Leptospira interrogans Binds to Human Cell Surface Receptors Including Proteoglycans

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Abstract

Leptospirosis is a global public health problem, primarily in the tropical developing world. The pathogenic mechanisms of the causative agents, several members of the genus *Leptospira*, have been understudied. The exception to this has been the demonstration of pathogenic leptospires binding to the extracellular matrix (ECM) and its components. In this work, interactions of *L. interrogans* with mammalian cells, rather than ECM, were examined. The bacteria bound more efficiently to the cells than to the ECM, and a portion of this cell binding activity was attributable to attachment to glycosaminoglycan (GAG) chains of proteoglycans (PGs). Chondroitin sulfate B proteoglycans appeared to be the primary targets of *L. interrogans* attachment, while heparan sulfate proteoglycans were much less important. Inhibition of GAG/PG-mediated attachment resulted in partial inhibition of bacterial attachment, suggesting that additional receptors for *L. interrogans* await identification. GAG binding may participate in the pathogenesis of leptospirosis within the host animal. In addition, because GAGs are expressed on the luminal aspects of proximal tubule epithelial cells, this activity may play a role in targeting the bacteria to this critical site. Because GAGs are shed in the urine, GAG binding may also be important for transmission to new hosts through the environment.
Introduction

Leptospirosis is an important zoonotic disease caused by spirochetes of the genus \textit{Leptospira}. This genus contains free-living non-pathogenic species as well as pathogenic species, but the essential elements of biology and infection are thought to be similar among most of the pathogens. Leptospirosis has emerged as the most widespread zoonotic disease worldwide (8). The disease in humans varies from a self-limited flu-like illness to an acute life-threatening infection (Weil’s disease) with pulmonary hemorrhage, myocarditis, and kidney and liver failure. Virtually every species of mammal, both domesticated and wild, can serve as carriers of the disease, harboring the spirochete in the proximal convoluted tubules of the kidney and chronically excreting \textit{Leptospira} through the urine. Humans acquire the disease directly from exposure to urine, indirectly from fresh water sources contaminated by urine, or through occupational exposure to contaminated tissues and body fluids (15). \textit{Leptospira} species enter the body through mucous membranes of the eyes, nose or throat and via cuts or abrasions in the skin. The severity of disease in humans varies with the \textit{Leptospira} species and serovar involved, and the age, health and immune status of the patient.

Adhesion to host cells and extracellular matrix (ECM) is a critical feature in the infectious process of virtually all pathogens that require active interaction with the host to cause disease. This is likely to be true of \textit{Leptospira}, as well, yet the adhesion mechanisms that \textit{Leptospira} uses during infection have not been as thoroughly studied as those of many other bacterial pathogens. It is likely that, like \textit{Borrelia burgdorferi}, another pathogenic spirochete that can disseminate widely and chronically infect mammalian hosts, pathogenic \textit{Leptospira} species bind to multiple receptors on host cells and in ECM to establish and maintain infection. Several groups have investigated the adhesion of \textit{Leptospira interrogans} to endothelial, fibroblast, kidney epithelial, and monocyte-macrophage cell lines cultured \textit{in vitro} (3, 22, 30, 32, 37, 38, 40). The majority of these studies have shown more efficient adherence and/or entry of virulent
strains vs. avirulent or non-pathogenic (saprophytic) strains. Migration through MDCK cell layers was also associated with an infectious vs. a saprophytic *Leptospira* strain (5). In one study, adherence was decreased following pre-treatment of cell monolayers with proteases, but there was no statistically significant change in adherence when cells were pre-treated with neuraminidase, sodium metaperiodate, or lipase (37). Most studies of *Leptospira* adherence, however, have focused on attachment of the bacteria to ECM components. For example, adherence of infectious strains of *Leptospira* to the ECM components type I collagen, fibronectin, and laminin has been documented, and in some cases, one or more bacterial proteins that bind to these substrates have been identified (2, 4, 7, 9, 20, 23, 36). For example, *Leptospira interrogans* serovar Copenhageni binds to the extracellular matrix proteins fibronectin, laminin, and collagen type IV, and the plasma protein fibrinogen; adherence to these proteins is mediated largely by LigA and LigB, particularly under physiologically relevant conditions (9).

The Lig proteins are members of the bacterial immunoglobulin-like family of proteins, and were identified by screening a leptospiral gene expression library with convalescent sera from human leptospirosis patients (27, 33). Physiologic osmolarity has been found to be a powerful inducer of the expression of the Lig proteins as well as a number of additional genes (28, 29), and to increase leptosporal adhesion to the extracellular matrix proteins to which LigA and LigB bind. A *ligB* knockout strain of *L. interrogans*, however, retained infectivity and virulence (11), suggesting that the technically challenging generation of a *ligA ligB* double mutant may be required to assess the roles of the Lig proteins during infection. Previous studies had identified a 36 kDa fibronectin binding protein with properties different from the Lig proteins (31). In addition, a leptospiral outer membrane lipoprotein that binds both laminin and factor H has been described (4, 39); further work demonstrated that this protein is a member of a larger family that can also bind fibronectin (36). Together, these studies have focused on interactions between...
*Leptospira* species and the extracellular matrix, and have identified several bacterial proteins involved in these interactions.

Interactions of *Leptospira* species with glycosaminoglycans, however, have not yet been reported. Glycosaminoglycans (GAGs) are long, unbranched, disaccharide polymers that may be modified by sulfation and/or epimerization, resulting in considerable functional heterogeneity. Proteoglycans (PGs) consist of glycosaminoglycan (GAG) chains covalently linked to core proteins. GAGs and PGs are substrates for attachment by many bacterial pathogens, including another pathogenic spirochete, *Borrelia burgdorferi* (17-19, 21, 25). Because GAG binding in *vitro* is a property of many bacterial pathogens (reviewed in (35)), binding of *Leptospira* to GAGs and PGs was investigated.

**Materials and Methods**

**Bacterial and Mammalian Culture**

*Leptospira biflexa* serovar Patoc, strain 23582 (the reference strain for the serovar; non-pathogenic) and *L. interrogans* serovar Canicola (strain 23606, “known to be virulent”) were obtained from ATCC. Early experiments were also performed using *L. biflexa* serovar Patoc from Dr. David Haake, (UCLA, Los Angeles, CA) and *L. interrogans* serovar Canicola from Dr. Richard Zuerner (USDA, Ames, IA), which behaved similarly. *L. interrogans* serovar Copenhageni, strain Fiocruz L1-130 (pathogenic, LD$_{50}$ $\cong$ 10 bacteria (11)) was provided by Dr. David Haake. The bacteria utilized for this study were at low passage ($\leq$8 from animals for the virulent strains, or from the supplier if passage number was not specified), and initially cultured in semi-solid EMJH medium (2.0g/L agarose) supplemented with 100 µg/ml of 5-fluorouracil (Sigma, St. Louis, MO) and 1% heat-inactivated rabbit serum (Sigma) (15) at 30°C. After Dinger zone formation in semi-solid medium, bacteria were transferred to liquid supplemented EMJH
medium in plastic screw cap tubes and incubated at 30°C. Radioactive *Leptospira* were prepared by growing the bacteria in liquid medium with 1 mCi of $^{35}$S-methionine per 45ml culture. The bacteria were then washed to remove excess $^{35}$S, then suspended in medium supplemented with glycerol to 20% and stored in aliquots at -80°C, essentially as previously described (10).

The epithelial cell lines HEp-2 (human), HEK293 (human), MDCK (canine), and CHO K1 (and its mutant derivatives) were obtained from the ATCC, and were grown at 37°C under 5% CO$_2$ in the media recommended by the ATCC. The human microvascular endothelial cell line HMEC-1 (1) was cultured in endothelial basal medium (Clonetics, San Diego, CA) supplemented with 15% heat-inactivated fetal bovine serum (Hyclone, Logan, UT), 1 µg/ml hydrocortisone (Sigma-Aldrich, St. Louis, MO) and 10 ng/ml epidermal growth factor (Sigma-Aldrich). The human endothelial cell line EA.hy926 (13, 14) was grown at 37°C under 5% CO$_2$ in DMEM with 4.5 g/L glucose, supplemented with 10% FBS, HAT (Sigma) to final concentrations of 100 µM hypoxanthine, 0.4 µM aminopterin and 16 µM thymidine, and 2mM L-glutamine. All cell lines were also grown in the presence of 100 units/ml penicillin and 100 µg/ml streptomycin. The cell layers were washed prior to the addition of the bacteria to remove the antibiotics.

**Adhesion assays**

Mammalian cell lines were plated in 96 well tissue culture plates, adjacent to control wells containing cell culture medium only. One hour before addition of the bacteria the cell culture medium was removed, and the cell layers were washed in PBS, then returned to the incubator in medium supplemented with 3% BSA but without antibiotics. After one hour, this medium was removed, and radiolabeled bacteria suspended in the cell culture medium without antibiotics were added at a multiplicity of infection (MOI) of 10 (approximately 3 x 10$^5$/well) and centrifuged onto the cell layers for 10 min. at ambient temperature at 800 x g. The plates were then incubated for 1 hour at 37°C under 5% CO$_2$. At the end of the incubation, the motility and
integrity of the bacteria were assessed by dark-field microscopy in randomly chosen wells in multiple independent experiments. Unbound bacteria were removed by washing 3 times with 200 µl/well cell culture medium (without antibiotics). Because the LD$_{50}$ for one of the strains used (L. interrogans Fiocruz L1-130) is very low in hamsters (11), all washes were taken to bleach to kill the unbound bacteria. The washed wells were solubilized with 1% SDS, and the contents transferred to Luma Plates® scintillation plates (Packard, Meriden, CT), and dried before counting in a Packard 96 well plate scintillation counter. All experiments were performed on at least three independent occasions, with each condition in quadruplicate.

For experiments in which cell layers at different degrees of confluence were tested, the cells were plated 4 days in advance to achieve “confluence + 2 days”, 2 days in advance to achieve confluence, and 6 hours prior to the addition of the bacteria for the “sub-confluent” condition. For experiments in which cells vs. extracellular matrix were compared, confluent layers in 96 well plates were lifted with either 5 mM EDTA in PBS, or with Trypsin-EDTA (Gibco-Invitrogen, Gaithersburg, MD). The lifted cells were taken to 96 well v-bottom plates containing culture medium without antibiotics and washed twice in the same medium prior to the addition of bacteria and incubation in suspension for 1 hour as described above. The extracellular matrix (ECM remaining in the wells after the cells had been lifted) was washed twice with cell culture medium without antibiotics; each well was visually inspected to ensure that no cells remained. The bacteria were then added to the ECM containing wells and centrifuged, or to the wells containing cells in suspension without a centrifugation step, and incubated as described above. Washing to remove unbound bacteria and quantification of binding were then performed for the cells and the ECM as described above, with the exception that the cells were washed using centrifugation. In parallel experiments, ECM proteins remaining in the wells were detected by ELISA using polyclonal antisera at the dilutions recommended by the manufacturer (Chemicon, Temecula, CA) after verification of specificity. After washing, primary antibody bound was
detected using anti-rabbit IgG secondary antibody conjugated to alkaline phosphatase (Promega, Madison, WI) at 1:10,000, and development in phosphatase substrate (Sigma Chemical, St. Louis, MO).

The roles of glycosaminoglycans and proteoglycans in adhesion of *Leptospira* strains were tested using the following modifications of the adhesion assay protocol. To inhibit transfer of the GAGs to protein cores, cells were preincubated overnight in medium containing 5 mM p-nitrophenyl-β-D-xyloside (β-xyloside), an inhibitor of the xylosyltransferase required for the initial modification of the serine residues that serve as acceptors of GAG chains (Sigma-Aldrich, St. Louis, MO). Control cells were left untreated, or were incubated with an analog, p-nitrophenyl-α-D-galactoside (α-galactoside) (Sigma-Aldrich). The cell monolayers were washed and the infection performed as described above. To test the competitive effect of exogenous GAGs or GAG analogs on leptospiral attachment to cells, the bacteria were incubated for 30 minutes at room temperature in cell medium containing 1% BSA supplemented with purified GAGs (Sigma-Aldrich) at concentrations ranging between 0.01 and 1000 µg/ml. The attachment assay was then performed as described above. The IC$_{50}$ was determined using the four parameter logistic model (Hill-Slope model). To determine the effect of enzymatic removal of different classes of GAGs from cell surface proteoglycans on leptospiral attachment, cell monolayers were incubated with 35 µl/well of 0.5 U of a lyase (heparinase I, heparitinase, chondroitinase AC, or chondroitinase ABC) (Sigma-Aldrich) at 37°C for 2 hours in cell medium supplemented with 1% BSA, plus the protease inhibitors aprotinin at $10^{-2}$ trypsin inhibitory units/ml, and phenylmethylsulfonyl fluoride at 150 µg/ml. The monolayers were then washed with PBS 3 times and the attachment assay was performed as described.

**Purified GAG binding assay**

To screen for GAGs recognized by *L. interrogans*, 5 mg/ml of purified GAGs (Sigma-Aldrich) solubilized in water or PBS the day of plating were added to non-tissue culture 96-well plates.
and incubated at 4°C overnight. The plates were washed with PBS and blocked for two hours at room temperature in 50 mM HEPES, 100 mM NaCl, 1 mM MgCl$_2$, 1 mM MnCl$_2$, 1 mM CaCl$_2$, and 3.5% BSA. Bacteria were added at 3 x 10$^5$/well in the same buffer containing 1% BSA. The plates were centrifuged for 15 minutes and rocked for 45 minutes at room temperature. The wells were washed with PBS+0.2% BSA, and then treated with 1% SDS. The well contents are transferred to Luma plates and analyzed by liquid scintillation counting, as described above.

**Gel Electrophoresis and Immunoblotting**

The ECM proteins associated with mammalian cells lifted with EDTA were compared to those associated with intact monolayers by SDS-PAGE on 10% polyacrylamide gels under non-reducing conditions. Cells were harvested either by lifting with EDTA, as described above, or by washing the monolayers three times with PBS, then vigorously scraping the cells off the plastic in the presence of 2x SDS-loading buffer without reducing agent. The cells lifted with EDTA were suspended in the same loading buffer. The viscosity of all samples was reduced by brief sonication on ice. Aliquots of each sample were then diluted further in gel loading buffer and heated to 95°C for 5 min, and loaded immediately onto the gels. After separation by electrophoresis, the proteins were transferred to Immobilon membranes (Millipore, Bedford, MA). The membranes were either stained with Coomassie blue, or blocked in Tris-buffered saline (TBS: 25 mM Tris, 150 mM NaCl, pH 7.5) containing 5% w/v non-fat dry milk. The latter membranes were probed with the following rabbit antisera, all from Chemicon (Temecula, CA): anti-fibronectin (AB1945, 100 ng/ml), anti-laminin (AB19012, 200 ng/ml), anti-collagen type 1 (AB745, 1:200), anti-collagen type IV (AB748, 1 µg/ml), or anti-vitronectin (AB19014, 1 µg/ml). After development as described below, all were re-probed with anti-actin (A-2066, Sigma Chemical, St. Louis, MO, 1:10,000) as a loading control in addition to the general Coomassie stain. The primary antibodies were detected with goat anti-rabbit IgG (Promega, Madison, WI, 1:10,000) followed by colorimetric development.
Statistical Analyses

The methods used to assess statistical significance varied with the type of experiment, and so are described in the Figure legends. P values of < 0.05 were considered to denote significant differences.
Results

The degree of confluence of the mammalian cells affects Leptospira attachment

Cells in culture express different receptors at different stages of growth, so we determined how the growth stage of the mammalian cells might influence adhesion by a few representative Leptospira strains (Figure 1). The non-pathogenic L. biflexa serovar Patoc showed relatively inefficient cell-specific binding activity under all conditions tested. L. interrogans serovar Canicola bound most efficiently to both HEp-2 and MDCK cells just at confluence, while L. interrogans serovar Copenhageni bound very efficiently to HEp-2 cells just at confluence, and less efficiently to HEp-2 cells post confluence. Binding of this strain to MDCK cells was maximal when the cells were subconfluent. For both of the pathogenic strains tested, binding to HEp-2 cells decreases after the cells reach confluence (compare 2 days post confluent to confluent); for the other cell lines shown this trend was not apparent (HMEC cells) or differed between bacterial strains (MDCK cells). These results suggest that the HEp-2 cell receptors for pathogenic Leptospira may be masked, become inaccessible, or be down-regulated as the cells form completely confluent layers. Some of the variability with the MDCK cells may be due to differential loss of cells from the wells; the same was true in some experiments for the human kidney epithelial cell line HEK-293 (data not shown). HEp-2 and endothelial cells, in contrast, were efficiently retained, and so were used for most subsequent studies.

Leptospires bind to mammalian cells as well as to the extracellular matrix (ECM) deposited by the cells

One question resulting from the results shown in Figure 1, and from the literature, is whether the Leptospira are binding primarily to the extracellular matrix or to mammalian cell surface receptors. We therefore lifted cells with EDTA, which disrupts the functions of integrins and
other cell-attachment molecules, resulting in detachment of the cells from the extracellular matrix, or with trypsin-EDTA, which also degrades proteins that are involved in cell adhesion. We then tested both the ECM remaining in the wells and the cells in suspension for *Leptospira* attachment. Both the ECM and the cells were washed with cell culture medium (without antibiotics) to remove the EDTA and restore the divalent cations that might be required for bacterial-cell interactions, prior to the addition of the bacteria. We found that binding to intact cell layers, in which both cells and the ECM are present, is most efficient, but that binding to cells lifted with EDTA is more efficient than is binding to the remaining ECM alone (Figure 2A). This cannot be attributed simply to the presence of ECM proteins associated with the cells after lifting with EDTA, as most of those analyzed are diminished or undetectable in comparisons of cells lifted with EDTA as opposed to scraped off the well in SDS buffer (Figure 2B); this was more pronounced for the HEp-2 cells than for the Ea.hy926 cells. The increased binding to the cells vs. the ECM is particularly notable in light of the lack of centrifugation of the bacteria and cells in suspension, but that facilitates bacterial interactions with intact cell layers and the ECM.

The ECM components detected after removal of the cells varied with the cell line, as determined by ELISA (Table 1). These results suggest that, as has been demonstrated abundantly in the literature, *Leptospira* do attach to ECM components, but here we demonstrate that these bacteria also bind to mammalian cell surface-specific receptors. At least a portion of the complement of cell surface receptors for *Leptospira* is proteinaceous in nature, as binding to HEp-2 cells lifted with trypsin-EDTA was less than 10% of the level of binding to cells lifted with EDTA alone (data not shown). This is consistent with previous results obtained by other investigators (37). The effect of trypsin-EDTA vs. EDTA alone on binding to ECM was not statistically significant. This may be due to the overall less efficient attachment to the ECM. Alternatively, the brief trypsin-EDTA treatment required to remove the cells may be insufficient to disrupt interactions between the ECM components, leaving partially digested protein fragments associated in the relatively insoluble matrix available for bacterial attachment. As
many ECM proteins have functionally separable domains that retain adhesion activity after proteolytic digestion of the intact protein, these results are consistent with previous data showing that the bacteria recognize specific domains of extracellular matrix proteins (9, 36).

One notable result in this experiment is that the different strains of pathogenic leptospires appear to depend on the presence of fibronectin in the ECM to different degrees. While serovar Canicola has lower overall cell-binding activity than does serovar Copenhageni, and binding to the HEK-293 ECM is approximately equal for the two strains, binding to the HEP-2 ECM is lower for Copenhageni than for Canicola. Since HEP-2 cells do not express fibronectin (12, 16), one possible explanation is that the serovar Copenhageni bacteria rely more on the presence of fibronectin secreted and deposited by the cells into the ECM, although it is clear that these bacteria must be able to bind to other ECM components, or to bind to soluble fibronectin that has been captured from the serum in the growth medium, as suggested by the results shown in Table 1 and by previous work by other groups (9, 36).

**Proteoglycans account for some, but not all, of *L. interrogans* attachment to cells.**

A number of pathogenic bacteria bind to particular glycosaminoglycans (GAGs), which may be conjugated to proteins to form proteoglycans (PGs). We therefore tested two mutant CHO-K1 derivatives that either do not synthesize PGs, or that express reduced levels in comparison to wild-type cells (35), in our standard *L. interrogans* adherence assay. As shown in Figure 3, both of the *pgs* mutants tested, which are defective in PG synthesis, showed statistically significant reductions in attachment by both *L. interrogans* strains tested. However, the reduced binding efficiencies were still high, suggesting that other, non-PG receptor(s) may have significant roles in *L. interrogans* attachment to cells. Binding of the bacteria to three *lec* mutants tested (*lec1*, *lec2*, and *lec8*) was not significantly different for both bacterial strains (data not
shown), suggesting that other glycoconjugates play a relatively minor role in *L. interrogans* attachment to CHO cells.

As a second approach to determining whether *L. interrogans* binds to proteoglycans on mammalian cells, wild type HEp-2, Ea.hy926, and HEK293 cells were incubated overnight with β-xyloside, an inhibitor of GAG transfer to protein cores. As a control, a different sugar analog, α-galactoside was tested. As shown in Figure 4, β-xyloside treatment of all three mammalian cell lines significantly decreased attachment by *L. interrogans*, confirming the role of proteoglycans suggested by the results with the CHO pgs mutants.

*Leptospira interrogans binds to purified glycosaminoglycans.*

To build on the results suggesting that *L. interrogans* recognizes proteoglycans expressed by mammalian cells, we determined which GAGs are recognized by *L. interrogans*. Purified GAGs, the polysaccharides dextran and dextran sulfate, or the buffer control, were immobilized in plastic wells, then probed with *L. interrogans*. The results shown in Figure 5 demonstrate that *L. interrogans* binds to high molecular weight dextran sulfate but not to high molecular weight dextran or to low molecular weight dextran sulfate, suggesting that sulfation and polymer size (and consequently, binding sites per molecule) are important determinants of *L. interrogans* binding to GAGs. This is supported by the attachment of the bacteria to heparin, which is more highly sulfated than is heparan sulfate, which did not appear to be recognized by the bacteria. The more efficient binding of *L. interrogans* to chondroitin sulfates B and C than to heparin is indicative of specific *Leptospira* recognition of particular GAGs.

**Roles of particular glycosaminoglycans in Leptospira attachment to cells**
The profile of GAGs recognized by *L. interrogans* presented in Figure 5 may be influenced by the efficiency of coating of the plastic wells particular to each GAG. To determine whether the apparent preference of GAGs is consistent with GAGs recognized by *L. interrogans* when expressed by mammalian cells, two approaches were taken. First, exogenous GAGs in solution were preincubated with *L. interrogans* to determine whether they could serve as competitive inhibitors of bacterial attachment. As shown in Figure 6A, dextran sulfate (high molecular weight) competed with cellular GAGs for recognition by *L. interrogans* (IC$_{50}$ ≈ 0.007 mg/ml), while high molecular weight dextran did not. While these results are consistent with the binding to immobilized GAGs shown in Figure 5, these polysaccharides are model compounds that are not biologically relevant. Chondroitin sulfates B and C, however, are biologically relevant and inhibited *L. interrogans* attachment to mammalian cells. The IC$_{50}$ for chondroitin sulfate B was ≈ 0.023 mg/ml, while that of chondroitin sulfate C was greater than 0.3 mg/ml, the maximum concentration tested, suggesting either that chondroitin sulfate B is the primary GAG recognized by *L. interrogans* serovar Copenhageni on HEp-2 cells, or that this GAG interacts with the leptospiral GAG-binding adhesin(s) with the highest affinity. Heparin, like dextran sulfate, efficiently inhibited leptospiral attachment to HEp-2 cells (IC$_{50}$ ≈ 0.002), which suggests that sulfation, charge, or both may be important in attachment activity, but heparan sulfate and chondroitin sulfate A did not significantly affect bacterial binding to the mammalian cells. With the exception of heparin, these data are also consistent with the binding to purified GAGs shown in Figure 5. Heparin, however, is the most negatively charged of the naturally occurring GAGs tested, so like dextran sulfate may affect bacterial attachment for reasons other than specific competition for particular cell-surface GAGs.

As a second approach to identification of cell-associated GAGs that are recognized by *L. interrogans*, cell layers were treated with GAG lyases, which cleave GAG chains, prior to the addition of the bacteria. As shown in Figure 6B, heparinase, which degrades heparin, did not
affect *L. interrogans* attachment to epithelial or endothelial cells. In contrast, the two chondroitinases both significantly inhibited subsequent bacterial attachment to the treated cells. The abilities of the chondroitinases to diminish *L. interrogans* more effectively than heparinase or heparatinase is consistent with the ability of the chondroitin sulfate GAGs to inhibit *L. interrogans* attachment to cells (Figure 6A) and to bind the bacteria (Figure 5).

**Discussion**

Pathogenic leptospires have been shown previously to bind to mammalian cell layers in culture, and to particular extracellular matrix components, more efficiently than non-pathogenic leptospires. Here, we report that binding to ECM molecules does not account for all of the *Leptospira* cell-layer binding activity. Cell-surface receptors also serve as targets for *Leptospira* attachment. In addition, the effects of the confluence of the mammalian cell layers suggest that the availability of cell surface and/or ECM molecules that serve as substrates for attachment of *Leptospira* may change as the cells in *in vitro* monolayers grow. This may be due to differences in production, modification, and turnover of particular substrates. The identities of these molecules, however, will require further investigation.

Because many bacterial pathogens bind to proteoglycans, and in particular to the GAG chains that form the major surface-exposed component of proteoglycans, we tested *L. interrogans* for this activity. The data presented here demonstrate that *L. interrogans* strain L1-130 (serovar Copenhageni), which is a significant pathogen of humans, binds most efficiently to chondroitin sulfate B and C proteoglycans and the corresponding GAGs. Glycosaminoglycans are displayed by diverse cell types, and are present in the extracellular matrix as well as associated with the mammalian cell surface. They are therefore widely available for pathogen attachment. In fact, preliminary data presented here suggest that a different *L. interrogans* strain, of serovar Canicola, also binds to proteoglycans. Although our results must be expanded...
to include additional strains and serovars in the future, the data suggest that *L. interrogans* utilizes proteoglycans as mammalian substrates for attachment. Our results also suggest that additional, non-GAG or PG receptors on the mammalian cell surface await identification.

It is apparent from the aggregate of the data presented in Figures 5 and 6 that *L. interrogans* L1-130 binds to chondroitin sulfate proteoglycans more efficiently than to heparan sulfate proteoglycans. This is in contrast to the situation with *B. burgdorferi*, another pathogenic spirochete that recognizes heparin, heparan sulfate, and chondroitin sulfate B, but does not efficiently bind to chondroitin sulfate C (25). It should be noted, however, that although heparin is widely used as a tractable model GAG, it is produced by, and released from, mast cells. It is therefore not typically present in the ECM and available to serve as a target for bacterial attachment to the ECM (34). In contrast, the heparan sulfate and chondroitin sulfate proteoglycans are widely distributed, and accordingly, are substrates for attachment by a variety of pathogens. In studies such as those described here, the possibility that heparin serves more as a non-specific inhibitor of interactions based on the high negative charge cannot be dismissed. Additional GAGs that are biologically relevant can, in contrast, assist in the identification of specific receptors for pathogens.

Although both *Borrelia* and *Leptospira* species are spirochetes, they are different at the molecular, cellular, and ecologic levels, and cause different disease manifestations. For example, the *Borrelia* do not contain the genes required for LPS synthesis, while the leptospires express LPS that is variable and determines serovar type. It is unlikely that the LPS, however, contributes to GAG and proteoglycan binding, as interactions between carbohydrate polymers would be unlikely due to mutual repulsion by the negative charges. It is far more likely that the GAGs and proteoglycans are recognized by one or more *Leptospira* proteins that are expressed on the surface of the bacteria.
Several proteins have been identified as *Leptospira* adhesins mediating attachment to extracellular matrix molecules, i.e. MSCRAMMs (34). As is the case for other bacterial proteins that bind to extracellular matrix components, these proteins bind to several different mammalian substrates. Since many extracellular matrix molecules contain repeated elements, it is possible that the proteins that bind to repeating units may also bind to the repeats of GAGs. This was shown to be the case for the fibronectin binding protein of *B. burgdorferi*, which also binds GAGs (16). The identities of leptospiral proteins that mediate interactions with GAGs will be determined in future experiments, but the known adhesins, such as LigA, LigB, LipL32, TlyC, and the family of proteins known by several names (LenA-F, Lsa24 and LfhA) represent a partially characterized subset for prioritized testing (4, 7, 9, 20, 27, 36).

The role of GAG and proteoglycan binding in the pathogenesis of *Leptospira* infections remains to be tested, but several possibilities exist. For example, it is possible that binding to GAGs facilitates leptospiral infection, either initially at mucosal surfaces, or as the bacteria disseminate to diverse tissues. Because these bacteria disseminate from the site of inoculation to virtually any site in the body, tissue tropism may be affected by the GAG-binding preferences of the bacteria and the GAGs available to the bacteria in the tissues. This mechanism has been implicated in the different rates of CNS colonization by relapsing fever *Borrelia* that express surface proteins with different GAG preferences (26). The initial encounter of *Leptospira* species with mammalian hosts is typically at mucous membranes. Epithelia are known to express both heparan sulfate and chondroitin sulfate proteoglycans, including syndecans 1 and 4 and epican. It is therefore possible that the initial host-bacterial interaction is, at least in part, mediated by *Leptospira* binding to the GAG moieties of chondroitin sulfate proteoglycans.

In the case of *Leptospira* infection, in particular, a specific site of GAG-bacterial interaction will be interesting to examine. Kidney proximal tubule epithelial cells produce both heparan sulfate and chondroitin sulfate GAGs in culture (6) and *in vivo* (24), although different animal
species show different GAG profiles in the urine. It is possible that GAG binding facilitates colonization of this epidemiologically critical site. In addition, bacteria bound to GAGs that are released from the epithelial cells of the proximal tubules of the kidney may provide a mechanism for shedding of the bacteria in the urine. The bacteria making the transition from mammalian host to environmental soil and water may then be associated with host-derived GAGs. In addition, binding to GAGs released into the extracellular environment within the host may allow the bacteria to cloak themselves, which would potentially facilitate the persistent infection established in maintenance hosts. This may also be a key function for ECM proteins with soluble forms, e.g. fibronectin. Identification of the GAG binding adhesin(s) of *Leptospira interrogans* will facilitate further analyses of the roles of attachment to GAGs and proteoglycans in the ability of these bacteria to cause infection and disease in mammals.

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Figure Legends

Figure 1: The degree of confluence of mammalian cell layers affects attachment of different Leptospira strains differently. $^{35}$S-labeled bacteria were added to wells containing cells that had reached confluence 2 days before the experiment, wells containing cells that were just reaching confluence (95-100% well area coverage), and wells expected to reach confluence the day after the experiment (45-50% well area coverage). Shown are the means of cell-specific attachment, ± standard deviations from a single experiment, representative of multiple experiments. For the HEp-2 cells, attachment of both L. interrogans strains was significantly less efficient to post-confluent vs. confluent layers ($P < 0.0008$); a similar difference ($P < 0.0001$) was observed in comparison of confluent to subconfluent layers, although this result is likely affected by the approximately 2-fold differences in the numbers of cells in the wells. For the other cell lines, confluence did not significantly affect efficiency of bacterial attachment ($P > 0.05$). L. interrogans sv. Canicola bound to confluent MDCK more efficiently than to HEp-2 cell layers ($P < 0.016$). L. interrogans sv. Copenhageni, in contrast, bound more efficiently to HEp-2 than to MDCK confluent layers ($P < 0.0001$). All comparisons were analyzed by the Student’s two-tailed t test.

Figure 2: Attachment of Leptospira to cells vs. ECM. Panel A: Confluent cell layers were left as is (intact layers), or lifted with EDTA twice to remove all cells from the ECM. The cells were collected by centrifugation, and both the cells and the ECM were washed in medium without antibiotics to remove the EDTA and restore the divalent cations prior to the addition of $^{35}$S labeled Leptospira at MOI = 10. After incubation for 1 hour at 37°C, all wells were washed to remove unbound bacteria, and bound bacteria were quantified by scintillation counting. Results are shown after subtraction of background binding to wells without cells or ECM. The means and standard deviations of 4 replicates from representative experiments (of ≥ 4 for each cell line) are shown. By the Student’s two-tailed t test, comparisons of binding to lifted cells vs. the
ECM left behind were as follows: for HEp-2 cells, Patoc P > 0.1, for Canicola and Copenhageni, P = 0.0002; for 293 cells, Patoc P = 0.008, Canicola P > 0.06, for Copenhageni P < 0.0001; for Ea.hy926 cells only Copenhageni was tested, for cells vs. ECM P < 0.0001. Panel B: Immunoblots of ECM proteins associated with cells after either scraping the cell layers in SDS gel loading buffer, or lifting with EDTA prior to solubilizing in gel loading buffer. As a positive control for the anti-fibronectin antibody, 0.1 µg of purified fibronectin (soluble form) was loaded. The first lane of the blot probed with anti-vitronectin was taken from one of the other panels after re-probing with anti-vitronectin, as the original blot was mis-cut between lanes.

Figure 3: Binding of *L. interrogans* to wild type and mutant CHO cells. Cells were plated in 96 well plates the attachment assay performed as described in Materials and Methods. To allow incorporation of all data from multiple experiments, binding to the mutant cell lines in each experiment was normalized to binding to the wild-type CHO K1 cells in the same experiment. Shown are the means and standard deviations of all data points. Binding of both *L. interrogans* strains to the *pgsA* and *pgsB* mutant cells was statistically significantly different from that to wild type CHO K1 cells by the Student’s two-tailed t test, p < 0.001.

Figure 4: Inhibition of GAG transfer to proteoglycans reduces *L. interrogans* attachment to mammalian cells. Three cell lines, HEp-2, HEK293, and Ea.hy926 were plated in 96 well dishes and allowed to grow and adhere to the wells. The day before the attachment assay, the media were replaced with fresh media containing no additions (no treatment control), 5 mM α-galactoside (a control sugar analog), or 5 mM β-xyloside (a sugar analog that competitively inhibits the xylosyltransferase required to transfer the xylose root of GAGs to protein cores). On the day of the attachment assay, the cell layers were washed, and bacterial attachment was quantified as described in Materials and Methods. Shown are the means and standard deviations of all data points. Binding of both *L. interrogans* strains to the *pgsA* and *pgsB* mutant cells was statistically significantly different from that to wild type CHO K1 cells by the Student’s two-tailed t test, p < 0.001.
deviations of attachment normalized to the control; data are from multiple experiments. By the
Student’s two-tailed t test, for all cell lines, control vs. β-xyloside P < 0.01, control vs. α-galactoside P > 0.1.

Figure 5: Attachment of *L. interrogans* serovar Copenhageni Fiocruz L1-130 to purified GAGs.
Glycosaminoglycans (GAGs) were plated in plastic wells at 1 mg/ml, then probed with 35S-
labeled bacteria. After incubation, then washing to remove unbound bacteria, the percent
inoculum bound was calculated. Shown are the means ± standard deviations of 4 replicates for
two independently grown and radiolabeled batches of bacteria (black and gray bars). These
data represent multiple experiments. CSB = chondroitin sulfate B, CSC = chondroitin sulfate C.
The molecular weights of the dextran molecules used are indicated. Binding to heparan sulfate
has not been detected in any experiment. In comparison to the PBS control by the Students
two-tailed t test, P > 0.5 for dextran and low molecular weight dextran sulfate, P = 0.016 for
heparin, and P < 0.001 for CSB, CSC, and high molecular weight dextran sulfate.

Figure 6: Roles of particular glycosaminoglycans in attachment of *L. interrogans* to cells. Panel
A: GAGs at the concentrations indicated were incubated for 30 min. with the bacteria prior to the
addition of the bacteria to the immobilized mammalian cells. Shown are the means and
standard deviations of all data from at least 3 independent experiments, each of which
consisted of 4 technical replicates. Statistical significance was determined for the entire dose
range for each GAG in comparison to dextran by Tukey’s Multiple Comparison test; P > 0.05
(not significant) for heparan sulfate, P < 0.01 for CSA, and P < 0.001 for dextran sulfate,
heparin, CSB, and CSC. CSB, CSC, heparin, and dextran sulfate were not significantly different
from each other. Panel B: Mammalian cell layers were treated with 35 µl of 0.5 u/ml of the GAG
lyases heparinase (HA), heparatinase (HTA), chondroitinase AC (CAC), or chondroitinase ABC
(CABC) for 2 hours, or were left untreated (NT), then washed prior to the addition of bacteria. Shown are the means and standard deviations of 4 replicates from a single experiment representative of 7 experiments. For each experiment, in comparison to the no treatment control, the P values for HA were > 0.1, for HTA were 0.01-0.001, and for CAC and CABC were <0.001 by the Student's two-tailed t test for both cell lines.
References Cited


8. CDC 2001, posting date. Leptospirosis. [Online.]


Table 1: Representative ECM proteins remaining in wells after removal of cells with EDTA.

<table>
<thead>
<tr>
<th>ECM protein, OD&lt;sub&gt;405&lt;/sub&gt; &lt;sup&gt;a&lt;/sup&gt;</th>
<th>HEP-2</th>
<th>HEK-293</th>
</tr>
</thead>
<tbody>
<tr>
<td>fibronectin</td>
<td>0.028 ± 0.006</td>
<td>0.268 ± 0.008</td>
</tr>
<tr>
<td>laminin</td>
<td>0.027 ± 0.002</td>
<td>0.078 ± 0.004</td>
</tr>
<tr>
<td>vitronectin</td>
<td>NS&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.015 ± 0.004</td>
</tr>
<tr>
<td>collagen type IV</td>
<td>NS</td>
<td>NS</td>
</tr>
</tbody>
</table>

<sup>a</sup>Values are OD<sub>405</sub> readings for wells in which confluent cell layers that had been plated 2 days prior to treatment with EDTA as described in Materials and Methods – OD<sub>405</sub> readings for control wells that had contained medium without cells (background). Shown are means ± standard deviations of duplicate experiments, each of which contained 4 replicate wells. The values for HEP-2 cells in these experiments were always low compared to those for other cell lines tested after the same incubation times in colorimetric substrate.

<sup>b</sup>NS: not significantly above background.
Figure 1

Effects of mammalian cell confluency on Leptospira 

patoc

confluence/Leptospira strain

canicola

copenhageni

HEp-2 cells
MDCK cells
HMEC cells
Figure 2

A

B

Coomassie stain

α-fibronectin

α-laminin

α-vitronectin

α-collagen type I

α-collagen type IV

α-actin
Figure 3
Figure 4

![Bar chart showing relative binding efficiency for different treatments and cell lines.

- **待遇**: no treatment, α-galactoside, β-xyloside
- **细胞类型**: HEp-2, 293, Ea.hy926

Y-axis: Relative binding efficiency
X-axis: Cell lines
Figure 5

[Image: A bar graph showing the percent inoculum bound for different samples including PBS, Heparin, CSB, CSC, Dex (MW 500,000), Dex SO4 (MW 500,000), and Dex SO4 (MW 9,000-20,000).]
Figure 6
Effects of mammalian cell confluency on Leptospira

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>2 d post confluent</th>
<th>confluent</th>
<th>sub-confluent</th>
<th>2 d post confluent</th>
<th>confluent</th>
<th>sub-confluent</th>
</tr>
</thead>
<tbody>
<tr>
<td>HEp-2 cells</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MDCK cells</td>
<td></td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HMEC cells</td>
<td></td>
<td></td>
<td></td>
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<td></td>
<td></td>
</tr>
</tbody>
</table>
A intact layers ECM only cells only intact layers ECM only cells only intact layers ECM only cells only cell condition Leptospira

HEp-2 cells HEK-293 cells Ea.hy926 cells

B α-vitronectin α-actin α-laminin α-fibronectin α-collagen type I α-collagen type IV α-collagen type IV

Abnormal patterns of extracellular matrix proteins in infected Hep-2 cells compared to uninfected HEK-293 and Ea.hy926 cells.
PBS Heparin CSB CSC Dex (MW 500,000)
Dex SO4 (MW 500,000)
Dex SO4 (MW 9,000-20,000)

percent inoculum bound

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