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Changes in Lipopolysaccharide O Antigen Distinguish Acute versus Chronic *Leptospira interrogans* Infections

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Leptospirosis is the most geographically widespread zoonotic disease in the world. A severe pulmonary form of leptospirosis (SPFL) is being recognized with increased frequency. We have reported that human SPFL isolates of *Leptospira* cause acute lethal infection with prominent pulmonary hemorrhage in guinea pigs. We have found that the same SPFL strains cause asymptomatic infection and chronic renal shedding in rats, where infection is restricted to the renal tubules. To address the antigenic composition of host tissue-derived *Leptospira* (HTL), motile leptospires were purified from guinea pig liver by centrifugation on Percoll density gradients and compared to Percoll-purified in vitro-cultivated *Leptospira* (IVCL). The lipopolysaccharide O antigen (Oag) content of guinea pig liver-derived HTL was markedly reduced compared to that of IVCL, as demonstrated both by immunoblotting with a monoclonal antibody that was serovar specific for Oag and by periodate-silver staining. Confocal microscopy of HTL in guinea pig liver and kidney with the Oag-specific monoclonal antibody provided further evidence that diminution of the Oag content occurred in situ during lethal infection. In contrast, the Oag content of HTL in chronically infected rat renal tubules was indistinguishable from that of IVCL. These findings suggest that there may be regulation of Oag synthesis by *Leptospira* specific to the animal host infected. The hypothesis that the Oag content is related to whether lethal infection or chronic renal tubular colonization occurs remains to be tested.

Leptospirosis is the most geographically widespread zoonotic disease in the world (4, 21). Two distinct outcomes may result from infection with *Leptospira*. Rodents, pigs, cattle, sheep, and dogs may become clinically asymptomatic carriers, and these animals constitute the reservoir of spirochete infection. This carrier state consists of chronic infection restricted to renal tubules and shedding of *Leptospira* into the environment with urine. In other instances, leptospiral infection results in acute infection with a wide range of clinical severity. The hepatic and renal failure seen with human Weil's disease is well known. A severe pulmonary form of leptospirosis (SPFL) has emerged relatively recently, and cases have been reported from Brazil, Argentina, Nicaragua, India, Thailand, Korea and Australia (29, 31, 32, 34, 35, 37, 43). SPFL is characterized by an often-fatal pulmonary hemorrhage.

Reasoning that there must be fundamental differences in the leptospire-host relationship that distinguish acute lethal infection from chronic carriage, we have used blood isolates from human SPFL cases to establish experimental infections in which one of these distinct outcomes predictably occurs. Recently, we reported rapidly disseminated infection and fatal pulmonary hemorrhage in a guinea pig model (27). While few *Leptospira interrogans* serovar Copenhageni organisms were found in lung tissue, large numbers were present in the liver, kidneys, spleen, and intestines. There was no bleeding diathesis underlying the pulmonary hemorrhage. The presence of antibodies and complement and the paucity of spirochetes along alveolar septa suggested that the infection may have precipitated an autoimmune process that led to pulmonary hemorrhage through damage to the alveolar septa.

In this report we describe establishment of chronic aymptomatic renal infection in rats using the same SPFL serovar Copenhageni isolate that caused fatal pulmonary hemorrhage in guinea pigs. Experimental study of the carrier state in rats or other rodents has been very limited. Thiermann described an experimental infection of *Rattus norvegicus* established with intraperitoneal injections of serovar Icterohaemorrhagiae (36). Rats infected with serovar Icterohaemorrhagiae were clinically asymptomatic and shed leptospires throughout the 220-day duration of the study. No further microbiologic or histopathologic study was conducted. Faine reported experimental induction of the carrier state in mice, but histopathology was not reported; the major findings were that mice infected with strains that caused carriage first lost weight, suggesting systemic illness, and then gained weight (12, 13).

We compared features of the antigenic composition of *L. interrogans* serovar Copenhageni found in guinea pig liver and in rat renal tubules and cultivated in vitro. The lipopolysaccharide O antigen (Oag) content of leptospires in guinea pig liver was markedly reduced compared to that of organisms found in rat renal tubules or cultivated in vitro. Thus, there is an association between diminished O-antigen content and acute lethal infection, while the O-antigen content during renal tubular colonization approximates that of in vitro-cultivated leptospires.

MATERIALS AND METHODS

Bacteria. An isolate of *L. interrogans* serovar Copenhageni, RJ16441, was obtained from blood cultures of a patient suffering from SPFL who was admitted to Antonio Pedro University Hospital in Rio de Janeiro, Brazil (27, 34). Cultures were maintained in EMJH liquid medium (Becton Dickinson, Cockeysville, MD)

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or EMJH semisolid medium (EMJH liquid medium containing 0.2% Noble agar) at 30°C. Isolates were passaged through guinea pigs to maintain virulence as previously described (27). A clonal isolate of strain RJ16441, generated by plating on solid agar medium, was used for certain experiments in this study, as indicated below.

Animals. Hartley male guinea pigs (Charles River Laboratories, Kingston, NY) that weighed 200 g and were 12 to 15 days old were injected intraperitoneally with 10^5 organisms of low-passage clonal or nonclonal *Leptospira* isolate RJ16441 in a final volume of 500 μl as previously described (27). Sprague-Dawley rats (Charles River Laboratories, Kingston, NY) that weighed 130 to 150 g and were approximately 6 weeks old were injected intraperitoneally with 10^7 organisms of low-passage RJ16441 in a final volume of 500 μl. Negative control animals were injected with EMJH medium alone. Animals were monitored daily for signs of illness, including weight loss and loss of mobility. Guinea pigs were euthanized on day 5 or 6, and rats were euthanized on day 21. Animal tissues, including the liver and kidney, were processed as previously described (27). Immunohistochemistry was performed as previously described (27). All animal studies were approved by the Animal Research Committee of the University of California Los Angeles.

Antisera. The generation of antiserum against outer membrane vesicles (OMV), raised against purified OMV of strain RJ-15858, has been described previously (27, 28). Chronic rat serum (CRS) was obtained from Sprague-Dawley rats approximately 5 months after infection with RJ16441.

Purification of *Leptospira* from infected guinea pig liver. Intact motile *Leptospira* organisms were extracted from infected guinea pig liver as previously described for extraction of the syphilis spirochete, *Treponema pallidum*, from infected rabbit testes, with some modifications (14). In brief, infected guinea pig liver (~8 to 10 g) was triturated and divided into two samples, and each sample was resuspended in 12 ml phosphate-buffered saline (PBS), pH 7.4. Samples were shaken vigorously for at least 2 h before centrifugation at 400 × *g* for 5 min to remove crude tissue debris. Each supernatant was gently overlaid onto a 30-ml Percoll (Amersham Biosciences, Piscataway, NJ) density gradient (1.08 g/ml), and this was followed by centrifugation for 20 min at 30,000 × *g* in a fixed-angle rotor. Fractions were collected from the bottom of the gradient, the approximate numbers of *Leptospira* cells per fraction were determined by dark-field microscopy, and samples were processed directly for gel electrophoresis and immunoblotting. As a control, in vitro-cultivated *Leptospira* (IVCL) organisms were also separated with a Percoll density gradient and fractions were collected as described above. Samples were stored at −20°C until analysis. Samples were boiled for 10 min in 5× final sample buffer (Immunopure lane marker reducing sample buffer; Pierce, Rockford, IL) before Percoll removal by centrifugation for 15 min at 16,000 × *g*. Supernatant was either analyzed directly or treated with 200 μg/ml proteinase K (Roche Diagnostics Corporation, Indianapolis, IN) overnight at 55°C before analysis.

Gel electrophoresis and immunoblotting. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis was performed using the discontinuous buffer system described by Laemmli (20) and 15% acrylamide gels. Electrophoresis was performed using a Mini-PROTEAN II cell (Bio-Rad Laboratories, Richmond, CA) for 60 min at 180 V in Tris-glycine running buffer (25 mM Tris, 192 mM glycine, 0.1% sodium dodecyl sulfate, pH 8.3). Samples were transferred to an Immobilon-P transfer membrane (Millipore, Bedford, MA) and blocked with 5% (wt/vol) nonfat dry milk in PBS-0.1% Tween 20 (PBS-T). Membranes were individually incubated for 1 h either with antiserum raised against OMV (1:2,000 dilution), followed by a 1-h incubation with horseradish peroxidase–donkey anti-rabbit immunoglobulin G (IgG) conjugate (1:2,500 dilution; Amersham Biosciences, Piscataway, NJ); with CRS (1:2,000 dilution), followed by incubation with horseradish peroxidase–goat anti-rat IgG conjugate (1:2,000 dilution; Amersham Biosciences, Piscataway, NJ); or with serovar Copenhageni-specific monoclonal antibody F70C24 (1:50 dilution; WHO/FAO/OIE/RIVM Leptospirosis Reference Laboratory, KIT Royal Tropical Institute, The Netherlands), followed by incubation with horseradish peroxidase–sheep anti-mouse IgG conjugate (1:2,500 dilution; Amersham Biosciences, Piscataway, NJ). Bound conjugates were detected with SuperSignal West Dura extended duration substrate (Pierce Biotechnology Inc., Rockford, IL) and an Alpha Innotech Fluorchem 8000 imager. Integrated density values of immunoblots were obtained using the FluorChem software, version 3.04A (Alpha Innotech Corporation, San Leandro, CA).

Periodate-silver staining of lipopolysaccharide. Lipopolysaccharide (LPS) of smooth and rough *Salmonella enterica* serovar Minnesota was obtained from Sigma (St. Louis, MO). An enrichment of *Leptospira* LPS was prepared as previously described (7). Periodate-silver staining of polyacrylamide gels was performed as previously described (38).

Immunohistochemistry and immunofluorescence. OMV were prepared as previously described (28) and used to immunize 3.5- to 4-kg New Zealand White rabbits in order to generate anti-OMV antiserum. Double-immunofluorescence labeling was performed with animal tissues as follows. Four-micrometer sections of paraffin-embedded tissue were treated for 30 min at 60°C. The sections were subsequently incubated in xylene (twice for 5 min), 100% ethanol (twice for 1 min), 95% ethanol (twice for 1 min), 70% ethanol (twice for 1 min), H_2O (twice for 5 min), and PBS (for 5 min). Each tissue section was then treated with trypsin (0.2 mg/ml in 0.05 M Tris-Cl, pH 8.0) for 9 min at 37°C. After washing in PBS (three times for 5 min), sections were blocked with 10% normal goat serum (NGS) for 30 min. Sections were washed in PBS-Tween 20 (0.05%) (PBS-T) (three times for 5 min) before incubation with monoclonal antibody F70C24 (1:1,000) in 3% NGS overnight at 4°C. Sections were again washed in PBS-T (0.05%) (three times for 5 min), and the remaining reactions were performed in the dark. Goat anti-mouse Cy-3 (Jackson ImmunoResearch Laboratories, West Grove, PA) was added (1:500 dilution in PBS) for 1 h. Sections were again washed in PBS-T (0.05%) (three times for 5 min), and anti-OMV (1:500 dilution) in 3% NGS was added for 90 min. After washing in PBS-T (0.05%) (three times for 5 min), sections were incubated in goat anti-rabbit Alexa 488 (Molecular Probes, Eugene, OR) at a 1:500 dilution in PBS for 1 h. After washing in PBS-T (0.05%) (three times for 5 min) and PBS (once for 5 min) and a final rinse in H_2O, sections were mounted with VectaShield mounting medium for fluorescence (Vector Laboratories, Burlingame, CA). Fluorescence confocal images were scanned on a Leica TCS-SP spectral confocal inverted or fixed-stage upright microscope equipped with argon (488-nm blue excitation) and krypton (568-nm yellow excitation) lasers (Leica Microsystems, Heidelberg, Germany).

Control samples included noninfected tissues and infected tissues processed as described above without primary antiserum. Additionally, infected tissues were processed with just monoclonal antibody F70C24 alone. Confocal microscopy was also performed on in vitro-cultivated *Leptospira* by resuspending live cultures in molten 2% agarose which was solidified, fixed, and embedded in paraffin such that they could be processed in the same way as tissue obtained from infected animals, as described above.

RESULTS

Chronic leptospiral infection in rats. A total of 10^5 organisms of *L. interrogans* serovar Copenhageni human SPFL isolate RJ16441 caused rapidly lethal infection in guinea pigs, as previously described (27). In contrast, experimental infection of Sprague-Dawley rats with 10^7 organisms of the same isolate resulted in asymptomatic chronic renal infection. Infected rats gained weight at the same rate as noninfected controls. Shedding of *Leptospira* into urine was first detected by dark-field microscopy at 8 to 10 days after infection, and the peak level of shedding reached 10^7 *Leptospira* organisms/ml of urine within 28 days; shedding ceased by 72 days after infection (data not shown; E. Chow, J. E. Nally, X.-Y. Wu, M. C. Fishbein, D. R. Blanco, and M. A. Lovett, unpublished data). A striking feature of the infected rat kidneys was the absence of histopathology; there was normal anatomy without inflammatory infiltrates at day 21 (Fig. 1 D). We have previously described the few inflammatory cells despite the large numbers of leptospires found in infected guinea pig liver and kidneys (27).

To compare the distributions of spirochetes in lethally infected guinea pigs and chronically infected rats, antiserum specific for outer membrane vesicles (OMV) of in vitro-cultivated *Leptospira* (IVCL) was used to demonstrate *Leptospira* as previously described (27) (Fig. 1). In contrast to the dissemination of *Leptospira* observed in acutely infected guinea pigs, in which large numbers of spirochetes were seen in the liver and kidneys, the presence of intact spirochetes was restricted to renal tubules in rats sacrificed on day 21 after infection. No *Leptospira* cells were seen in other rat tissues, including the liver, heart, lung, and intestine, but the rat spleen contained

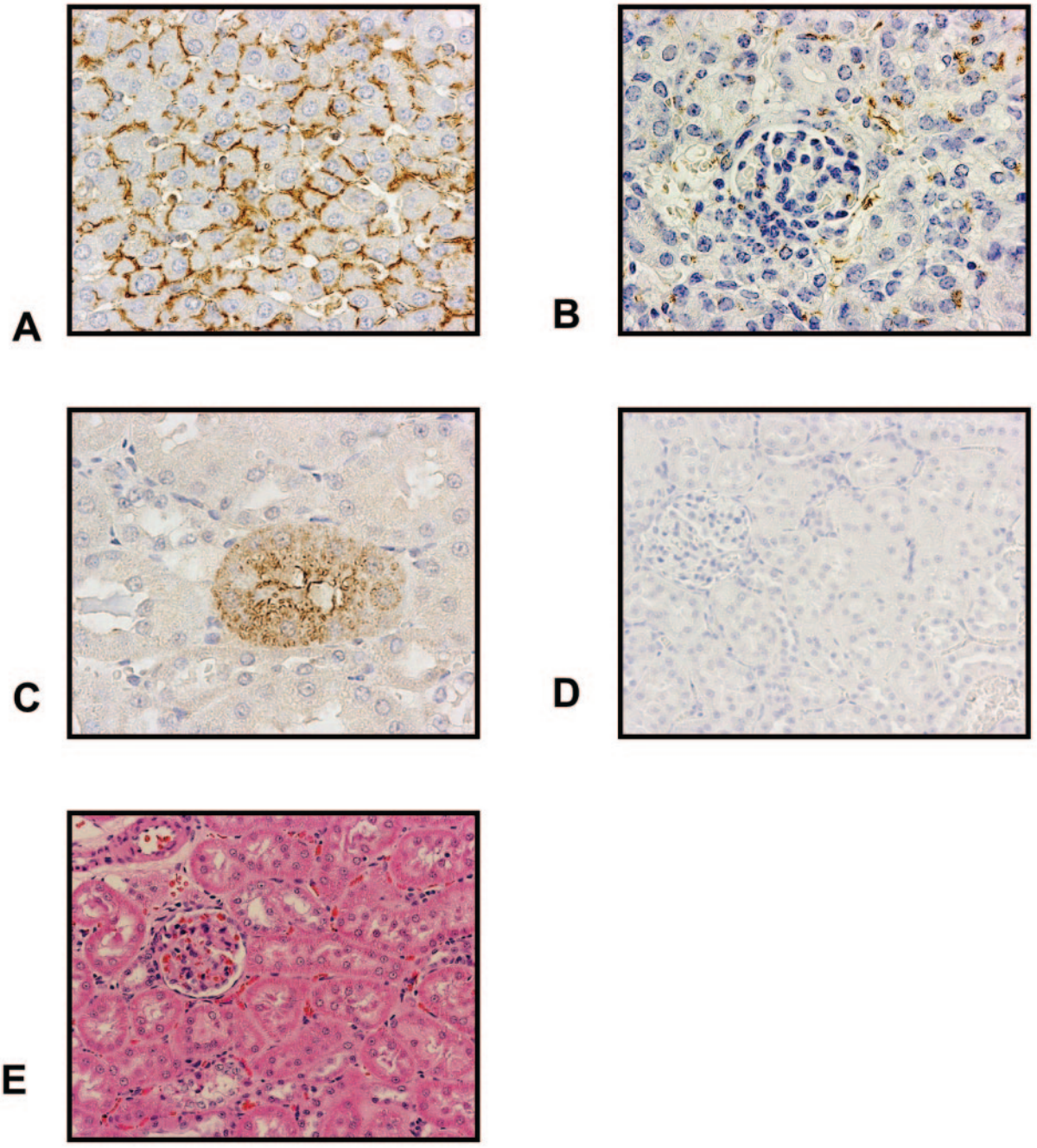

FIG. 1. Features of acute guinea pig and chronic rat infections with SPFL strain RJ16441. Acutely infected guinea pigs have large numbers of leptospires disseminated in the liver (original magnification, ×400) (A) and the kidney (original magnification, ×400) (B), as demonstrated by immunohistochemistry with antiserum specific for OMV of *Leptospira*. Few inflammatory cells are present. In contrast, immunohistochemistry showed that in chronically infected rats at day 21, the presence of leptospires was restricted to renal tubules (original magnification, ×100) (C). (D) Hematoxylin and eosin staining of infected rat kidney revealed the absence of inflammatory cells (original magnification, ×200). (E) Uninfected rat kidney showed no binding of OMV antiserum.

some granular debris that was reactive with the OMV antiserum (data not shown).

Purification of *Leptospira* from guinea pig liver by Percoll density gradient centrifugation. The large numbers of intact *Leptospira* cells visualized in guinea pig liver suggested that *Leptospira* could be extracted from this tissue using a method that has been used for purification of *T. pallidum* from infected rabbit testes (14). Percoll purification yielded fractions containing from 10^7 to $>10^8$ motile host tissue-derived *Leptospira* (HTL) organisms from guinea pig liver in guinea pigs that weighed ~200 g and were sacrificed 5 to 6 days after infection with SPFL isolate RJ16441. Percoll purification of HTL from guinea pigs infected with 10^5 cells of RJ15958 yielded significantly lower numbers, as expected

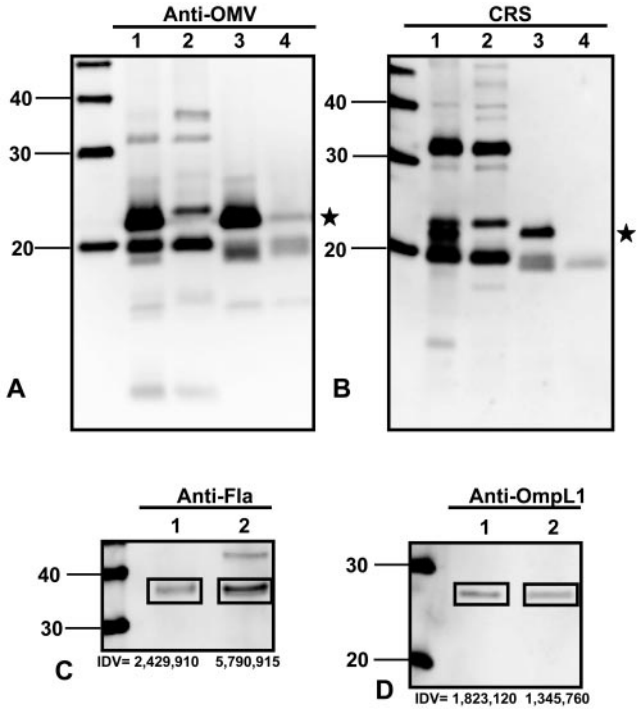

FIG. 2. Antigenic composition of guinea pig liver-derived HTL and demonstration that the content of a proteinase K-resistant 22-kDa antigen is diminished. Immunoblots of equal numbers of whole IVCL cells purified over Percoll (lane 1), whole guinea pig liver-derived HTL cells purified over Percoll (lane 2), Percoll-purified IVCL cells treated with proteinase K (lane 3), and Percoll-purified HTL cells treated with proteinase K (lane 4) were probed with either antiserum specific for OMV of *Leptospira* (A) or serum from chronically infected rats (CRS) (B). In addition, Percoll-purified IVCL or HTL was immunoblotted with antiserum specific for flagella (C) or OmpL1 (33) (D) to verify that similar amounts of *Leptospira* cells were loaded in each lane. The integrated density values (IDV) demonstrate that the diminished content of the 22-kDa proteinase K-resistant antigen was not due to smaller amounts of HTL being compared to IVCL. The positions of molecular mass markers (in kilodaltons) are indicated to the left of each blot. An star indicates the position of the 22-kDa antigen expressed in smaller amounts in HTL than in IVCL.

based on detection of lower numbers of intact leptospires by immunohistochemistry (27), suggesting that the optimal time for recovery of HTL from infected animals has to be determined for each isolate.

Antigenic analysis of guinea pig liver-derived *Leptospira* and marked reduction of a 22-kDa proteinase K-resistant antigen in HTL. OMV antiserum was used to compare the antigenic profiles of Percoll-purified IVCL and HTL on immunoblots (Fig. 2A, lanes 1 and 2). Since OMV antiserum was raised against outer membrane vesicles of IVCL, a parallel immunoblot analysis was also performed using antiserum from chronically infected rats (CRS), which might recognize antigens better expressed during infection than in IVCL, and other antigens not contained in the outer membrane of IVCL (Fig. 2B, lanes 1 and 2). The most striking difference between the IVCL and HTL profiles obtained with both anti-OMV and CRS was the absence or marked diminution of a 22-kDa antigen in the HTL sample (Fig. 2A and B, lane 2).

In order to determine whether the 22-kDa antigen whose expression was diminished in HTL was proteinaceous or lipopolysaccharide in nature, samples were treated with proteinase K and immunoblotted with anti-OMV and CRS. Immunoblotting of proteinase K-treated samples demonstrated the presence of the 22-kDa antigen in IVCL and its diminution in HTL, indicating that the 22-kDa antigen was resistant to proteinase K treatment, suggesting that it was likely LPS in composition (Fig. 2A, lanes 3 and 4, and Fig. 2B, lanes 3 and 4). Complete digestion of IVCL and HTL samples was confirmed by Sypro Ruby protein staining (data not shown).

To obtain confirmation that the differences in the quantity of leptospiral antigen obtained from IVCL and HTL do not explain why different amounts of the 22-kDa antigen were detected, immunoblots containing the same IVCL and HTL samples were probed with antiserum to flagella, a protein expected to be present in similar amounts of motile IVCL and HTL (Fig. 2C), and antiserum specific for OmpL1, an outer membrane porin of *Leptospira* (Fig. 2D). The integrated density values for the resulting flagellum and OmpL1 bands provided an independent way to verify that the greatly reduced content of the 22-kDa proteinase K-resistant antigen of the HTL observed was not the result of smaller amounts of HTL being compared to the amounts of IVCL.

Lipopolysaccharide serovar Copenhageni O antigen of liver-derived HTL is the proteinase K-resistant band diminished in HTL. In order to address the identity of the 22-kDa proteinase K-resistant antigen diminished in liver-derived HTL, characterization of the LPS of two serovar Copenhageni strains and other *Leptospira* species was performed. As shown in Fig. 3A, the sodium dodecyl sulfate-polyacrylamide gel electrophoresis profile of LPS from *Leptospira* differs significantly from that of typical enterobacterial rough or smooth LPS, as previously reported (7, 40). In contrast to the ladder-like appearance of the smooth LPS of *Salmonella*, which ranged in size from approximately 10 to >80 kDa, the periodate-silver-stained bands of the two serovar Copenhageni strains were two major bands, one at 22 kDa and one at approximately the size of the core LPS seen in the rough *Salmonella* strain (Fig. 3A, lane 1). A 22-kDa band was identified as the serovar Copenhageni O antigen by immunoblotting with monoclonal antibody F70C24 from the WHO/FAO/OIE/RIVM Leptospirosis Reference Laboratory (Fig. 3B). As shown in Fig. 3A, the periodate-silver-stained putative O antigen of *Leptospira kirschneri* is smaller than that of serovar Copenhageni, and *Leptospira biflexa* lacks an O antigen, as previously reported (7).

Monoclonal antibody F70C24 was then used to probe the Percoll-purified liver-derived HTL and IVCL samples. Figure 4A demonstrates that the 22-kDa proteinase K-resistant antigen was markedly reduced or absent in HTL compared with IVCL (Fig. 2A and B), which bound monoclonal antibody F70C24 and was therefore identified as the serovar Copenhageni Oag; thus, it was Oag that was reduced or absent in liver-derived HTL compared with IVCL. CRS antibodies bound both to the Oag in IVCL and to an 18-kDa proteinase K antigen not reactive with monoclonal antibody F70C24 present in both IVCL and HTL (Fig. 4B). The identity of this band has not been established. Periodate-silver staining of Percoll-purified IVCL and HTL demonstrated the absence of detectable 22-kDa Oag in the HTL (Fig. 4C). The abundant higher-molecular-weight proteins observed in the HTL sample (lane

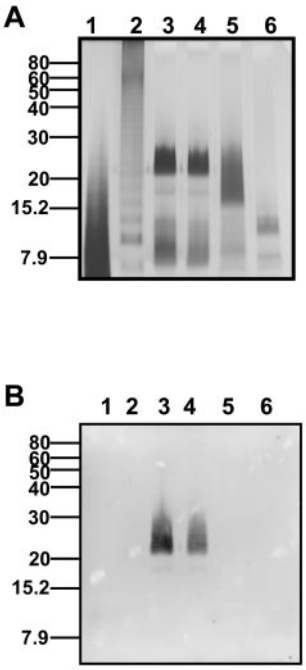

FIG. 3. Comparison of enterobacterial and leptospiral LPS and identification of serovar Copenhageni Oag. (A) Periodate-silver staining of *Salmonella* rough LPS (2.5 μg) (lane 1), *Salmonella* smooth LPS (7.5 μg) (lane 2), *L. interrogans* serovar Copenhageni strain RJ16441 LPS from approximately 7.5×10^7 cells (lane 3), *L. interrogans* serovar Copenhageni strain RJ15958 LPS from approximately 7.5×10^7 cells (lane 4), *L. kirschneri* LPS from approximately 10^8 cells (lane 5), and *L. biflexa* LPS from approximately 7.5×10^7 cells (lane 6). The positions of molecular mass markers (in kilodaltons) are indicated on the left. (B) Immunoblot of the same samples as in panel A probed with monoclonal antibody F70C24 specific for LPS of serovar Copenhageni.

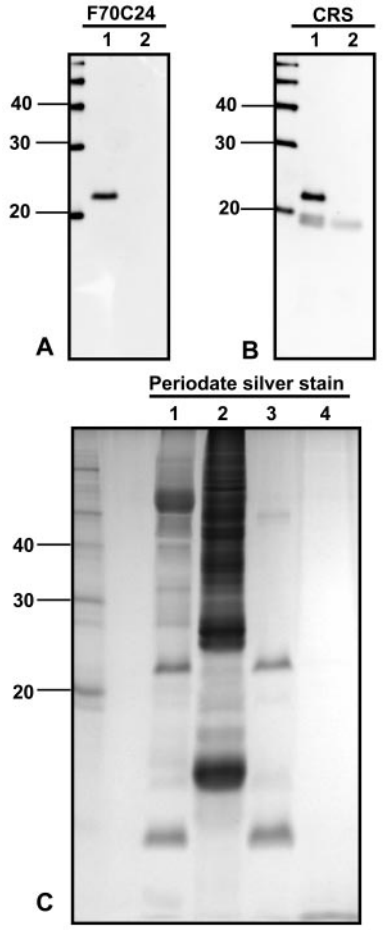

FIG. 4. Reduced Oag content in guinea pig liver-derived HTL compared to IVCL. Parallel immunoblots of proteinase K-treated Percoll-purified IVCL (lane 1) and HTL (lane 2) were probed with monoclonal antibody F70C24 (A) or CRS (B). In both cases the diminution in Oag content of HTL is apparent. (C) Periodate-silver staining of whole IVCL (lane 1) and HTL (lane 2), proteinase K-treated IVCL (lane 3), and proteinase K-treated HTL (lane 4) shows that no 22-kDa Oag band is seen in proteinase K-treated HTL.

2) were likely due to contaminating guinea pig liver protein during the extraction process. This is evidence which indicates that the diminution of O antigen observed in the liver-derived HTL was due to the physical absence of Oag rather than to the appearance of a new Oag antigenic species. While the use of monoclonal antibody F70C24 and CRS (Fig. 2 and 4) demonstrated the absence of serovar Copenhageni Oag in HTL, it should be noted that the loss of Oag was not absolute, as demonstrated by the weak reactivity of the OMV antiserum with a 22-kDa band in the HTL (Fig. 2A, lane 4).

Detection of Oag expression in situ by confocal microscopy. To address whether the diminished Oag content of Percoll-purified liver-derived HTL corresponded to the Oag content of HTL in situ during infection, confocal microscopy using OMV antiserum (1:500) and monoclonal antibody F70C24 (1:1,000) was employed to assess Oag expression by HTL in guinea pig liver and in rat renal tissues, as well as expression by IVCL. IVCL organisms were first suspended in molten 2% agarose, which was allowed to solidify, and then fixed and finally embedded in paraffin so that they could be processed in the same way as infected tissue obtained from infected animals. IVCL bound both anti-OMV and monoclonal antibody F70C24 (Fig. 5A and B). Figure 5C shows an exact overlap in the pattern of reactivity shown in Fig. 5A and B. In contrast, OMV antiserum bound HTL in guinea pig liver (Fig. 5D), while monoclonal antibody F70C24 did not bind the HTL (Fig. 5E). As shown in Fig. 5F, there was no overlap in the reactivities of the two antibodies. Similar results were observed in guinea pigs infected with a clonal isolate of RJ16441. These observations are consistent with the results obtained by the direct antigenic and compositional analyses of liver-derived HTL and IVCL described above which indicated that there was diminution of Oag by HTL.

Similar results were also observed when infected guinea pig kidney was probed with anti-OMV and monoclonal antibody F70C24 (Fig. 5G, H, and I) in that acutely infected kidney was reactive with anti-OMV but not with monoclonal antibody F70C24. In contrast, when infected rat kidney was examined, HTL cells were reactive with both anti-OMV (Fig. 5J) and monoclonal antibody F70C24 (Fig. 5K), with the expected reactivity overlap (Fig. 5L). In addition, there appeared to be reactivity specific for OMV but not monoclonal antibody

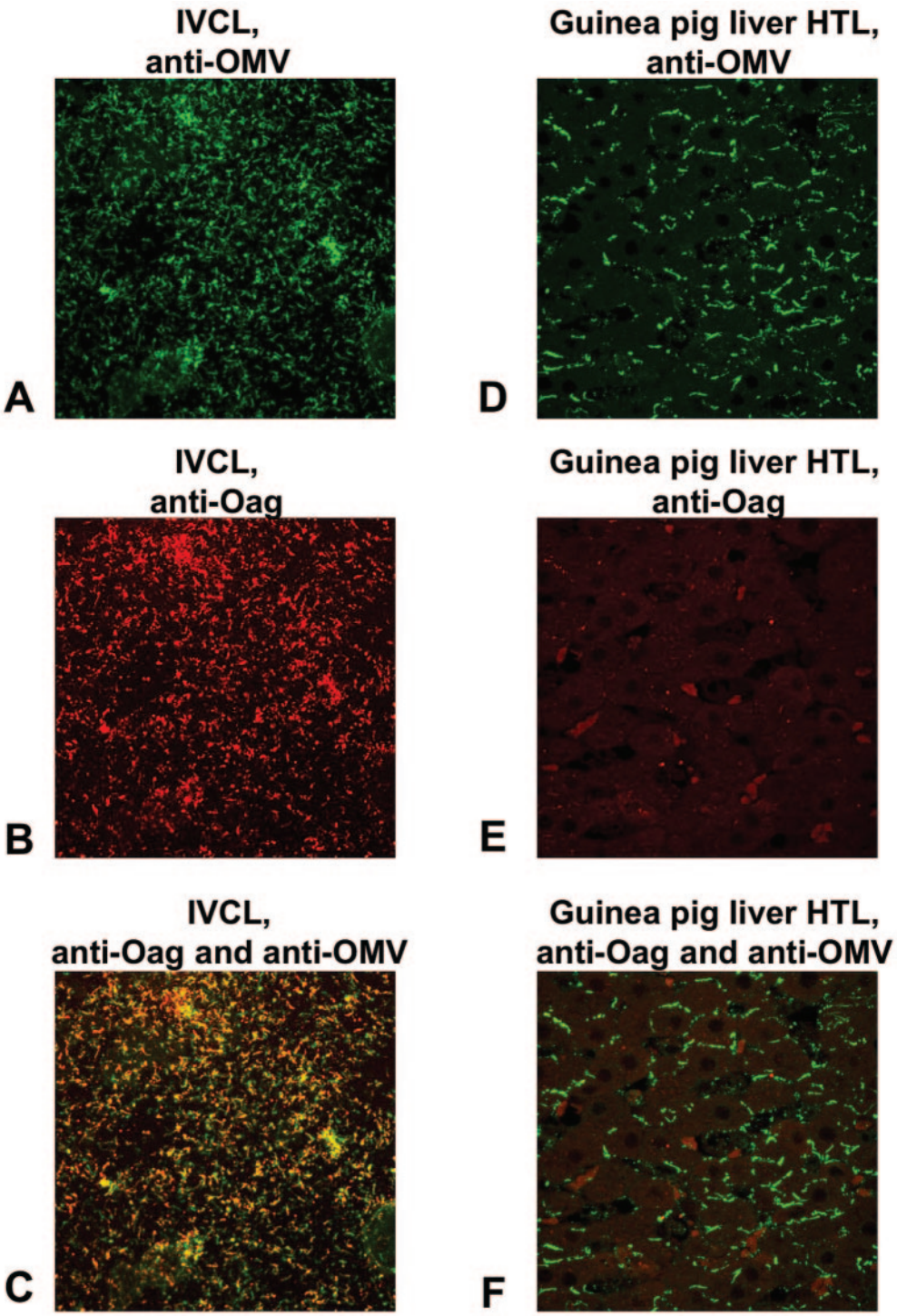

FIG. 5. Oag content of *Leptospira* in situ in rat renal tubules and in guinea pig livers and kidneys: confocal microscopy of IVCL and HTL. Double-immunofluorescence labeling of IVCL embedded in agar (A, B, and C), HTL in guinea pig liver (D, E, and F), HTL in guinea pig kidney (G, H, and I), and HTL in chronically infected rat kidney (J, K, and L) was performed with anti-OMV, which was detected with an Alexa fluor 488 conjugate (A, D, G, and J), or with monoclonal antibody F70C24, which was detected with a Cy-3 conjugate (B, E, H, and K). Detection of both reagents in the same panel for each sample is also shown (C, F, I, and L). Control images of normal tissues and infected tissue stained without primary antibody are not shown. The length of each side of each panel corresponds to 158.75 μm.

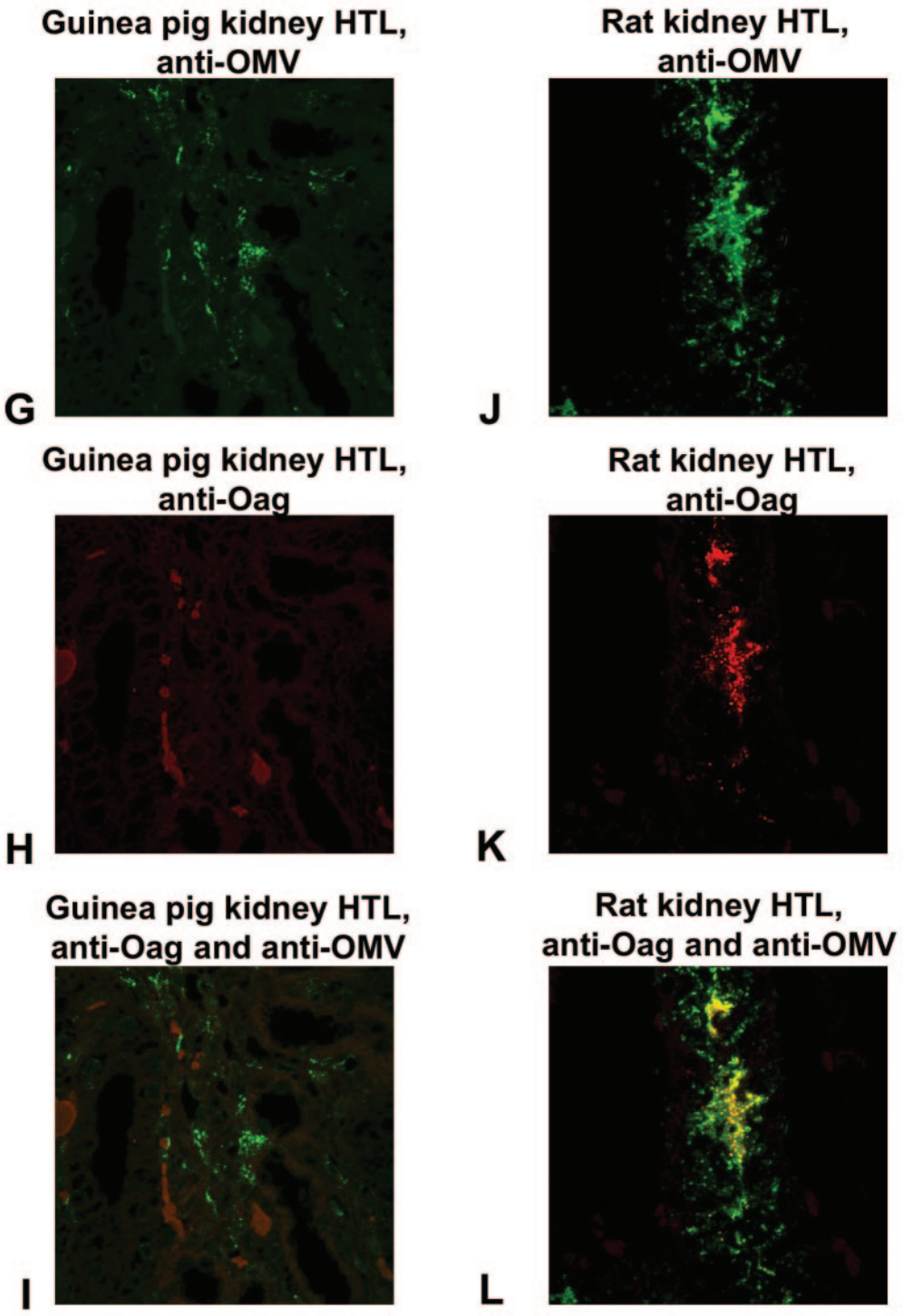

FIG. 5—*Continued.*

F70C24, suggesting that there was a mixed population of *Leptospira* expressing different amounts of Oag. Alternatively, this may have been due to the increased sensitivity of anti-OMV for detection of leptospiral antigens compared to other reagents (Fig. 2A and B). These results suggest that the HTL cells that are widely disseminated in infected guinea pig kidney and liver tissue during acute lethal infection differ significantly in the amounts of Oag compared to IVCL cells or to HTL cells that are restricted to the renal tubules of chronically infected rats.

DISCUSSION

Pathogenic *Leptospira* species infect a wide range of mammalian hosts. While acute infection can result from exposure to

many serovars of *Leptospira*, chronic infection is more evident with leptospiral serovars which are commonly associated with particular animal reservoirs (e.g., serovar Hardjo in cattle, serovar Canicola in dogs, serovar Bratislava in horses, and serovar Copenhageni in rats) (4). In this study, we used two animal models of leptospirosis that are susceptible to the same infecting isolate of *L. interrogans* serovar Copenhageni, in which experimental infection results in acute versus chronic asymptomatic infections. Specifically, experimental infection of guinea pigs emulates the acute severe pulmonary form of leptospirosis observed in human patients (27), while experimental infection of rats emulates the natural classic chronic carrier of infectious leptospires which are shed from infected rat renal tubules into the environment and await contact with new hosts.

Transmission of leptospirosis requires that *Leptospira* survives and adapts to environmental conditions that range from renal tubules to rivers and from conjunctiva eye tissue to mud. It seems likely then that the spirochetes have evolved specific methods to adapt to individual environments. However, the characteristics of the adaptations are not clear, primarily since most studies to date have focused on characterization of IVCL. In addition, pathogenic species of *Leptospira* have not yet been proven to be amenable to genetic manipulation. With this in mind, we used Percoll purification to recover *Leptospira* cells from infected guinea pig liver (HTL) so that they could be characterized and compared to their in vitro-cultivated counterparts. An unexpected finding resulting from the study of Percoll-purified HTL was that the content of the lipopolysaccharide Oag of *Leptospira* was markedly less in HTL derived from acutely infected guinea pigs than in IVCL and in HTL in chronically infected rats.

Aside from being a major component of the outer membrane of gram-negative bacteria, LPS and its associated Oag repeats are regarded as an important virulence factor in gram-negative pathogens. For example, Oag has been reported to play a role as an adhesin (3) and to provide resistance to complement (26). LPS is also a primary target of the innate arm of the mammalian immune response (11). It is noteworthy that few inflammatory cells were present in livers and kidneys of lethally infected guinea pigs (27) and in chronic infections of rat renal tubules 21 days after infection. This may have been a function of the fact that leptospiral LPS differs from typical gram-negative LPS in that it activates macrophages through Toll-like receptor 2 instead of Toll-like receptor 4 (41). Additionally, the endotoxic activity of leptospiral LPS is considerably less than that of typical gram-negative LPS (15, 16, 23), which perhaps is a function of the unique structure of leptospiral lipid A (30).

LPS is also a major component in the outer membrane of leptospires cultivated in vitro. Electrophoretic profiles of pathogenic strains of *Leptospira* differ between species and typically comprise two or three major bands (7, 40). The *rfb* locus of *Leptospira* has been extensively characterized (5, 6, 9, 10, 18, 25). Aside from being a protective antigen against homologous challenge, LPS Oag forms the basis for the reference diagnostic assay for leptospirosis, the microscopic agglutination test, in which live leptospires representing a panel of serovars undergo reaction with patient serum samples to detect agglutinating antibodies. Agglutination is thought to depend primarily on the presence of antibodies specific for surface LPS, and the specificity for individual serovars of the test panel often is interpreted to infer the infecting isolate, although this is reported to be of little predictive value (8, 22).

Our findings related to the Oag content of serovar Copenhageni during lethal infection, during chronic infection, and during in vitro cultivation suggest that the following events occur in the passage of leptospires from an infected carrier to the environment and to a host susceptible to disease. When *Leptospira* cells are shed in the urine of a chronically infected host into the environment, the Oag content is similar to that of in vitro-cultivated organisms. This is the form of *Leptospira* encountered by its incidental hosts. LPS is highly immunogenic, which explains the initial microscopic agglutination test titer in such cases. Additionally, the expression of Oag by in vitro-cultivated *Leptospira* during experimental challenge also explains why anti-Oag monoclonal antibodies provide protection against homologous but not heterologous infection (17). We have not yet established at what point during lethal infection the diminution of Oag content occurs, as the liver-derived HTL data were obtained from guinea pigs sacrificed when they were moribund. It should also be noted that while the Oag content was greatly reduced in these guinea pigs, some Oag was present, as demonstrated by the weak reactivity of the 22-kDa proteinase K-resistant Oag band in HTL with anti-OMV antiserum shown in Fig. 3A, lane 4.

We termed the diminished Oag content of guinea pig liver-derived leptospires "Oag loss." This term does not imply that no Oag is present but rather that the amount of Oag is markedly reduced. Furthermore, it does not address whether core forms of LPS are absent as well as Oag. The evidence that we have obtained on this point is consistent with the possibility that the amount of core LPS forms is also reduced in guinea pig liver-derived HTL. Figure 4A shows that purified LPS from in vitro-cultivated serovar Copenhageni produces a periodate-silver-stained band whose mobility is comparable to that of rough *Salmonella* LPS; this finding is in accord with previous reports (7, 39). However, neither anti-OMV nor CRS bound this putative core band (Fig. 3A and B). The comparison of periodate-silver-stained Percoll-purified liver-derived HTL and IVCL shown in Fig. 5C again shows the presence of a putative core LPS band in IVCL but not in the HTL. However, structural characterization of the putative LPS forms found in IVCL and HTL and their precise quantitation need to be performed before any conclusion can be drawn regarding whether only Oag content or whether Oag content and core LPS content are reduced in the liver-derived HTL.

Our findings show an association of Oag loss with disseminated lethal infection and an association of Oag with renal tubular colonization. Whether these associations are causal remains to be determined. In addition, our studies focused solely on serovar Copenhageni, and whether Oag loss is a property of additional serovars must be addressed. However, in this regard, it has been reported that hamsters infected with host-derived *L. kirschneri*, where hamsters were infected directly with tissue from an acutely infected hamster, failed to produce antibodies which reacted with Oag (2). This suggests a lack of Oag on *L. kirschneri* in acutely infected hamster tissue. In contrast, the hamsters which survived acute infection had kidneys which were positive for the presence of Oag as

determined by immunohistochemistry (2). With these caveats, the potential significance of Oag loss can be considered in the context of the association of LPS and Oag with other spirochetal pathogens and in the broader context of gram-negative bacterial pathogens. While a specific reason for the diminution of Oag content during lethal leptospirosis is not yet clear, *T. pallidum* subspecies and pathogenic borreliae lack LPS and cause infections like that caused by *Leptospira*, in which dissemination into many tissues is a prominent feature. Intestinal *Serpulina* and oral treponemes contain smooth LPS and inhabit distinct niches in their hosts.

There are cases where the absence of an Oag or an alteration in Oag content has been related to pathogenesis. The absence of an Oag in *Yersinia pestis* has recently been related to facilitation of Pla-mediated plasminogen activation and invasiveness (19). Since plague is a disease marked by disseminated infection, it is tempting to speculate that a loss of Oag in *Leptospira* may also facilitate dissemination of the spirochete. Similarly, the presence of smooth LPS in *S. enterica* renders potential plasminogen activation by the Pla-related omptin PgtE cryptic (19).

Several pathogens have been reported to regulate expression of Oag in response to environmental cues. Optimum expression of *Yersinia enterocolitica* Oag occurs at 22 to 25°C, compared to growth at 37°C, the host temperature, which results in only trace amounts of Oag (1). Increased expression of the putative glycosyltransferase *migA* gene of *Pseudomonas aeruginosa* is observed in response to mucus in lungs of patients with cystic fibrosis. This results in a loss of core-plus-one LPS but has no effect on the long-chain Oag bearing LPS (42). Interestingly, the genome of *L. interrogans* serovar Lai encodes nine significant homologs of MigA, and several of the genes are outside the *rfb* locus characterized to date. In fact, 24 *Leptospira* genes are identified as putative glycosyl transferase genes, as annotated by The Institute of Genomic Research (www.tigr.org).

Animal models that delineate acute from chronic infections provide novel avenues of research for clarifying not only virulence factors per se but also specific mechanisms that allow microbes to persist within the host environment (24). The observation of Oag loss by guinea pig liver-derived HTL and the "normal" Oag content of leptospires on rat renal tubular surfaces may provide key insights into the pathogenesis of leptospirosis. Additionally, there are extensive differences in the proteomes of HTL and IVCL (Nally, Chow and Lovett, unpublished observations). Of further interest is the elucidation of all differentially expressed genes and the associated regulatory networks involved in such regulation using DNA microarrays.

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