) and immunoblot (B) of 7.5×10^7 whole, virulent (WV) and whole, attenuated (WA) *L. interrogans*. Fractions were analyzed with (+) and without (−) proteinase K treatment (PK). Arrows indicate locations of LLS bands which are unique or present in greater amounts in the virulent or attenuated strain of serovar grippotyphosa. Molecular size standards are in the left lane (in kilodaltons).

antigens of 36, 38, and 41 kDa were prominently detected in both the virulent and attenuated strains. By comparison, an antigenic 33-kDa protein was detected in significantly greater amounts in the attenuated strain (Fig. 6B). Further comparisons between the detergent phases of each strain were not possible because of the abundant amounts of LLS which obscured the identification of antigenic proteins.

Identification of surface-exposed protein antigens on virulent and attenuated *L. interrogans*. In order to identify surface-exposed antigens on virulent and attenuated *L. interrogans*, immunoprecipitation utilizing homologous and heterologous antisera with motile organisms was employed. In this procedure, unbound antibody is removed from motile organisms by washing prior to solubilization and immunoprecipitation, thereby ensuring the detection of only surface-exposed antigens. This was further confirmed by the inability to immunoprecipitate the 35-36-kDa doublet endoflagellar filament proteins (Fig. 7A). The results of immunoprecipitation showed that several prominent antigens, ranging from 17 to 44 kDa, were detected in both virulent and attenuated strains by using either homologous or heterologous antisera. Antigens immunoprecipitated in equal amounts from both the virulent and attenuated strains included the 41- and 44-kDa antigens, as well as a diffusely reacting band with an apparent molecular mass ranging from 24 to 28 kDa (R_f = 0.60 to 0.65). By contrast, an antigen with an apparent molecular mass of 30 kDa (R_f = 0.57) was immunoprecipitated in greater amounts from the virulent strain, whereas the 33-kDa antigen and the antigen with an apparent molec-

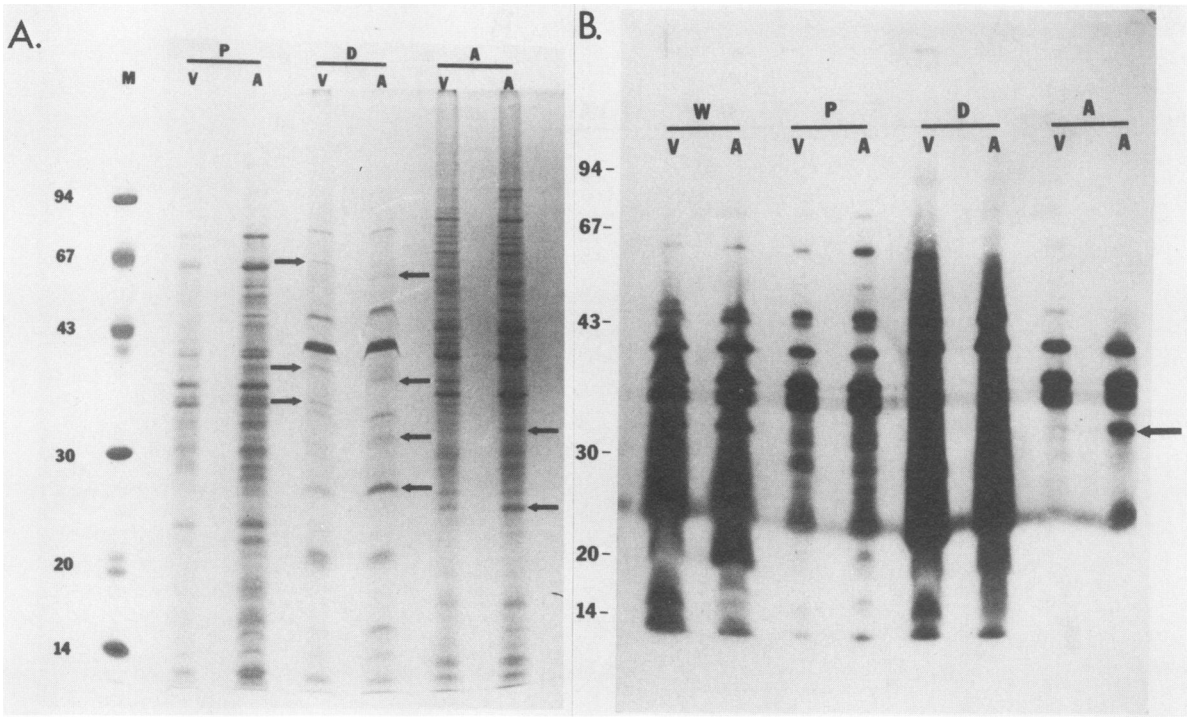

FIG. 6. Comparison of TX-114 fractions from virulent (V) and attenuated (A) *L. interrogans* serovar grippotyphosa extracted with 0.1% Triton X-114 and phase partitioned. Fractions compared were the protoplasmic cylinders (P), detergent phase (D), and aqueous phase (A). (A) Analysis of TX-114 protein fractions stained with Coomassie brilliant blue. Protoplasmic cylinder lane contained material from 2×10^9 organisms. Detergent and aqueous lanes contained material from 5×10^9 organisms. Molecular size standards (M) are in the left lane (in kilodaltons). Arrows indicate locations of proteins which are unique or present in greater amounts in the virulent or attenuated strain of serovar grippotyphosa. (B) Immunoblot of TX-114 fractions probed with rabbit antiserum to whole, virulent serovar grippotyphosa. Whole organism (W) and pellet (P) lanes contained material from 5×10^8 organisms. Detergent (D) and aqueous (A) phases contained material from 1.3×10^9 organisms. Location of molecular size standards are shown (in kilodaltons) on the left. Arrow indicates location of 33-kDa aqueous-phase protein which is present in greater amounts in the attenuated strain of serovar grippotyphosa.

ular mass of 20 kDa ($R_f = 0.74$) were immunoprecipitated in greater amounts from the attenuated strain.

The protein nature of the immunoprecipitated antigens was determined by treatment of these fractions with proteinase K. The 33-, 41-, and 44-kDa antigens are proteins as indicated by their sensitivity to proteinase K (Fig. 7B). Immunoprecipitated antigens with an apparent molecular mass of less than 33 kDa were not sensitive to proteinase K and likely represent the leptospiral LLS.

In order to determine the fate of the 33-, 41-, and 44-kDa surface-exposed proteins following TX-114 phase partitioning, affinity-purified antibody was prepared against each of these immunoprecipitated proteins and used to probe immunoblots containing TX-114 detergent and aqueous leptospiral fractions. The 33-kDa protein partitioned preferentially into the aqueous phase, whereas the 44-kDa protein partitioned preferentially into the detergent phase (data not shown). In contrast, the 41-kDa protein did not phase partition selectively and was found in both the detergent and aqueous phases.

Freeze-fracture electron microscopy and particle density quantitation. Comparison of the particle density between virulent and attenuated strains of *L. interrogans* serovar grippotyphosa revealed significant differences. The concave outer membrane fracture face (OMF) particle density of the attenuated strain (Fig. 8B) was twice that of the virulent strain (Fig. 8A). A total of 27 OMF profiles of virulent *L. interrogans* serovar grippotyphosa were quantified and yielded 443 particles per μm^2, with a standard deviation of 214 particles per μm^2. A total of 26 OMF profiles of attenuated *L. interrogans* serovar grippotyphosa were quantified and yielded 990 particles per μm^2, with a standard deviation of 245 particles per μm^2. There was a highly significant difference in particle density between the concave OMF of the virulent and attenuated strains by using the Student's *t* test ($P < 0.000001$). In contrast, both the virulent and attenuated strains contained a similar number of particles in the convex OMF, 88 and 55 particles per μm^2, respectively.

DISCUSSION

Characterization of the surface of pathogenic leptospires will be essential to understand the pathogenesis of leptospiral diseases. The present approach to definition of the surface molecules of *L. interrogans* was designed to address problems inherent in the known fragility of its cell envelope (23) by using methods which have proven useful in studying other fragile pathogenic spirochetes, such as *T. pallidum* (15, 38, 39, 41, 47, 59) and *Borrelia burgdorferi* (8, 58). As in the cases of *T. pallidum* (15, 39) and *B. burgdorferi* (8), several lines of evidence presented here indicate that the nonionic detergent TX-114 solubilizes the outer membrane of *L. interrogans* serovar grippotyphosa. TX-114 treatment reduced the diameter of the spirochetes with loss of discernable outer membrane structure and resulted in almost quan-

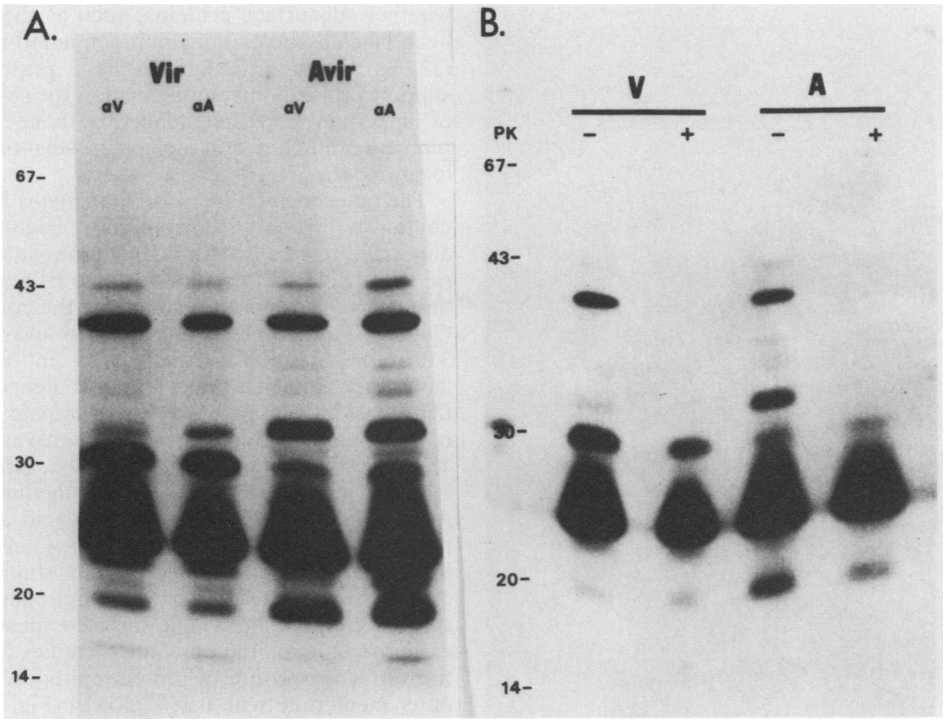

FIG. 7. Identification of surface antigens on virulent (Vir, V) and attenuated (Avir, A) *L. interrogans* serovar grippotyphosa. (A) Immunoblot of surface-immunoprecipitated material by using antiserum to whole virulent (αV) and attenuated (αA) serovar grippotyphosa. Location of molecular size standards are shown (in kilodaltons) on the left. (B) Immunoblot of surface-immunoprecipitated material with (+) and without (−) proteinase K treatment (PK). Location of molecular size standards are shown (in kilodaltons) on the left.

titative release of LLS. TX-114 treatment did not solubilize the periplasmic endoflagella or penicillin-binding proteins from the resulting cytoplasmic cylinders. These findings represent the first demonstration of epitopic conservation between endoflagella of *T. pallidum* and *L. interrogans*, as well as the first demonstration of *L. interrogans* penicillin-binding proteins, whose location is in the cytoplasmic membrane of most bacterial species (53).

Although it is reasonable to conclude that among the molecules released by TX-114 are the transmembrane proteins of the *L. interrogans* outer membrane, the information provided by the TX-114 fractionation procedure is insufficient to reveal their identities. Despite the quantitative retention of endoflagella and the penicillin-binding proteins by the cytoplasmic cylinders after TX-114 treatment, there is no evidence at present to judge whether the fate of these proteins on TX-114 treatment is reflective of the fate of other periplasmic, cytoplasmic membrane, and cytoplasmic proteins of *L. interrogans*. Even demonstration of the integrity of the cytoplasmic membrane by electron microscopy of thin sections after TX-114 treatment (15) does not rigorously rule out partial solubilization of the cytoplasmic membrane or leak of cytoplasmic contents. It should also be noted that appearance of proteins in the detergent phase after partitioning does not necessarily indicate the presence of a transmembrane peptide domain. Lipoproteins of *T. pallidum* which lack predicted transmembrane sequences partition into the TX-114 phase (9, 10, 49). Procedures useful for radiolabeling the lipid moiety of lipoproteins of *T. pallidum* and *B. burgdorferi* with fatty acids such as palmitate cannot be employed for *L. interrogans* because unlike these other spirochetes, *L. interrogans* utilizes free fatty acids as the substrate for oxidative metabolism; we therefore have not determined which of the 16 or more proteins which partition into the detergent phase are lipoproteins.

The surface immunoprecipitation procedure provided information corroborating the surface location of certain polypeptides released by TX-114. This technique involved the addition of heat-inactivated antisera directly to a culture of fully motile spirochetes to avoid the possibility that any physical manipulation of the fragile spirochetes, for example, centrifugation, might lead to the artifactual exposure of subsurface antigens. No appreciable amounts of the 35-36-kDa doublet endoflagellar protein were detected, supporting the selectivity of the immunoprecipitation procedure for the identification of surface-exposed proteins. Evidence for surface exposure of LLS and three protein antigens of 33, 41, and 44 kDa was obtained.

We found that the 33-kDa polypeptide identified by the surface immunoprecipitation procedure corresponded to a 33-kDa polypeptide antigen released by TX-114 which partitioned into the hydrophilic aqueous phase. While most transmembrane proteins partition selectively into the TX-114 hydrophobic detergent phase (7), there is at least one example of a porin which partitions selectively into the hydrophilic aqueous phase (34). It is important to note that the hydrophilic aqueous phase can be more accurately described as a detergent-poor phase (0.03% TX-114) compared with the hydrophobic detergent phase, which is detergent rich (20% TX-114). Therefore, if a membrane protein has a large proportion of hydrophilic domains (such as a porin) or is otherwise relatively incompatible with high concentrations of TX-114, it may partition either partly or completely into the detergent-poor aqueous phase (34). It is possible that the 33-kDa protein is such a molecule.

Earlier studies, using other *L. interrogans* serovars, have

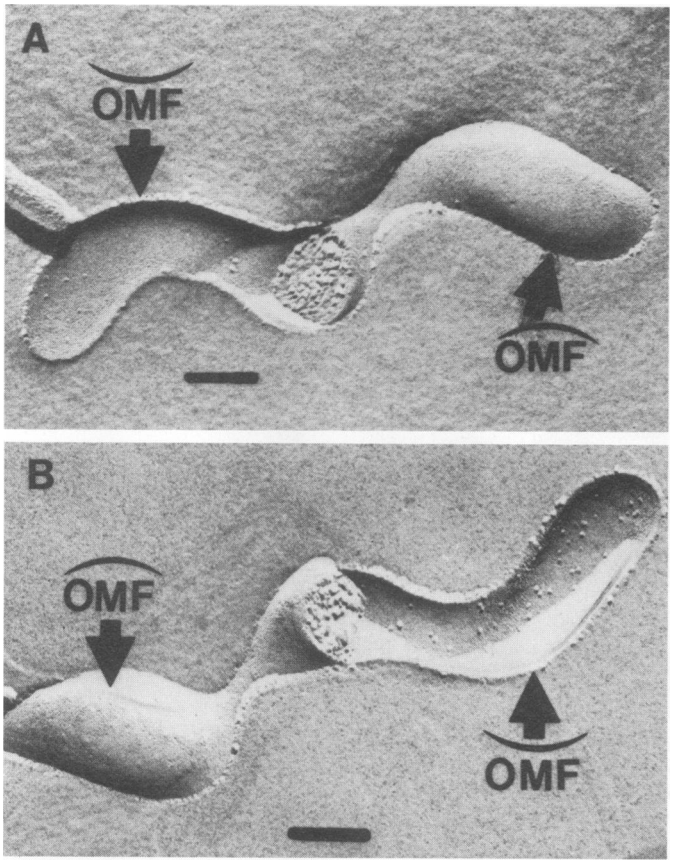

FIG. 8. Comparison of outer membrane particle density of virulent (A) and attenuated (B) *L. interrogans* serovar grippotyphosa by freeze-fracture electron microscopy. Note the intramembranous particles, representing transmembrane outer membrane proteins, seen in the concave outer membrane fracture faces (OMF) of both virulent and attenuated strains. Bars, 0.1 μm.

identified larger sets of polypeptides including those of 33, 41, and 44 kDa as surface protein candidates; some of these larger sets included antigens with molecular masses of 35 and 36 kDa (36, 37). Because the 35-36-kDa doublet probably represents endoflagellar protein (31), there is concern as to whether the additional polypeptides identified might have a subsurface rather than a surface location. A variety of methods have been used by earlier investigators. Nunes-Edwards et al. (37) used radioimmunoprecipitation and cell fractionation schemes based on the use of the ionic detergent SDS. The leptospires used in their radioimmunoprecipitation procedure were subjected to high-speed centrifugation (20,000 × *g*) prior to the addition of antibody. We have found that such high centrifugal forces cause disruption of the leptospiral outer membrane (data not shown). Niikura et al. (36) immunoprecipitated SDS-solubilized extracts of virulent and avirulent strains of *L. interrogans* serovar copenhageni that had been labeled by lactoperoxidase-catalyzed surface radioiodination. Among the putative outer membrane proteins that were immunoprecipitated, a 33-kDa protein was identified which appeared to be present in greater amounts in the avirulent strain (36). A follow-up study using the same technique found that the 33-kDa protein could be identified in some leptospiral strains but not in others (22). However, neither of these studies directly addressed the question of whether subsurface proteins, such as the endoflagella, had been labeled and/or immunoprecipitated by their technique (22, 36). Other investigators have presented evidence in support of a cell envelope location for several polypeptides of uncertain correspondence to those described in this report, both in terms of molecular weight and native cellular location (16, 27).

The outer membrane of *L. interrogans* has been known to contain a glycolipid antigen (60). High-titer agglutinating antiserum results from immunization with purified leptospiral LLS (1, 18). Like gram-negative LPS, leptospiral LLS is thought to be important in evading the complement cascade (24, 54). Passive protection (19, 29) and immunization (28, 35) studies suggest that anti-LLS antibodies can protect laboratory animals against lethal challenge. Despite the fact that considerable attention has been paid to the importance of leptospiral LLS in determining serovar specificity (2, 30, 45), its precise structure is unknown. Although leptospiral LLS is a glycolipid and is active in the limulus lysate assay, it lacks 2-keto-3-deoxyoctulosonic acid and the endotoxic properties of gram-negative LPS (11, 43, 44, 57). The surface immunoprecipitation results of our studies have provided evidence demonstrating that LLS epitopes of *L. interrogans* serovar grippotyphosa have surface exposure on living cells; earlier radioimmunoprecipitation studies focused entirely on the surface exposure of protein epitopes. Extraction of the outer membrane with 0.1% TX-114 (Fig. 1) was associated with essentially complete solubilization of the periodate silver-staining, proteinase K-resistant material, which subsequently partitioned exclusively into the hydrophobic phase, suggesting that leptospiral LLS is a hydrophobic, outer membrane component (Fig. 3). This result is also consistent with the hydrophobic nature of the long-chain fatty acid component of LLS (11, 43, 57).

Our studies also have provided new information regarding LLS structure and antigenicity. Periodate silver stains of material separated by SDS-PAGE detected four distinct bands in a pattern which was unlike the typical step-ladder pattern of gram-negative smooth LPS. Previous silver stains of leptospiral LLS separated by SDS-PAGE have shown a pattern containing two or three low-molecular-weight bands, with an apparent molecular mass of less than 25 kDa which could be immunoprecipitated with serovar-specific monoclonal antibodies (13). The pattern and number of leptospiral LLS bands seen by silver staining varied, depending on the *L. interrogans* serovar studied (12). Comparison of the periodate silver stain (Fig. 5A) and immunoblot (Fig. 5B) patterns of leptospiral LLS revealed several differences. In addition to the four LLS bands detected by periodate silver staining, immunoblotting detected a fifth, larger band with an apparent molecular mass of 30 kDa (R_f = 0.57). This result is consistent with our impression that larger forms of leptospiral LLS are more antigenic. An alternative explanation is that the band with an apparent molecular mass of 30 kDa (R_f = 0.57) is a proteinase K-resistant protein; however, Coomassie brilliant blue-stained gels of proteinase K-treated material were negative. This LLS band (apparent molecular mass of 30 kDa, R_f = 0.57) was immunoprecipitated in greater amounts from the virulent strain than from the attenuated strain, whereas the LLS band with an apparent molecular mass of 20 kDa (R_f = 0.74) was immunoprecipitated in greater amounts from the attenuated strain than from the virulent strain (Fig. 7A). Further study is needed to determine the potential relationship of these findings to the pathogenicity of an *L. interrogans* isolate.

We also studied the outer membrane of *L. interrogans* by

using freeze-fracture electron microscopy (Fig. 8). The attenuated strain had 990 particles per μm^2 in the concave OMF. This particle density is in agreement with that recently reported for an attenuated strain by Chinese investigators (25). The virulent strain, which had been passaged five times in vitro, had a significantly lower content of transmembrane protein, 443 particles per μm^2. In comparison, *T. pallidum*, subsp. *pallidum*, the agent of syphilis, has 70 particles per μm^2 in the concave OMF, whereas the human commensal spirochete *Treponema phagedenis* biotype Reiter has 800 particles per μm^2 in the concave OMF (41, 59). In contrast the nonpathogen *Spirochaeta aurantia*, which has 5,250 particles per μm^2 in the concave OMF (59), more closely resembles the outer membrane particle density of typical gram-negative bacteria, such as *Escherichia coli*, which have 6,000 to 10,000 particles per μm^2 in the concave OMF (33). The rarity of transmembrane outer membrane protein in *T. pallidum* is an important factor in delaying the activation of the classical complement pathway (6).

We found that the attenuated *L. interrogans* strain contained considerably more of the surface-exposed 33-kDa polypeptide than the virulent strain from which it was derived by repeated in vitro passage. It is possible that this increase in amount of the 33-kDa polypeptide reflects direct correspondence to the increased transmembrane outer membrane protein content of the attenuated strain. The amount of 33-kDa protein we detected in the aqueous phase of TX-114-fractionated material is consistent with this idea. If one subtracts the number of particles found per square micrometer in the virulent strain OMF (443 in the concave plus 88 in the convex equals 531) from the number found per square micrometer in the attenuated strain OMF (990 in the concave plus 55 in the convex equals 1,045), there is a net increase of 514 particles per μm^2 in the OMF. Multiplying this number by the surface area, 41 μm^2, for a spirochete whose size is approximately that of *T. pallidum* (4), there is an increase of 2.1×10^4 particles per leptospire; by using Avogadro's number, the increase is 3.5×10^{-20} mol per spirochete. If these particles represent a single species of 33-kDa molecular mass, this would be evident as 5.8 μg per 5×10^9 leptospires. In Fig. 6A the intensity of the Coomassie-stained 33-kDa polypeptide band derived from 5×10^9 leptospires suggests that several micrograms are present. While the 33-kDa polypeptide is therefore a candidate transmembrane outer membrane protein(s) of *L. interrogans*, further study is needed to define its actual relationship to the leptospiral outer membrane. If, in contrast, the transmembrane particles of the outer membrane reflect many protein species, each may be present in amounts insufficient for demonstration by conventional means such as Coomassie staining.

Further study is needed to examine the relative contributions of surface protein and LLS to killing of *L. interrogans* in vivo as well as in vitro. In vivo down-regulation of transmembrane outer membrane protein content could be a factor in the pathogenicity of *L. interrogans* if down-regulation contributed to the ability of the spirochete to delay complement activation, as in the case of *T. pallidum*. Leptospires also have the capacity to survive for long periods of time in environmental conditions (20), an important factor in the transmission of leptospirosis. Increased outer membrane particle density during in vitro cultivation may represent regulation of outer membrane protein expression through sensory transduction (48), coordinating adaptation to the nutritional and osmotic changes which occur on excretion of *L. interrogans* from the host.

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REFERENCES

1. **Adachi, Y., and R. Yanagawa.** 1977. Inhibition of leptospiral agglutination by the type-specific main antigens of leptospiras. Infect. Immun. **17**:466–467.
2. **Adler, B., and S. Faine.** 1979. Serological cross-reactions of leptospiral lipopolysaccharide (F4) antigen. Zentralbl. Bakteriol. Parasitenkd. Infektionskr. Hyg. Abt. 1 Orig. Reihe A **244**:291–301.
3. **Alexander, A. D., E. F. Lessel, L. B. Evans, E. Franck, and S. S. Green.** 1972. Preservation of leptospiras by liquid-nitrogen refrigeration. Int. J. Syst. Bacteriol. **22**:165–169.
4. **Baughn, R. E.** 1987. Role of fibronectin in the pathogenesis of syphilis. Rev. Infect. Dis. **9**:S372–S385.
5. **Bey, R. F., and R. C. Johnson.** 1978. Humoral immune response of dogs vaccinated with leptospiral pentavalent outer envelope and whole cultured vaccines. Am. J. Vet. Res. **39**:831–836.
6. **Blanco, D. R., E. M. Walker, D. A. Haake, C. I.Champion, J. N. Miller, and M. A. Lovett.** 1990. Complement activation limits the rate of in vitro treponemicidal activity and correlates with antibody-mediated aggregation of *Treponema pallidum* rare outer membrane protein. J. Immunol. **144**:1914–1921.
7. **Bordier, C.** 1981. Phase separation of integral membrane proteins in Triton X-114 solution. J. Biol. Chem. **256**:1604–1607.
8. **Brandt, M. E., B. S. Riley, J. D. Radolf, and M. V. Norgard.** 1990. Immunogenic integral membrane proteins of *Borrelia burgdorferi* are lipoproteins. Infect. Immun. **58**:983–991.
9. **Chamberlain, N. R., M. E. Brandt, A. L. Erwin, J. D. Radolf, and M. V. Norgard.** 1989. Major integral membrane protein immunogens of *Treponema pallidum* are proteolipids. Infect. Immun. **57**:2872–2877.
10. **Chamberlain, N. R., J. D. Radolf, P.-L. Hsu, S. Sell, and M. V. Norgard.** 1987. Genetic and physicochemical characterization of the recombinant DNA derived 47-kilodalton surface immunogen of *Treponema pallidum* subsp. *pallidum*. Infect. Immun. **56**:71–78.
11. **Cinco, M., E. Banfi, M. Giani, M.-L. Gundelach, and C. Galanos.** 1988. Chemical and biological properties of a phenol-water extract from *Leptospira interrogans*. Evidence for the absence of lipopolysaccharide. Infection **16**:238–241.
12. **Cinco, M., E. Banfi, and E. Panfili.** 1986. Heterogeneity of lipopolysaccharide banding patterns in *Leptospira* spp. J. Gen. Microbiol. **132**:1135–1138.
13. **Cinco, M., E. Banfi, and E. Panfili.** 1988. Leptospiral lipopolysaccharide presence in the outer envelope: electrophoretic evidence and immunological specificity. Zentralbl. Bakteriol. Parasitenkd. Infektionskr. Hyg. Abt. 1 Orig. Reihe A **269**:277–283.
14. **Cunningham, T. M., J. N. Miller, and M. A. Lovett.** 1987. Identification of *Treponema pallidum* penicillin-binding proteins. J. Bacteriol. **169**:5298–5300.
15. **Cunningham, T. M., E. M. Walker, J. N. Miller, and M. A. Lovett.** 1988. Selective release of the *Treponema pallidum* outer membrane and associated polypeptides with Triton X-114. J. Bacteriol. **170**:5789–5796.
16. **Doherty, J. P., B. Adler, J. I. Rood, S. J. Billington, and S. Faine.** 1989. Expression of two conserved leptospiral antigens in *Escherichia coli*. J. Med. Microbiol. **28**:143–149.
17. **Everard, C. O. R., G. M. Fraser-Chanpong, and J. D. Everard.** 1987. The incidence of severe leptospirosis in Trinidad. Trop. Geogr. Med. **39**:126–132.
18. **Faine, S., B. Adler, and A. Palit.** 1974. Chemical, serological and biological properties of a serotype-specific polysaccharide antigen in *Leptospira*. Aust. J. Exp. Biol. Med. Sci. **52**:311–319.
19. **Farrelly, H. E., B. Adler, and S. Faine.** 1987. Opsonic monoclonal antibodies against lipopolysaccharide antigens of *Leptospira*

interrogans serovar *hardjo*. J. Med. Microbiol. **23**:1–7.
20. **Gordon Smith, C. E., and L. H. Turner.** 1961. The effect of pH on the survival of Leptospires in water. Bull. W.H.O. **24**:35–43.
21. **Hansen, E. J., C. F. Frisch, and K. H. Johnston.** 1981. Detection of antibody-accessible proteins on the cell surface of *Haemophilus influenzae* type B. Infect. Immun. **33**:950–953.
22. **Hata, K., E. Ono, and R. Yanagawa.** 1988. Comparative studies of strains Ictero no. 1 and RGA as the type strain of *Leptospira interrogans*: agglutinin absorption test, protein and antigen profiles and enzyme activities. Microbiol. Immunol. **32**:817–832.
23. **Holt, S. C.** 1978. Anatomy and chemistry of spirochetes. Microbiol. Rev. **42**:114–160.
24. **Isogai, E., H. Isogai, and N. Ito.** 1986. Decreased lipopolysaccharide content and enhanced susceptibility of leptospiras to serum leptospiricidal action and phagocytosis after treatment with diphenylamine. Zentralbl. Bakteriol. Parasitenkd. Infektionskr. Hyg. Abt. 1 Orig. Reihe A **262**:438–447.
25. **Jianguo, X., and D. Baomin.** 1989. Ultrastructure of leptospires after freeze-etching. J. West China Univ. Med. Sci. **20**:262–265.
26. **Johnson, R. C., and V. G. Harris.** 1967. Differentiation of pathogenic and saprophytic leptospires. I. Growth at low temperatures. J. Bacteriol. **94**:27–31.
27. **Jost, B. H., B. Adler, and S. Faine.** 1988. Reaction of monoclonal antibodies with species specific determinants in *Leptospira interrogans* outer envelope. J. Med. Microbiol. **27**:51–57.
28. **Jost, B. H., B. Adler, and S. Faine.** 1989. Experimental immunisation of hamsters with lipopolysaccharide antigens of *Leptospira interrogans*. J. Med. Microbiol. **29**:115–120.
29. **Jost, B. H., B. Adler, T. Vinh, and S. Faine.** 1986. A monoclonal antibody reacting with a determinant on leptospiral lipopolysaccharide protects guinea pigs against leptospirosis. J. Med. Microbiol. **22**:269–275.
30. **Kawaoka, Y., M. Naiki, and R. Yanagawa.** 1982. Isolation of the the antigen-active components from leptospiral serovar-specific lipopolysaccharide antigen by mild acid hydrolysis. Jpn. J. Vet. Sci. Juigaku Zasshi **44**:473–478.
31. **Kelson, J. S., B. Adler, A. J. Chapman, and S. Faine.** 1988. Identification of leptospiral flagellar antigens by gel electrophoresis and immunoblotting. J. Med. Microbiol. **26**:47–53.
32. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the the head of bacteriophage T4. Nature (London) **227**:680–685.
33. **Lugtenberg, B., and L. Van Alphen.** 1983. Molecular architecture and functioning of the outer membrane of *Escherichia coli* and other gram-negative bacteria. Biochim. Biophys. Acta **737**:51–115.
34. **Maher, P. A., and S. J. Singer.** 1985. Anomalous interaction of the acetylcholine receptor protein with the nonionic detergent Triton X-114. Proc. Natl. Acad. Sci. USA **82**:958–962.
35. **Masuzawa, T., R. Nakamura, T. Shimizu, Y. Iwamoto, T. Morita, and Y. Yanagihara.** 1989. Immunological characteristics of the glycolipid antigen of *Leptospira interrogans* serovar lai. Infect. Immun. **57**:2502–2506.
36. **Niikura, M., E. Ono, and R. Yanagawa.** 1987. Molecular comparison of antigens and proteins of virulent and attenuated clones of *Leptospira interrogans* serovar *Copenhageni*, strain *Shibaura*. Zentralbl. Bakteriol. Parasitenkd. Infektionskr. Hyg. Abt. 1 Orig. Reihe A **266**:453–462.
37. **Nunes-Edwards, P. L., A. B. Thiermann, P. J. Bassford, and L. V. Stamm.** 1985. Identification and characterization of the protein antigens of *Leptospira interrogans* serovar *hardjo*. Infect. Immun. **48**:492–497.
38. **Penn, C. W., A. Cockayne, and M. J. Bailey.** 1985. The outer membrane of *Treponema pallidum*: biological significance and biochemical properties. J. Gen. Microbiol. **131**:2349–2357.
39. **Radolf, J. D., N. R. Chamberlain, A. Clausell, and M. V. Norgard.** 1988. Identification and localization of integral membrane proteins of virulent *Treponema pallidum* subsp. *pallidum* by phase partitioning with the nonionic detergent Triton X-114. Infect. Immun. **56**:490–498.
40. **Radolf, J. D., C. Moomaw, C. A. Slaughter, and M. V. Norgard.** 1989. Penicillin-binding proteins and peptidoglycan of *Treponema pallidum* subsp. *pallidum*. Infect. Immun. **57**:1248–1254.
41. **Radolf, J. D., M. V. Norgard, and W. W. Schulz.** 1989. Outer membrane ultrastructure explains the limited antigenicity of virulent *Treponema pallidum*. Proc. Natl. Acad. Sci. USA **86**:2051–2055.
42. **Segers, R. P. A. M., A. Van der Drift, A. De Nijs, P. Corcione, B. A. M. Van der Zeijst, and W. Gastra.** 1990. Molecular analysis of a sphingomyelinase C gene from *Leptospira interrogans* serovar *hardjo*. Infect. Immun. **58**:2177–2185.
43. **Shimizu, T., E. Matsusaka, N. Nagakura, K. Takayanagi, T. Masuzawa, Y. Iwamoto, T. Morita, I. Mifuchi, and Y. Yanagihara.** 1987. Chemical properties of lipopolysaccharide-like substance (LLS) extracted from *Leptospira interrogans* serovar *canicola* strain Moulton. Microbiol. Immunol. **31**:717–725.
44. **Shimizu, T., E. Matsusaka, K. Takayanagi, T. Masuzawa, Y. Iwamoto, T. Morita, I. Mifuchi, and Y. Yanagihara.** 1987. Biological activities of lipopolysaccharide-like substance (LLS) extracted from *Leptospira interrogans* serovar *canicola* strain Moulton. Microbiol. Immunol. **31**:727–735.
45. **Shimono, E., K. Sugiyama, and R. Yanagawa.** 1979. Specificity of serovar-specific main antigens of leptospiras shown by the inhibition of leptospiral microscopic agglutination. Jpn. J. Vet. Sci. **41**:623–628.
46. **Spratt, B. G.** 1977. Properties of the penicillin-binding proteins of *Escherichia coli* K12. Eur. J. Biochem. **72**:341–352.
47. **Stamm, L. V., R. L. Hodinka, P. B. Wyrick, and P. J. Bassford, Jr.** 1987. Changes in the cell surface properties of *Treponema pallidum* that occur during in vitro incubation of freshly extracted organisms. Infect. Immun. **55**:2255–2261.
48. **Stock, J. B., A. J. Ninfa, and A. M. Stock.** 1989. Protein phosphorylation and regulation of adaptive responses in bacteria. Microbiol. Rev. **53**:450–490.
49. **Swancutt, M. A., B. S. Riley, J. D. Radolf, and M. V. Norgard.** 1989. Molecular characterization of the pathogen-specific, 34-kilodalton membrane immunogen of *Treponema pallidum*. Infect. Immun. **57**:3314–3323.
50. **Thiermann, A. B.** 1984. Leptospirosis: current developments and trends. J. Am. Vet. Med. Assoc. **184**:722–725.
51. **Thiermann, A. B., R. D. McClellan, and H. T. Hill.** 1984. Improved techniques for the isolation of leptospires from swine abortion cases. Annu. Proc. Am. Assoc. Vet. Lab. Diagn. **27**:233–244.
52. **Thomas, D. D., J. B. Baseman, and J. F. Alderete.** 1985. Putative *Treponema pallidum* cytadhesins share a common functional domain. Infect. Immun. **49**:833–835.
53. **Tomasz, A.** 1986. Penicillin-binding proteins and the antibacterial effectiveness of beta-lactam antibiotics. Rev. Infect. Dis. **8**:S260–S278.
54. **Torres, J., R. Yanagawa, and E. Ono.** 1981. Inhibition of leptospiricidal reaction by the type-specific main antigen of Leptospira. Zentralbl. Bakteriol. Parasitenkd. Infektionskr. Hyg. Abt. 1 Orig. Reihe A **250**:147–152.
55. **Towbin, H. T., T. Staehelin, and J. Gordon.** 1979. Electrophoretic transfer of proteins. Proc. Natl. Acad. Sci. USA **76**:4350–4354.
56. **Tsai, C.-M., and C. E. Frasch.** 1982. A sensitive silver stain for detecting lipopolysaccharides in polyacrylamide gels. Anal. Biochem. **119**:115–119.
57. **Vinh, T., B. Adler, and S. Faine.** 1986. Ultrastructure and chemical composition of lipopolysaccharide extracted from *Leptospira interrogans* serovar *copenhageni*. J. Gen. Microbiol. **132**:103–109.
58. **Walker, E. M., et al.** Submitted for publication.
59. **Walker, E. M., G. A. Zampighi, D. R. Blanco, J. N. Miller, and M. A. Lovett.** 1989. Demonstration of rare protein in the outer membrane of *Treponema pallidum* subsp. *pallidum* by freeze-fracture analysis. J. Bacteriol. **171**:5005–5011.
60. **Zeigler, J. A., and W. P. VanEseltine.** 1975. Isolation and chemical characterization of outer envelope of *Leptospira pomona*. Can. J. Microbiol. **21**:1102–1112.