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Hemagglutination and Proteoglycan Binding by the Lyme Disease Spirochete, *Borrelia burgdorferi*

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The ability of the Lyme disease spirochete to attach to host components may contribute to its ability to infect diverse tissues. We present evidence that the Lyme disease spirochete expresses a lectin activity that promotes agglutination of erythrocytes and bacterial attachment to glycosaminoglycans. Among a diverse collection of 21 strains of Lyme disease spirochete, hemagglutinating activity was easily detected in all but 3 strains, and these three strains were noninfectious. The ability to agglutinate erythrocytes was associated with the ability of the spirochete to bind to the sulfated polysaccharide dextran sulfate and to mammalian cells. Soluble dextran sulfate was a potent inhibitor of both hemagglutination and attachment to mammalian cells, while dextran had no effect on either activity, suggesting that dextran sulfate may inhibit attachment by mimicking host cell glycosaminoglycans. Consistent with this, the spirochete bound to immobilized heparin, and soluble heparin inhibited bacterial adhesion to mammalian cells. The bacterium did not bind efficiently to Vero cells treated with heparinase or heparitinase or to mutant CHO cell lines that are deficient in proteoglycan synthesis. Sulfation of glycosaminoglycans was critical for efficient bacterial recognition, as Vero cells treated with an inhibitor of sulfation, or a mutant CHO cell line that produces undersulfated heparan sulfate, did not mediate maximal spirochetal binding. Binding of the spirochete to extracellular matrix also appeared to be dependent upon this attachment pathway. These findings suggest that a glycosaminoglycan-binding activity which can be detected by hemagglutination contributes to the attachment of the Lyme disease spirochete to host cells and matrix.

Lyme disease is a chronic multisystemic illness that is caused by the tick-borne spirochetes *Borrelia burgdorferi* (sensu stricto), *B. garinii*, and *B. afzelii* (3, 4, 53). It is the most common arthropod-borne illness in the United States and causes significant morbidity in parts of Europe and Asia as well (31). *B. burgdorferi* (sensu stricto) comprises most North American and some European isolates, while *B. garinii* and *B. afzelii* comprise European and Asian strains (3, 57). The disease is characterized by a progression through clinical stages (53). The first stage, localized infection, includes the distinctive expanding rash, erythema migrans, at the site of the tick bite (54). The second stage, disseminated infection, occurs days to weeks after infection and can involve virtually all organ systems (12). Finally, persistent infection often begins after the first year of illness and commonly affects the skin, joints, and nervous system. The efficacy of antibiotic treatment for most patients at various stages of Lyme disease suggests that these manifestations are associated with ongoing bacterial infection (53).

It is clear from the course of infection that *B. burgdorferi* can spread from the site of the tick bite to a variety of organ systems and can avoid immune clearance to give rise to chronic manifestations of the illness. Although these features are undoubtedly the result of complex interactions between many bacterial and host factors, specific binding of host cells and/ or extracellular matrix by *B. burgdorferi* is likely to play an important role at several points during the infection. Most pathogenic bacteria possess adhesins that mediate attachment to host cells and that are necessary for successful colonization (5, 7, 17). It has been known for several years that *B. burgdorferi* binds to many types of mammalian cells (25, 55, 56) and to extracellular matrix (55).

B. burgdorferi has been shown to bind to platelets via the integrin $\alpha_{IIb}\beta_3$ (9), and $\alpha_{IIb}\beta_3$-binding activity was found among all three species of Lyme disease spirochete, including each of the 11 infectious strains tested (10). Integrins, which promote a wide variety of cell-cell and cell-extracellular matrix interactions (28), are expressed on most cell types, and integrins closely related to $\alpha_{IIb}\beta_3$ are likely to contribute to attachment of the Lyme disease spirochete to a wide variety of cells. Most pathogens, however, appear to possess multiple pathways for cell binding, a reflection of the key role that adherence plays in the establishment of infection (23, 27). Consistent with this, Garcia-Moncho et al. have shown that *B. burgdorferi* binds to purified glycolipids (19, 20), and more recently, Isaacs reported that host cell proteoglycans are recognized by *B. burgdorferi* (29). Proteoglycans consist of core proteins linked to glycosaminoglycans, which are negatively charged, linear, repeating disaccharides (34). Proteoglycans are expressed on the cell surface, as well as in extracellular matrix, and play a role in a wide variety of biological phenomena, such as cell adhesion and migration (30). They have also been shown to mediate adherence of diverse pathogenic microorganisms (44, 46, 51).

Agglutination of erythrocytes, which do not express integrins, has been widely used to detect microbial factors that mediate host cell attachment (11, 43). Therefore, to investigate binding pathways distinct from the integrin-mediated attachment previously described (9), we examined a hemagglutinat-

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ing activity of *B. burgdorferi* and have independently developed evidence for *Borrelia*-proteoglycan interaction. The hemagglutinating and proteoglycan-binding activities were both expressed by most strains analyzed, including representatives of all three species of Lyme disease spirochete, and the few strains that did not efficiently express these activities were noninfectious. Hemagglutination and proteoglycan binding were associated with attachment to extracellular matrix as well as to host cells, and a strain that did not bind integrin $\alpha_{IIb}\beta_3$ was shown to bind proteoglycans, suggesting that the integrin- and proteoglycan-mediated pathways are independent.

MATERIALS AND METHODS

Bacterial and cell culture. All strains of *B. burgdorferi* were cultured in MKP medium (47) supplemented with human serum (9). Briefly, 100 ml of 10× CMRL medium (G1BCO-BRL, Bethesda, Md.), 3 g of neopeptone, 6 g of *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES), 0.7 g of sodium citrate, 3 g of glucose, 0.8 g of sodium pyruvate, 0.8 g of *N*-acetylglucosamine, and 2 g of sodium bicarbonate were added to 900 ml of distilled H_2O, and the pH was adjusted to 7.6. Then, 200 ml of 7% gelatin, 35 ml of 35% bovine serum albumin (BSA), and 70 ml of heat-inactivated human serum were added prior to filtration through a 0.22-μm-pore-size filter.

Vero (monkey kidney epithelial) cells were cultured in RPMI 1640 supplemented with 10% Nu-Serum (Collaborative Research, Bedford, Mass.). 293 (human kidney embryonic carcinoma) cells (ATCC CRL1573) were cultured in RPMI 1640 medium plus 10% fetal bovine serum, and HEp-2 (human laryngeal carcinoma) cells were cultured in RPMI 1640 plus 5% newborn calf serum. CHO (Chinese hamster ovary)-K1 cells and the proteoglycan-deficient derivatives CHO-*pgsA*-745, *pgsB*-618, *pgsE*-606, and *pgsD*-677 (obtained from Jeffrey Esko [13]) were cultured in Ham's F12 medium plus 5% fetal bovine serum.

In parallel, wild-type and CHO-*ldlD* cells (33), were cultured under conditions that do not allow CHO-*ldlD* synthesis of proteoglycans or that promote the synthesis of one or both of the major CHO cell proteoglycans, heparan sulfate proteoglycan and chondroitin sulfate proteoglycan, as described previously (14, 36). Briefly, cells were cultured 16 h in Ham's F12 medium supplemented with 10% dialyzed newborn calf serum. Monolayers were washed once in Hanks' buffered saline and cultured for 48 h in Ham's F12 medium-ITS+ serum supplement (Collaborative Research) with or without 20 μM galactose or 200 μM *N*-acetylgalactosamine (GalNAc). In the absence of either sugar, neither heparan sulfate nor chondroitin sulfate is synthesized (14). In the presence of galactose, heparan sulfate is synthesized, and in the presence of both galactose and GalNAc, both heparan sulfate and chondroitin sulfate are synthesized. The *ldlD* defect also affects protein glycosylation, detectable by resistance to killing by the lectin wheat germ agglutinin (33). Wheat germ agglutinin sensitivity experiments were performed as described previously (33), and as expected, only when cells were cultured in the absence of galactose and GalNAc did CHO-*ldlD* cells survive a 48-h incubation in 5 or 50 μg of wheat germ agglutinin per ml. CHO-K1 cells were sensitive to wheat germ agglutinin after culture in all of the conditions.

Hemagglutination assays. A modification of the method described by Nowak et al. (45) was used to prepare trypsinized, fixed erythrocytes. Blood was drawn from a New Zealand White rabbit into 0.1 M citrate anticoagulant. Erythrocytes were separated from platelet-rich plasma and the buffy coat by differential centrifugation at 150 × *g* centrifugation for 15 min. The erythrocytes were then pelleted by centrifugation at 350 × *g* and washed three times in phosphate-buffered saline (PBS; 150 mM NaCl, 16.9 mM K_2HPO_4, 4.8 mM KH_2PO_4 [pH 7.4]). The erythrocyte pellet was resuspended to 4% (vol/vol) in PBS plus 10 μg of trypsin (Sigma Chemical Co., St. Louis, Mo.) per ml and incubated for 1 h at 37°C with rocking. The cells were pelleted, washed three times in PBS containing 0.2% BSA and once in PBS, and resuspended at ~17% (vol/vol) in PBS. Glutaraldehyde (EM Corp., Chestnut Hill, Mass.) was added to 0.625%, and cells were rocked for 1 h at 20°C. Fixation was stopped by adding 0.8 volume of PBS–0.1 M glycine, and the cells were incubated for 30 min at 4°C and washed twice in PBS–0.1 M glycine. These fixed erythrocytes were washed twice in PBS and resuspended at a final 17% suspension. Erythrocytes were washed in ethanol as described by Kobiler and Barondes (35). Briefly, 9 volumes of ethanol was added slowly to a 17% suspension of erythrocytes with stirring, and then the cells were incubated for 1 h at 20°C with gentle stirring. The erythrocytes were pelleted, washed once more in ethanol, and stored in ethanol at 4°C. Before use in hemagglutination assays, the erythrocytes were washed three times in PBS and resuspended in PBS at a final 2% suspension.

Bacteria were prepared as previously described (9). Briefly, aliquots of bacteria frozen at −80°C in MKP–20% glycerol were thawed, washed three times in PBS–0.2% BSA, and resuspended at 5×10^8/ml in PBS–0.2% BSA, as determined in a Petroff-Hauser bacterial counting chamber. For hemagglutination assays, 25-μl serial twofold dilutions of bacteria were prepared in 96-well round-bottom microtiter plates (Falcon 3911); 25 μl of a 2% suspension of trypsinized, fixed, ethanol-washed rabbit erythrocytes was then added. After 30 min at 20°C, each well was scored for agglutination as described previously (6). The hemagglutination titer of a given strain varied somewhat with the particular preparations of bacteria and erythrocytes; therefore, each strain was tested on at least three different occasions.

To test potential inhibitors of hemagglutination, bacteria were prepared at a concentration twofold higher than the minimum concentration required to give complete agglutination; 25 μl of this bacterial suspension was incubated with the inhibitor for 30 min at 20°C in PBS–0.2% BSA prior to assaying for hemagglutination as described above. Inhibitors tested included dextran sulfate, dextran, heparin, heparan sulfate, dermatan sulfate, chondroitin sulfate A, chondroitin sulfate C, hyaluronic acid, colominic acid, *N*-acetylglucosamine, GalNAc, *N*-acetylneuraminic acid, glucose-6-sulfate, glucosamine-2-sulfate, glucosamine-3-sulfate, glucosamine-2,3-disulfate, glucosamine-2,6-disulfate, and glucose.

Binding of *B. burgdorferi* to immobilized proteoglycans or dextran sulfate. (i) Radiolabeled bacteria. To coat plastic wells with potential substrates for bacterial attachment, 50 μl of PBS containing 5 mg of proteoglycan or dextran sulfate (molecular weight, ~500,000; Sigma) per ml was added to NUNC Maxi-Sorp 96-well break-apart microtiter wells and incubated for 16 h at 4°C. Wells were washed twice in PBS and blocked for 2 h at 20°C in 3.5% BSA in 50 mM Tris (pH 7.5)–100 mM NaCl–1 mM each $MgCl_2$, $MnCl_2$, and $CaCl_2$; 10^6 [^{35}S]methionine-labeled bacteria in 50 μl of MKP-S/3 (MKP bacteriological medium without serum, diluted 1:3 in 10 mM glucose–10 mM HEPES [pH 7.0] [9]) were then added to each well. Microtiter plates were centrifuged at 1,100 × *g* for 15 min and then rocked for 45 min at 20°C. Wells were washed three times with PBS–0.2% BSA and dried overnight. The radioactivity associated with each well was determined by scintillation counting. Wells containing 10^6 bacteria were counted to derive percent inoculum bound for each well.

(ii) DAPI staining of bound bacteria. Ninety-six-well black polystyrene Fluoroplates (Labsystems, Helsinki, Finland) were coated with dextran sulfate or chondroitin sulfate C as described above. Wells were washed three times in PBS and then blocked for 1 h with 3.5% BSA in 50 mM Tris HCl–100 mM NaCl–1 mM $MgCl_2$–1 mM $MnCl_2$–1 mM $CaCl_2$ (pH 7.5). Wells were washed three times in PBS, and 50 μl of 10 mM HEPES–10 mM glucose (pH 7.0) was added to each well. Freshly harvested bacteria or bacteria stored at −80°C were washed three times in PBS–0.2% BSA and resuspended at 10^9/ml in MKP-S (9, 47); 25 μl of the bacterial suspensions was added to each well, and the plates were centrifuged at 1,200 × *g* for 15 min and rocked for 45 min at 20°C. Wells were washed three times with PBS to remove unbound bacteria; 50 μl of 3% paraformaldehyde in PBS was added to each well, and the plates were dried overnight. The following day, 50 μl of −20°C methanol was added to each well, and the plate was incubated at −20°C for 10 min. Wells were dried and then incubated with 50 μl of 0.1 μg of 4′,6-diamidino-2-phenylindole (DAPI; Molecular Probes, Eugene, Ore.) per ml in PBS for 10 min at 20°C. After two washes in PBS, 50 μl of PBS was added to each well, and the plate was read in a microtiter plate fluorimeter (excitation wavelength of 355 nm and emission wavelength of 460 nm). Percent inoculum bound for each experiment was calculated by using absolute numbers of bacteria converted from fluorescence units, using a standard curve established with two *B. burgdorferi* strains over a wide concentration range. For any given strain, the percentage of the inoculum that bound to dextran sulfate varied from assay to assay. A number of factors, such as the particular source and lot of BSA in the growth medium and wash buffer, contribute to this variability (not shown). Additionally, we have previously shown that bacterial culture conditions can dramatically effect integrin-binding activity (10). To account for this experimental variability, each strain was tested two to five times, with strains N40 and HB19 included in each assay for comparison.

Binding of labeled *B. burgdorferi* to mammalian cells. NUNC 96-well Maxi-Sorp break-apart plates were UV sterilized for 1 to 2 h and then coated with the cell-adhesive protein MBP-Inv479 at 7.5 μg/ml in PBS for 2 h at 37°C. MBP-Inv479 carries the cell-binding domain of the *Yersinia pseudotuberculosis* invasin protein (37) and facilitates stable cell attachment to the plastic wells. Except when CHO-*ldlD* cells or $NaClO_3$-treated Vero cells (see below) were tested, cells were cultured in MBP-Inv479-coated microtiter wells for 16 h. Confluent cell monolayers were used for all bacterial adhesion assays. The monolayers were washed twice with 200 μl of PBS and blocked for 20 min at 37°C in 200 μl of blocking medium (RPMI 1640, 20 mM HEPES, [pH 7.0], 3.5% BSA). (Pilot experiments revealed that BSA that had been heated to 56°C for 1 h and filtered through a 0.2-μm-pore-size filter gave lower background bacterial binding to plastic, and so heat-treated BSA was routinely used.) Blocked monolayers were washed once in PBS, and 1×10^6 to 2×10^6 bacteria, labeled with [^{35}S]methionine as previously described (9), were added to each well in 50 μl of MKP-S/3. To assay binding to the various CHO cell lines, bacteria were centrifuged onto CHO cells at 1,200 × *g* for 15 min at 20°C to increase binding efficiency. After rocking for 1 to 2 h at 20°C, wells were washed three times in PBS–0.2% BSA to remove unbound bacteria. After washing, wells were examined microscopically to evaluate the integrity of the monolayer. Wells were dried overnight, and the radioactivity associated with each well was determined by liquid scintillation counting. Bacterial binding to wells in which no cells were plated was determined for each experiment, and wells containing the inoculum alone were also counted. All assays were performed in quadruplicate.

Inhibition of bacterial attachment to mammalian cells. To evaluate potential inhibitors of cell binding such as heparin, chondroitin sulfate C, and dextran sulfate, *B. burgdorferi* cells at 3×10^6 to 6×10^6/ml were incubated at various concentrations of inhibitor for 30 min at 20°C in MKP-S. After incubation with

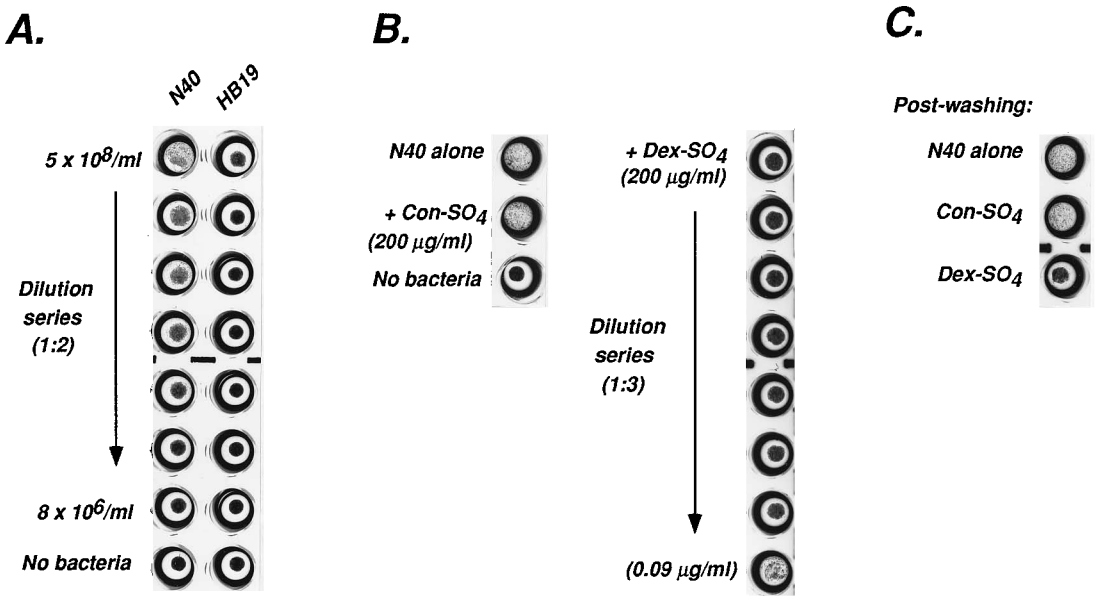

FIG. 1. Agglutination of rabbit erythrocytes by *B. burgdorferi* is inhibited by dextran sulfate. (A) The infectious strain N40 agglutinates rabbit erythrocytes more efficiently than does the noninfectious strain HB19. Erythrocytes were added to twofold serial dilutions of bacteria in microtiter wells. In the presence of $>5 \times 10^8$ of N40 cells per ml, a well-defined erythrocyte pellet at the bottom of the well was not formed because of the hemagglutinating activity that results in the formation of a diffuse lattice of erythrocytes. (B) Dextran sulfate (Dex-SO_4) inhibits hemagglutination by strain N40. In the absence of inhibitor, or after preincubation in 100 μg of chondroitin sulfate C (Con-SO_4) per ml, N40 agglutinated rabbit erythrocytes. Preincubation with dextran sulfate at concentrations as low as 0.24 μg/ml blocked hemagglutination by N40. (C) Dextran sulfate inhibition of hemagglutination is not reversed by washing. Strain N40 was incubated in 20 μg of inhibitor per ml for 20 min, then washed three times with PBS, and tested for hemagglutination. Washing the bacteria after incubation with dextran sulfate did not result in reappearance of hemagglutinating activity.

each inhibitor, the bacteria were evaluated by dark-field microscopy; none of the reagents tested affected bacterial motility or morphology. This mix of bacteria and inhibitor was then diluted 1:3 in 10 mM glucose–10 mM HEPES (pH 7.0), and 50 μl was added to monolayers in microtiter wells.

To examine the effect of platelet factor 4 on bacterial attachment, confluent Vero cell monolayers were incubated with 100 μl of platelet factor 4 (5 μg/ml; Sigma) in RPMI 1640–1% BSA for 2 h at 37°C. Wells were washed once with 200 μl of RPMI 1640–1% BSA, and bacterial binding was assessed as described above.

Chlorate inhibition of sulfation by mammalian cells. To inhibit sulfation of glycosaminoglycans, Vero cells were plated overnight in NUNC 96-well microtiter plates, prepared as described above, in Ham's F12 medium supplemented with 10% dialyzed newborn calf serum (for Vero cells) or fetal bovine serum (for CHO cells) as described previously (22). $NaClO_3$ (Baker Scientific, Medford, Mass.), or NaCl as a control, was added to a final concentration of 30 mM, and cells were grown for an additional 24 h. Cells were washed twice with 200 μl of Ham's F12 medium, blocked, and assayed for bacterial binding as described above. To show that the effect of chlorate treatment on bacterial attachment was due to an inhibition of sulfation, this inhibition was circumvented by the addition of 10 mM Na_2SO_4 to cultures at time of $NaClO_3$ addition (22). NaCl was also used as a control for Na_2SO_4 and had no effect on bacterial attachment in any condition tested.

Proteoglycan digestion by lyases. To test the effect of proteoglycan digestion on *B. burgdorferi* binding, confluent Vero or HEp-2 cell monolayers were washed once in RPMI 1640–1% BSA and then digested for 2 h at 37°C in a 5% CO_2 atmosphere with 0.5 U of heparinase I, heparitinase (heparinase III), or chondroitinase ABC (Sigma) per ml in 100 μl of RPMI 1640–1% BSA–0.1 mM phenylmethylsulfonyl fluoride–1.2×10^{-2} trypsin inhibitory unit of aprotinin per ml as previously described (26). Monolayers were washed once in RPMI 1640–1% BSA and then blocked and assayed for bacterial attachment. The activity of each lyase under above conditions was assessed by the parallel digestion of monolayers containing ^{35}S-labeled proteoglycans essentially as described previously (60). Briefly, cells were cultured for 48 h in Ham's F12 medium with 10% dialyzed newborn calf serum (22) and 20 μCi of [^{35}S]sulfate (DuPont-New England Nuclear, Wilmington, Del.) per ml. Monolayers were washed six to eight times in RPMI 1640–1% BSA to remove unincorporated [^{35}S]sulfate and digested with lyases as described above. Counts released into the medium by lyase or mock digestion were determined by scintillation counting, and each lyase was shown to release [^{35}S]sulfate-labeled material from the monolayer.

Bacterial attachment to extracellular matrix. To assay attachment of bacteria to extracellular matrix, confluent monolayers of mammalian cells were prepared as described above and then lifted in PBS–0.5 mM EDTA at 37°C for 15 to 30 min. After extensive washing, these wells were checked microscopically to ensure that all mammalian cells were removed by this treatment. Retention of Vero and CHO-K1 cell proteoglycans in microtiter wells with this method was confirmed by quantitating well-associated ^{35}S-labeled proteoglycans (see above for labeling method). The matrix was washed three times in PBS and then blocked and assayed for bacterial binding as described above.

RESULTS

An infectious strain of *B. burgdorferi* efficiently agglutinates rabbit erythrocytes and binds dextran sulfate and heparin. In an effort to uncover mechanisms of cell attachment distinct from the one mediated by integrins, we examined the interaction of *B. burgdorferi* with erythrocytes, which do not express integrins. An infectious clone of strain N40 (clone D10/E9 [38]) and a noninfectious clone of strain HB19 (clone 1 [9]) were screened for hemagglutination of human, sheep, chicken, or rabbit erythrocytes. Rabbit erythrocytes, but none of the other cells tested, were agglutinated by N40 at 5×10^8 bacteria per ml (Fig. 1A). HB19 agglutinated rabbit erythrocytes only at concentrations 10- to 50-fold higher than that required for N40 (Fig. 1A and data not shown).

Microbial hemagglutinins often recognize specific saccharides on the erythrocyte surface, so several sugars and glycoconjugates were tested for the ability to inhibit hemagglutination. The sulfated linear polysaccharide dextran sulfate was found to inhibit agglutination. Titration of dextran sulfate revealed that concentrations of less than 1 μg/ml were sufficient for inhibition (Fig. 1B). Sulfation of this glucose polymer was essential for inhibition, because dextran had no inhibitory activity, even at a concentration of 2.5 mg/ml (data not shown). Dextran sulfate did not affect the morphology or motility of the spirochete, as assessed by dark-field microscopy, and inhibition of hemagglutination by dextran sulfate was also not simply a function of negative charge, because of the several proteogly-

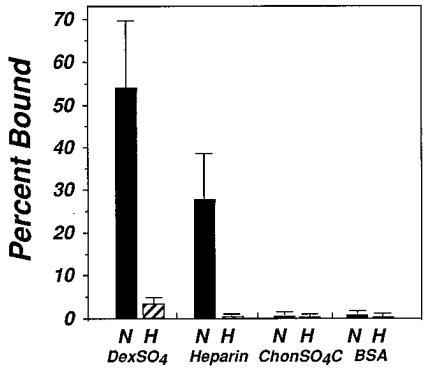

FIG. 2. *B. burgdorferi* binds to dextran sulfate and heparin. Microtiter wells were coated with dextran sulfate ($DexSO_4$), heparin, chondroitin sulfate C ($ChonSO_4C$), or BSA. Radiolabeled N40 (N; solid bars) or HB19 (H; hatched bars) cells were added, and after washing, stably bound bacteria were quantitated (see Materials and Methods). Shown are the means plus standard deviations of four replicates.

cans tested, only heparin partially inhibited hemagglutination (Fig. 1B and data not shown). Several unmodified and sulfated monosaccharides were tested (see Materials and Methods), and none inhibited hemagglutination (data not shown). Extensive washing of the bacteria after incubation with dextran sulfate did not restore hemagglutinating activity (Fig. 1C), suggesting that inhibition was due to stable binding of dextran sulfate by the spirochete.

To test for a direct interaction between N40 and dextran sulfate or heparin, radiolabeled bacteria were assayed for binding to sulfated polysaccharides immobilized on microtiter wells. N40 bound to dextran sulfate and heparin but not to the other negatively charged polysaccharides represented in the glycosaminoglycan chains of chondroitin sulfate C (Fig. 2) or keratan sulfate proteoglycan (data not shown). Binding to dextran sulfate was more efficient than binding to heparin, consistent with their relative efficiencies at inhibiting hemagglutination. HB19, which showed a much lower hemagglutinating activity than strain N40, bound only inefficiently to dextran sulfate (Fig. 2).

Hemagglutination, dextran sulfate binding, and attachment to mammalian cells by diverse Lyme disease spirochetes. If the dextran sulfate-inhibitable hemagglutinating activity that is expressed by *B. burgdorferi* N40 is important for infectivity, it should be found among diverse infectious strains of Lyme disease spirochetes. A collection of 19 other strains was tested for hemagglutinating activity (Table 1). These strains originated from diverse geographic locations and included infectious isolates representing all three species of Lyme disease spirochetes (3, 10, 57). All but 2 of the 19 strains consistently agglutinated rabbit erythrocytes at 10^9 bacteria per ml or less (Table 1). Dextran sulfate blocked hemagglutination by all of the 13 strains tested (Table 1), whereas chondroitin sulfate C had no effect. Thus, the hemagglutinating activity expressed by

TABLE 1. Diverse Lyme disease spirochetes agglutinate erythrocytes and bind dextran sulfate

Strain[a]	Clone	Species	Passage	Reference(s)	Osp expression[b]			Hemagglutination		$DexSO_4$ binding[c]
					A	B	C	$-DexSO_4$[d]	$+DexSO_4$[e]	
N40	D10/E9	*B. burgdorferi*	6–15	9, 38	+	+	−	+	−	+
HB19	1	*B. burgdorferi*	High[f]	9, 38	+	+	−	−	NA[g]	−
LP3	Uncl.[h]	*B. burgdorferi*	3–5	18	+	+	−	+	−	ND[i]
LP4	Uncl.	*B. burgdorferi*	3–5	18	+	+	ND	+	−	ND
LP5	Uncl.	*B. burgdorferi*	3–5	18	+	+	ND	+	−	ND
LP7	Uncl.	*B. burgdorferi*	3–5	18	+	+	+	+	−	+
CA-20	2A	*B. burgdorferi*	8–15	48	+	±	+	+	ND	+
CA-20	4A	*B. burgdorferi*	Low	42	+	+	+	+	ND	+
CA-11	1A	*B. burgdorferi*	Low	42	+	+	−	+	−	+
CA-17	14A	*B. burgdorferi*	High	42	+	+	+	+	ND	+
CA-17	13C	*B. burgdorferi*	8–15	48	+	±	+	+	ND	+
G39/40	A6	*B. burgdorferi*	High	21	+	+	+	±	ND	−
297	A11/B11	*B. burgdorferi*	15	38	+	+	+	+	−	+
PBi	Uncl.	*B. garinii*	8	58	+	+	+	+	−	+
VS102	Uncl.	*B. garinii*	8–12	41	+	−	+	+	−	+
G2	12	*B. garinii*	High	41, 48	+	+	−	+	−	+
G2	22	*B. garinii*	High	41, 48	−	+	−	±	ND	−
PBr	Uncl.	*B. garinii*	15	58	+	−	−	+	−	+
PKo	25	*B. afzelii*	5–7	58	+	+	+	+	ND	+
PBo	Uncl.	*B. afzelii*	5	58	+	+	+	+	−	+
VS461	Uncl.	*B. afzelii*	8–12	41	+	+	+	+	−	+

[a] For further strain details, see reference 10.
[b] Determined in a previous study by immunoblotting with polyclonal antisera 10. +, an antibody-reactive band of the appropriate apparent molecular mass was present; ±, a very weakly reactive band was present; −, no reactive band was detectable.
[c] +, 10 to 80% of the inoculum bound to immobilized dextran sulfate ($DexSO_4$) (see Materials and Methods); −, 0 to 3% of the inoculum bound; these strains bound 10- to 100-fold less efficiently than strain N40, which was routinely included as a positive control.
[d] Strains were tested for hemagglutination at a bacterial concentration of 10^9/ml. +, strain consistently promoted complete agglutination; ±, when assayed multiple times, agglutination was observed less than 50% of the time, and agglutination was often incomplete; −, strain was never observed to promote agglutination.
[e] −, dextran sulfate at 100 μg/ml inhibited agglutination. Chondroitin sulfate C at 100 μg/ml had no effect.
[f] Exact in vitro passage number unknown. Low passage is defined as fewer than 10 passages, at 1 to 2 passages per week in culture; high passage is defined as more than 50 passages.
[g] NA, not applicable.
[h] Uncl., not cloned.
[i] ND, not determined.

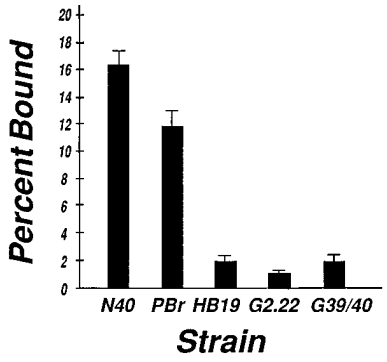

FIG. 3. The abilities of *Borrelia* strains to bind Vero cells parallel their abilities to bind erythrocytes and dextran sulfate. Vero cells grown in 96-well microtiter plates were infected with radiolabeled bacteria, and stably bound bacteria were quantitated (see Materials and Methods). G39/40 was tested on a different occasion from the other strains; in that assay, strain N40 bound at an efficiency of 12%. Shown are means and standard deviations of four replicates.

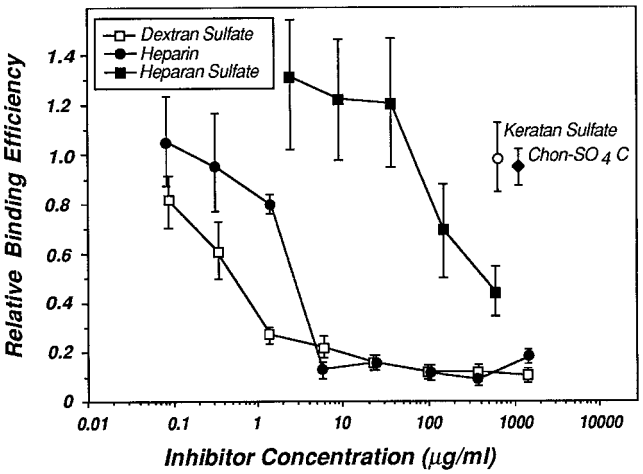

FIG. 4. Dextran sulfate, heparin, and heparan sulfate inhibit binding of *B. burgdorferi* to Vero cells. Radiolabeled strain N40 was preincubated with dextran sulfate, heparin, heparan sulfate, keratan sulfate, or chondroitin sulfate C (Chon-SO_4C) at the designated concentration and then diluted 1:3 just prior to infection of Vero cells (see Materials and Methods). Monolayers were washed, and bound radioactivity was determined. Binding relative to percent bound in the absence of inhibitor is shown. Shown are the means and standard deviations of four replicates.

B. burgdorferi N40 is also commonly found among other Lyme disease spirochetes (Table 1). Of note is the observation that PBr, a strain that does not bind the glycoprotein integrin $\alpha_{IIb}\beta_3$, agglutinated erythrocytes efficiently, suggesting that hemagglutination and integrin binding are not simply two measures of the same activity.

Of the entire collection of 21 strains examined in this study, the only strains that did not consistently agglutinate erythrocytes at 10^9 bacteria per ml were three noninfectious strains: HB19, G39/40, and G2 clone 22 (G2.22) (Table 1). To determine if the differences in hemagglutinating activity were associated with differences in dextran sulfate binding, the collection of strains was also tested for the ability to bind immobilized dextran sulfate. For binding-proficient strains such as N40, the percentage of inoculum bound to immobilized dextran sulfate varied considerably (i.e., 10 to 80%) among different experiments; therefore, each strain was tested two to five times, and N40 and HB19 were included in each assay for comparison. With the exception of HB19, G39/40, and G2.22, all of the strains tested consistently displayed greater than 10% binding to immobilized dextran sulfate (Table 1). Binding by the latter three strains never exceeded 3% and was always 10- to 100-fold less than that of the N40 control. These results indicate that low hemagglutinating activity was associated with the inability to bind dextran sulfate.

The hypothesis that the hemagglutinating and dextran sulfate-binding activities are related to adhesion of Lyme disease spirochetes to live cells was examined by measuring bacterial attachment to Vero cells, a fibroblast-like epithelial cell line derived from monkeys. *B. burgdorferi* has been observed previously to bind to this cell line (25), and strains N40 and PBr, which agglutinated erythrocytes and bound dextran sulfate, were found to bind Vero cells efficiently (i.e., >10% of inoculum bound; Fig. 3). In contrast, HB19, G39/40, and G2.22, the three strains that displayed low hemagglutinating and dextran sulfate-binding activities, bound to Vero cells much less efficiently. These three strains also bound inefficiently to several other cell lines, such as CHO cells, while N40 and PBr efficiently bound to all cells tested (see Table 2; also data not shown).

Dextran sulfate, heparin, and heparan sulfate inhibit binding of *B. burgdorferi* to mammalian cells. The findings presented above support the model that borreliae bind to the glycosaminoglycan chains of cell surface proteoglycans and that this activity is reflected by hemagglutination and binding to immobilized dextran sulfate. Consistent with this hypothesis, both heparin and dextran sulfate inhibited binding of radiolabeled N40 to Vero cells (Fig. 4). Titration revealed that dextran sulfate inhibited N40 binding more efficiently than did heparin, a result that parallels the relative potencies of these agents as inhibitors of hemagglutination. At high concentrations, heparan sulfate, which is structurally similar but less sulfated than commercial heparin (34), was also able to inhibit Vero cell binding by N40. Dextran had no effect on cell attachment (data not shown), indicating that, as was the case for hemagglutination, sulfation was critical for blocking activity. Inhibition was not solely a function of charge or sulfation, however, because the proteoglycans keratan sulfate and chondroitin sulfate C had no effect on cell attachment at concentrations more than 1,000-fold higher than that required for 50% maximal inhibition by dextran sulfate (Fig. 4). Binding of N40 to several other cell lines, such as HEp-2 (laryngeal carcinoma), 293 (kidney carcinoma), and CHO, was also inhibited by dextran sulfate (data not shown), suggesting that this binding pathway may contribute to attachment to a variety of cells.

Cell surface proteoglycans are required for attachment of Lyme disease spirochetes to mammalian cells. The role of mammalian cell proteoglycans in bacterial recognition was investigated further, by digesting proteoglycans associated with mammalian cells. Vero cell monolayers were digested with heparinase (which cleaves heparin-related glycosaminoglycans), heparitinase (which cleaves heparan sulfate-related glycosaminoglycans), or chondroitinase ABC and then tested for bacterial binding. Treatment of cells with heparinase or heparitinase greatly diminished the binding of strain N40, while chondroitinase treatment had no effect (Fig. 5A). Consistent with a role for heparan sulfate-related proteoglycans in *Borrelia* attachment, preincubation of the cell monolayer with platelet factor 4, which binds these proteoglycans (39), at 5 μg/ml resulted in a three- to eightfold decrease in N40 attachment to Vero cells (not shown). Although chondroitinase ABC treatment did not diminish N40 adhesion to Vero cells, analysis of bacterial binding to mutant CHO cell lines suggested that chondroitin sulfate proteoglycans expressed by CHO cells are

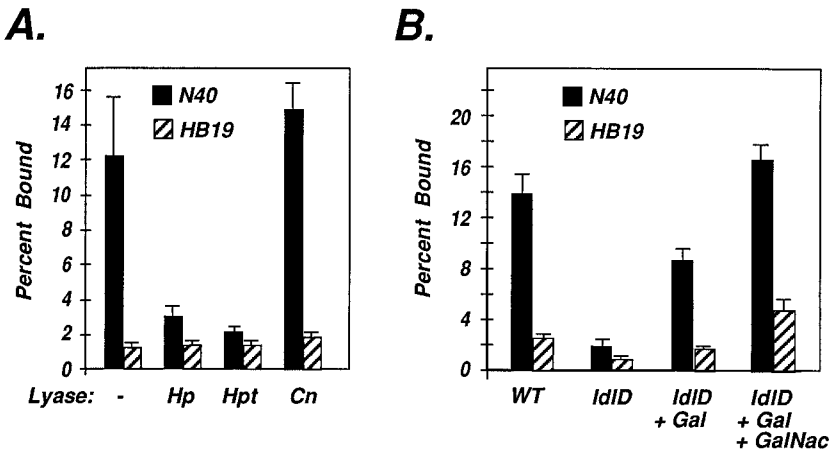

FIG. 5. Proteoglycans are required for efficient cell binding by *B. burgdorferi* N40. (A) Removal of heparin/heparan sulfate glycosaminoglycans diminishes N40 attachment to Vero cells. Vero cell monolayers were preincubated with buffer alone (−), heparinase (Hp), heparitinase (Hpt), or chondroitinase ABC (Cn) prior to infection with radiolabeled N40 or HB19. After washing, bound bacteria were quantitated; shown are the means and standard deviations of four replicates (see Materials and Methods). (B) CHO-*ldlD* cells that do not express proteoglycans are not efficiently bound by *B. burgdorferi* N40. CHO-*ldlD* (*ldlD*) cells were cultured under conditions that restrict the spectrum of proteoglycans expressed (see Materials and Methods) and compared with wild-type (WT) CHO cells for the ability to bind *B. burgdorferi* N40 or HB19. CHO-*ldlD* cells were cultured in the absence of exogenous galactose and GalNAc, conditions which do not promote the synthesis of heparan sulfate or chondroitin sulfate (14, 33); in the presence of galactose (+Gal), which promotes the synthesis of only heparan sulfate; or the presence of galactose and GalNAc (+ Gal, +GalNAc), which promote the synthesis of both heparan and chondroitin sulfate. Growth of CHO-K1 cells in galactose, GalNAc, or both had no effect on bacterial binding (data not shown). Shown are means and standard deviations of four replicates.

recognized by Lyme disease spirochetes and that the relative contribution of each class of proteoglycan to bacterial binding may vary from strain to strain (see below).

Mutant cell lines deficient in the synthesis of heparan and/or chondroitin sulfate proteoglycans have been derived from CHO cells (14, 33) and provided a genetic test for the role of these molecules in host cell recognition by N40. Heparan sulfate and chondroitin sulfate are the predominant classes of proteoglycans synthesized by wild-type CHO cells (14). CHO-*ldlD* cells are defective in the de novo synthesis of uridine 5′-diphospho (UDP)-galactose and UDP-GalNAc (33) and require exogenous galactose and/or GalNAc for the synthesis of glycosaminoglycans (14). In the absence of these sugars, CHO-*ldlD* cells express neither heparan sulfate nor chondroitin sulfate. When cultured with exogenous galactose, CHO-*ldlD* cells synthesize heparan sulfate but not chondroitin sulfate, and when cultured with both galactose and GalNAc, these cells express both heparan sulfate and chondroitin sulfate. We found that after culturing in the absence of galactose and GalNAc, N40 bound efficiently to only wild-type CHO-K1 cells, not to CHO-*ldlD* cells (Fig. 5B). N40 binding to CHO-*ldlD* cells was partially recovered after the CHO cells were cultured under conditions that promote expression of heparan sulfate (Fig. 5B, + Gal) and was equivalent to binding to wild-type CHO cells after culturing under conditions that promote the expression of both heparan and chondroitin sulfate (Fig. 5B, + Gal, + GalNac). The addition of galactose and GalNAc had no effect on N40 attachment to wild-type CHO cells (not shown). These findings are consistent with roles for both heparan sulfate and chondroitin sulfate proteoglycans in N40 adhesion.

In addition to affecting glycosaminoglycan synthesis, the *ldlD* mutation also inhibits N-linked and O-linked protein glycosylation (33); therefore, CHO cell mutants that are specifically defective in proteoglycan synthesis were also examined (15). N40 bound poorly to CHO-*pgsA* and *pgsB* cells, which are deficient in the synthesis of both heparan sulfate and chondroitin sulfate (15, 16) (Table 2). CHO-*pgsD* cells are deficient in heparan sulfate but express threefold more chondroitin sulfate than wild-type cells do, and N40 bound slightly more efficiently to these cells than to CHO-*pgsA* or CHO-*pgsB* cells.

To assess whether the proteoglycan-binding activity ex-

TABLE 2. Lyme disease spirochetes bind inefficiently to CHO cell mutants that are defective in proteoglycan synthesis

Cell type (strain)	Defect (reference)	Glycosaminoglycan synthesized		% Bound ± SD[a]			
		Heparan sulfate	Chondroitin sulfate	N40	PBr	VS461	G2.22
CHO wild type (K1)	None	+	+	40.7 ± 2.6	33.6 ± 1.0	27.3 ± 2.0	8.1 ± 2.1
CHO-*pgsA* (745)	Xylosyltransferase (15)	−	−	0.8 ± 0.2	2.7 ± 0.8	2.7 ± 0.6	0.9 ± 0.2
CHO-*pgsB* (761)	Galactosyltransferase (16)	−	−	0.8 ± 0.2	1.4 ± 0.5	2.2 ± 0.3	3.0 ± 0.4
CHO-*pgsD* (677)	*N*-Acetylglucosaminyl and glucuronosyltransferases (14)	−	+[b]	8.1 ± 2.0	23.0 ± 2.5	34.7 ± 1.6	3.1 ± 0.7
CHO-*pgsE* (745)	*N*-Sulfotransferase (2)	+[c]	+	3.3 ± 1.6	5.8 ± 0.5	8.2 ± 1.6	2.0 ± 0.9

[a] The ability of radiolabeled bacteria to bind wild-type and mutant CHO cells was determined (see Materials and Methods). Shown are means and standard deviations of four replicates from a representative experiment. Binding to wells without cells was less than 2.0% for all strains.
[b] Threefold higher levels of chondroitin sulfate than in wild-type cells (14).
[c] Heparan sulfate is undersulfated (2).

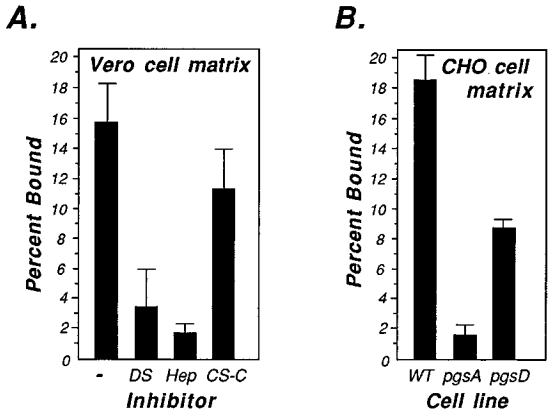

FIG. 6. Proteoglycans mediate attachment of *B. burgdorferi* N40 to extracellular matrix. (A) Radiolabeled strain N40 was preincubated with buffer alone (−) or with 300 μg of dextran sulfate (DS), heparin (Hep), or chondroitin sulfate C (CS-C) per ml prior to incubation with extracellular matrix (see Materials and Methods), and bound bacteria were quantitated. (B) Binding of radiolabeled N40 to matrix produced by CHO-K1 (wild type [WT]), CHO-*pgsA*-745 (*pgsA*), or CHO-*pgsD*-677 (*pgsD*) was quantitated. For both panels, shown are means and standard deviations of four replicates.

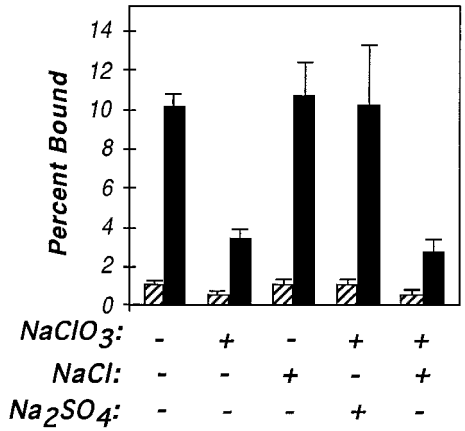

FIG. 7. Inhibition of Vero cell sulfation with chlorate inhibits binding by *B. burgdorferi* N40. Monolayers were pretreated in medium supplemented with $NaClO_3$, NaCl, or Na_2SO_4, as indicated, prior to infection with N40 (solid bars) or HB19 (hatched bars) (see Materials and Methods). After washing, bound bacteria were quantitated; shown are the means and standard deviations of four replicates. (See text for details).

pressed by N40 reflects a similar binding mechanism in other Lyme disease spirochetes, *B. garinii* PBr and G2.22 and *B. afzelii* VS461 were tested for attachment to the CHO-K1 and CHO-*pgs* mutants. Of all strains tested, G2.22 bound least efficiently to CHO cells, consistent with the low hemagglutinating and dextran sulfate-binding activities associated with this strain (Tables 1 and 2). All of the strains bound inefficiently to CHO-*pgsA* and CHO-*pgsB* cells, indicating that proteoglycan expression is critical for host cell attachment by all three species of Lyme disease spirochete (Table 2). Differences among N40, PBr, and VS461 were observed, however, in binding to CHO-*pgsD* cells, which overexpress chondroitin sulfate. PBr and VS461 bound to these cells more efficiently than did N40, suggesting that the relative contribution of chondroitin sulfate proteoglycans to binding by Lyme disease spirochetes may vary among from strain to strain.

Attachment of *B. burgdorferi* to extracellular matrix is mediated by proteoglycans. *B. burgdorferi* has previously been observed to bind extracellular matrix produced by endothelial cells (55). Proteoglycans are secreted into the extracellular matrix and are candidates for recognition by *B. burgdorferi*. Vero cells were lifted with EDTA to generate wells containing extracellular matrix. The hemagglutinin-expressing strain N40 bound to extracellular matrix more efficiently than did HB19 (data not shown), and this binding was significantly inhibited by dextran sulfate and heparin (Fig. 6A). Interestingly, although statistical significance was lacking, chondroitin sulfate C may have had a slight inhibitory effect on bacterial binding to extracellular matrix (Fig. 6A).

The CHO cell mutants described above provided another avenue to assess the role of matrix proteoglycan in *B. burgdorferi* attachment. Microtiter wells coated with matrix produced from wild-type CHO-K1 cells (containing both heparan sulfate and chondroitin sulfate), CHO-*pgsA* cells (containing neither proteoglycan), or CHO-*pgsD* cells (containing only chondroitin sulfate) were generated by lifting confluent monolayers of each cell line with EDTA as described above for Vero cells. Binding of *B. burgdorferi* N40 to matrix lacking both proteoglycans was diminished 10-fold compared with wild-type matrix, and binding to matrix lacking heparan sulfate was diminished 2-fold (Fig. 6B).

Sulfation of proteoglycans is required for maximal recognition of mammalian cells by *B. burgdorferi*. The inability of dextran to inhibit hemagglutination or cell attachment suggested that sulfation of the polysaccharide is critical for recognition by the bacterium. To test this, strains N40, PBr, and VS461 were tested for attachment to the mutant cell line CHO-*pgsE*, which produces heparan sulfate that is two- to threefold undersulfated (Table 2 and reference 2). None of the strains bound to this cell line as efficiently as to wild-type CHO cells, a result that is consistent with the importance of sulfation in spirochetal binding.

Chlorate is a competitive inhibitor of ATP-sulfurylase and reduces the sulfation of proteins and proteoglycans (1, 32). Pretreatment of Vero cells with sodium chlorate resulted in a threefold decrease in N40 binding (Fig. 7). HB19, which bound Vero cells less efficiently than did N40, was similarly affected by chlorate treatment. The addition of sulfate along with chlorate during the pretreatment should restore sulfation of proteoglycans (32), and efficient N40 binding was recovered under these conditions (Fig. 7). The effects of chlorate and sulfate treatments on spirochetal binding were not simply a result of Vero cell growth in a higher-ionic-strength medium, as pretreatment of the cells with sodium chloride had no effect (Fig. 7).

DISCUSSION

We have shown here that diverse Lyme disease spirochetes express a hemagglutinating activity and bind to proteoglycans located on host cell surfaces and in extracellular matrix. Hemagglutinating activity was associated with binding to the sulfated linear polysaccharide dextran sulfate in that the 18 strains that agglutinated rabbit erythrocytes efficiently also bound to immobilized dextran sulfate, while the 3 strains that displayed the lowest hemagglutinating activity did not. The latter three strains were also deficient in binding to mammalian cells. Dextran sulfate, the most potent inhibitor of hemagglutination, inhibited bacterial attachment to cultured mammalian cells and extracellular matrix. These correlations between hemagglutination and cell attachment are consistent with the model that a lectin activity that can be detected by the agglutination of erythrocytes promotes adhesion of the Lyme disease spirochete to host cells and matrix. Rigorous testing of this model

will require identification and isolation of the bacterial factor(s) involved.

Dextran sulfate presumably blocks *Borrelia* attachment to host cells by mimicking host cell glycosaminoglycan chains, and several lines of evidence indicate that proteoglycans are required for maximal cell binding by *B. burgdorferi*. CHO-*pgsA*, -*pgsB* and -*ldlD* cells, which are deficient in the synthesis of proteoglycans, were deficient for bacterial attachment. Bacterial binding to several cell lines was also diminished by pretreatment of cells with the proteoglycan synthesis inhibitor β-D-xylopyranoside (44a, 50). The sulfation of proteoglycans was critical for efficient bacterial recognition, because the cell lines that did not fully sulfate proteoglycans, due to a genetic defect or growth in the presence of an inhibitor, did not mediate maximal spirochetal binding.

Both heparin/heparan sulfate and chondroitin sulfate proteoglycans appeared to contribute to bacterial binding. *B. burgdorferi* N40 bound to immobilized heparin, and bacterial attachment to several cell lines could be inhibited almost completely with soluble heparin. Enzymatic removal of heparin- or heparan sulfate-related glycosaminoglycans from Vero cells diminished bacterial binding, as did pretreatment of cells with platelet factor 4, which binds heparan sulfate proteoglycans (39). CHO-*pgsE* cells, which are deficient in the sulfation of heparan sulfate specifically, did not support maximal bacterial adhesion. Chondroitin sulfate appeared to also mediate cellular attachment by the spirochete. CHO-*ldlD* cells bound *B. burgdorferi* at wild-type levels only when cultured under conditions that allowed for the synthesis of both heparan sulfate and chondroitin sulfate proteoglycans. Overexpression of chondroitin sulfate by CHO-*pgsD* cells partially (in the case of N40) or fully (in the case of VS461 and PBr) compensated for the absence of heparan sulfate. Isaacs found that binding of HeLa cells by *B. burgdorferi* 297 could be inhibited by chondroitinase ABC but not by chondroitinase AC, suggesting that chondroitin sulfate B (dermatan sulfate) can mediate attachment of this strain (29). Consistent with this observation, Isaacs showed that chondroitin sulfate B inhibited binding of strain 297 to HeLa cells. *B. burgdorferi* has also recently been demonstrated to bind decorin, a dermatan sulfate/chondroitin sulfate proteoglycan (22a), and to aggrecan, a chondroitin sulfate proteoglycan (54a). Our observation that chondroitinase ABC treatment of Vero cells had no effect on N40 attachment and that a commercial preparation of chondroitin sulfate C was unable to block hemagglutination or bacterial attachment to mammalian cells may reflect *Borrelia* recognition of only a specific subset of chondroitin sulfate proteoglycans. Glycosaminoglycans are heterogeneous and can differ in the degree and position of O sulfation, in the length of the polysaccharide chain, and in the extent of glucuronic acid epimerization (34). Additionally, differences in the efficiency of binding to CHO-*pgsD* cells by strains N40, PBr, and VS461 suggest that individual strains may differ somewhat in their abilities to recognize specific proteoglycans. N40, PBr, and VS461 represent the three species of Lyme disease spirochete, but further studies are required to determine if the differences observed here reflect differences in the lectin specificities of each of the species.

The analysis of many isolates in this study did reveal that the abilities to hemagglutinate and bind dextran sulfate are widespread among Lyme disease spirochetes. Of the 21 strains tested, representing each of the three species of Lyme disease spirochete, only 3 noninfectious strains did not efficiently express these activities. This finding is consistent with the hypothesis that proteoglycan-mediated attachment plays a role during *Borrelia* infection. Proteoglycans are ubiquitous components of host cell surfaces and extracellular matrix, and this bacterial attachment mechanism might facilitate infection of diverse tissues. For example, *B. burgdorferi* has been observed to penetrate between endothelial cells in confluent monolayers in vitro (55), and binding to glycosaminoglycans in extracellular matrix could promote such intercellular migration. We have shown here that proteoglycans are involved in *Borrelia* attachment to Vero and CHO cells; in addition, bacterial binding to rat glioma, human kidney carcinoma, and human melanoma cells was also disrupted by inhibitors of the proteoglycan-mediated pathway (44a). Little is known concerning glycosaminoglycans expressed by cells of the *Ixodes* tick, but given that some proteoglycans are highly conserved evolutionarily (52), this attachment mechanism might play a role in the behavior of the spirochete within its arthropod vector.

The analysis of a large collection of strains also allows for a comparison of the expression of known outer surface proteins (Osps) with proteoglycan binding activity. OspA, OspB, and OspC, because of their cellular location, are potential adhesin candidates. The pattern of Osp expression among the 21 strains was determined in a previous study using polyclonal antisera directed against these proteins (10), and when this was compared with the pattern of hemagglutination and dextran sulfate binding (Table 1), no correlations were found. Although we cannot rule out the possibility that some strains designated OspA, OspB, or OspC nonproducers actually express the given Osp, but one that is not recognized by the antiserum used for detection, these results suggest that expression of these proteins is not sufficient for hemagglutination or dextran sulfate binding.

Although heparin can inhibit ligand binding by some integrins (24), the finding that strain PBr, which does not bind integrin $\alpha_{IIb}\beta_3$ (10), agglutinated erythrocytes suggests that proteoglycan-mediated attachment is distinct from attachment mediated by integrins. The observation that proteoglycan-binding by *B. burgdorferi* mediates attachment not only to host cell surfaces but also to extracellular matrix, which does not contain integrins, is further evidence that this pathogen expresses at least two different binding pathways. *B. burgdorferi* has been previously shown to bind to purified glycolipids (19), and it is possible that proteoglycan binding and glycolipid binding reflect the same lectin activity. In any case, the integrins and/or glycolipids expressed by Vero or CHO cells are apparently not sufficient to promote stable bacterial attachment by the clone of strain N40 analyzed in this study, because inhibition of proteoglycan binding almost completely inhibited N40 attachment to these cells. In contrast, HeLa cell binding by several strains, including an uncloned population of N40, was only partially inhibited by treatments that blocked proteoglycan binding (29), consistent with the hypothesis that two (or more) pathways contribute to HeLa cell attachment by these strains. It is likely that the relative contribution of each mechanism to bacterial adherence depends upon both the specific target cell and the *Borrelia* strain.

An attractive model is that the integrin- and proteoglycan-mediated pathways act in concert, e.g., to promote tissue invasion. Migration of leukocytes from the bloodstream to inflammatory sites initially involves lectin-mediated interactions with endothelial cells, followed by integrin-mediated movement between endothelial cells (40). The Lyme disease spirochete might utilize a similar sequential, multistep process during hematogenous dissemination. Alternatively, the two binding activities might act simultaneously to promote avid bacterial attachment to particular host cells, analogous to the formation of focal adhesion plaques, the sites on the plasma membrane that provide the tightest adhesion between cells and

extracellular matrix (8). Extracellular matrix proteins such as fibronectin and vitronectin contain distinct integrin-binding and heparin-binding domains (49), and both domains are required for focal plaque formation (59). Future studies to delineate the precise roles of the different binding pathways and their significance to *Borrelia* infection will require the identification of the specific bacterial molecules that mediate these interactions with integrins and proteoglycans.

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