Heparin is a functional and structural analog of the Chlamydia trachomatis heparan sulfate-like attachment ligand that mediates infectivity by bridging chlamydiae to eukaryotic cells. The binding of heparin to the Chlamydia organism’s surface was characterized by a direct binding assay. Although for two C. trachomatis biovars the binding by heparin was saturable, trachoma biovar organisms bound twice the amount of heparin than lymphogranuloma venereum biovar organisms. To probe the structural nature of the heparan sulfate-like ligand interactions, a range of heparin derivative oligosaccharides and sulfation-modified species of heparin were compared for their ability to compete with [3H]heparin for binding to chlamydial organisms and for inhibition of chlamydial attachment and infection of eukaryotic host cells. The assays revealed that a deacetylated casaccharide was the minimal chain length required to effectively bind C. trachomatis organisms, compete with the host cell receptor and rescue infectivity. In addition, a moderately sulfated heparin analog, N-desulfated, N-acetylated heparin, was able to compete with chlamydial organisms for host cell receptors, whereas this derivative could not compete with [3H]heparin for binding to chlamydial organisms. These results indicate that the specificity of the eukaryotic cell receptor and the chlamydial surface acceptor differ in their fine-structure requirements of ligand binding, and that the size and sulfation density of the heparan sulfate-like ligand each contribute to its ability to bind and bridge chlamydiae to eukaryotic cells.

A broad range of microbial pathogens that infect humans bind heparan sulfate moieties of eukaryotic cell surface proteoglycans. Heparate-containing proteoglycans on eukaryotic cells have been shown to facilitate microbial adherence and/or cellular invasion for human immunodeficiency virus (1), herpes simplex virus (2, 3), cytomegalovirus (4, 5), varicella zoster virus (6), Bordetella pertussis (7), Leishmania donovani (8, 9), Trypanosoma cruzi (10), and Plasmodium circumsporozoites (11). Unlike each of these other microbial pathogens, it has recently been shown that Chlamydia trachomatis attachment to, and subsequent infectivity of, eukaryotic cells is dependent upon the presence of a heparan sulfate-like ligand on the surface of the organism (12, 13). Treatment of C. trachomatis organisms with a specific heparan sulfate lyase, heparitinas, abolishes chlamydial infectivity, yet exogenous heparin or heparan sulfate rescues chlamydial attachment and infectivity for heparitinase-digested organisms (13). Because exogenous heparin or heparan sulfate can restore attachment and rescue infectivity, it is thought that the organism binds the heparan sulfate-like glycosaminoglycan (GAG) by a specific surface receptor molecule. Characterization of the structural requirements of heparin binding to microbial pathogens has significant implications for understanding the basic biology of both the pathogen and the eukaryotic host as well as for development of antimicrobial strategies based upon the essential adhesion step of infectivity.

C. trachomatis is phylogenetically deeply separated from other eubacteria because chlamydiae grow only within eukaryotic host cells and have a developmental cycle (14). The developmental cycle is characterized by two forms of the organism: a metabolically active intracellular form, and a metabolically inactive extracellular form, called the elementary body (EB), that is capable of infecting mammalian cells. There are two biovars of C. trachomatis that cause a spectrum of important diseases in humans (15). The trachoma biovar is responsible for most genital tract and ocular infections of mucosal surfaces, whereas the lymphogranuloma venereum (LGV) biovar is much more invasive and primarily grows in lymphoid tissue. It has been shown for both biovars that a heparan sulfate-like ligand on the surface of the C. trachomatis EB is essential for infection of mammalian cells (12, 13). It has been proposed that the heparan sulfate-like adhesin is synthesized by chlamydiae (13), but the structure of the native heparan sulfate-like ligand that decorates the chlamydial cell surface is not known. The ligand can be produced and labeled with [35S]sulfate in infected cells incapable of GAG synthesis (16), the product is susceptible to heparitinase digestion, but it is not bound by heparan sulfate-specific monovalent antibodies (13). Although the two biovars are equally dependent upon the heparan sulfate-like ligand for infectivity (12), the biovars differ in surface charge (17) and in their susceptibility to heparin inhibition of attachment to host cells (18). The interaction of the heparan sulfate-like GAG with a chlamydial EB acceptor has not been established, nor has a specific GAG acceptor on chlamydiae been identified.

The major interactions between GAG and GAG-binding proteins involve negatively charged sulfates and carboxylates on
GAG and positively charged residues of proteins (19). Such an interaction is often expected to be relatively nonspecific in nature and of low affinity; however, type IV collagen contains three separate heparin binding domains, each of which has a distinct affinity (20). Likewise, the coagulation inhibitor anti-thrombin III binds specifically to a defined pentasaccharide sequence within heparin (21, 22). Thus, it is possible that there is a ligand-specific acceptor on the chlamydial cell surface that specifically binds the functional analog, heparin. Unfortunately, the diverse nature of heparin- or heparan sulfate-binding proteins that have been identified makes it difficult to predict structural or functional motifs that are conserved among this class of molecules (19).

The analysis of GAG-protein interactions is complicated by the inherent complexity of heparin and heparan sulfate structure (23). Three outstanding structural features of most GAG oligosaccharides are their 1) length; 2) high negative charge, and 3) constituent carbohydrate composition (24). The unbranched carbohydrate backbone of heparin or heparan sulfate consists of disaccharide repeats of hexuronic, \( \alpha \)-glucuronic, or \( \alpha \)-iduronic acids, and \( \alpha \)-glucosamine units, joined by 1,4-glycosidic linkages. The variable location of \( N \)-acetyl, \( N \)-sulfate, and \( O \)-sulfate groups on these three units can give rise to at least 10 different monosaccharide building blocks that can be combined into a large number of different oligosaccharide sequences (24). An entire GAG chain may contain approximately 100 disaccharide repeats. The large number of possible combinations of sulfation, backbone composition, and chain length complicates the structural and functional analysis of GAGs. Nevertheless, a better understanding of the nature of the heparan sulfate-like adhesion involved in chlamydial attachment and infection would not only contribute to defining a primary chlamydial virulence determinant but also would facilitate the identification, characterization, and isolation of the active chlamydial GAG ligand, GAG acceptor, and host cell receptor that are essential for productive infection of eukaryotic cells.

In this study, heparin and heparin derivatives were used as analogs of the chlamydial heparan sulfate-like ligand to circumvent the structural requirements of binding to chlamydial EBs and for mediating infectivity of eukaryotic cells. Heparin rather than heparan sulfate was used as the comparative standard for these studies as it appeared to be functionally equivalent in previous studies of chlamydial attachment and infectivity (12, 13), and each of the GAG derivatives used was obtained from heparin. By a direct binding assay it was shown that binding of exogenous \({ }^{3} \text{H}\)heparin to EBs representing both C. trachomatis biovars was saturable. Using derivatives of heparin, productive interaction with the EB and the eukaryotic cell differed and was dependent upon sulfate position, sulfation density, and oligosaccharide length.

**EXPERIMENTAL PROCEDURES**

**Cell Lines and Bacterial Strains—**L929 mouse fibroblast and HeLa 229 cell lines were grown in RPMI 1640 medium supplemented with 10% fetal calf serum, 0.1 mg/ml streptomycin, and 1 \( \mu \)g/ml cycloheximide added. At 10–12 h after infection, 0.5 \( \mu \)Ci of \( ^{35} \text{S} \)-labeled amino acid mixture (Trans-35S-label, ICN Biomedicals, Inc., Costa Mesa, CA) was added to each flask. At 48 or 72 h following infection, \( ^{35} \text{S} \)-labeled C. trachomatis organisms were harvested and purified as described above. Inhibition of C. trachomatis attachment was determined using confluent HeLa 229 cell monolayers (2 \( \times \) 105 cells/well) grown in 24-well tissue culture plates (Corning, NY). EBs were suspended in Hanks' balanced salt solution (HBSS) (Life Technologies, Inc.) density gradients as described previously (25). Organisms were dispensed in duplicate onto the delimited areas of toxoplasmosis slides (Bellco Glass, Inc., Vineland, N.J.) in 5- \( \mu \)l aliquots, air-dried, and fixed with methanol. EBs were visualized by staining with C. trachomatis species-specific murine monoclonal antibody conjugated with fluorescein isothiocyanate (Syva Corporation, Palo Alto, CA) and enumerated by counting all EBs contained in 5 \( \mu \)l.

**Heparin Binding to Chlamydia**

Heparin Binding to C. trachomatis Organisms—\( ^{3} \text{H} \)Heparin (specific activity, 0.44–0.48 mCi/mg) was obtained commercially (DuPont NEN). Designated concentrations of \( ^{3} \text{H} \)heparin were added to triplicate ali- quots of EBs (250 EBs) in a total of 150 \( \mu \)l. Incubated 1 h at 4 °C, centrifuged, and washed three times with ice-cold phosphate-buffered saline. The EB pellet was suspended and transferred to a scintillation vial and the amount of \( ^{3} \text{H} \)heparin bound was determined by scintillation counting of triplicate assessments. The assay was repeated a minimum of three times.

**Heparin Binding Inhibition Assay—**C. trachomatis EBs used in attachment assays were prepared in monolayer cultures of HeLa 229 cells (5 \( \times \) 107 cells) in 150-mm tissue culture flasks. After inoculation with \( 1 \times 10^{9} \) inclusion forming units (IFU) of C. trachomatis and incubation at room temperature for 2 h, 10 ml of RPMI 1640 medium supplemented with 10% fetal calf serum, 0.1 mg/ml streptomycin, and 1 \( \mu \)g/ml cycloheximide were added. At 48 h following infection, 0.5 \( \mu \)Ci of \( ^{35} \text{S} \)-labeled amino acid mixture (Trans-35S-label, ICN Biomedicals, Inc., Costa Mesa, CA) was added to each flask. At 70°C the mixture was added to triplicate HeLa cell monolayers and incubated at 4 °C for 1 h. The sizes of the sulfation-modified heparin derivatives were not significantly different compared to heparin (data not shown) as estimated by agarose-polyacrylamide gel electrophoresis migration as described previously (27). The pool of monosaccharide units of heparin were prepared as described previously (27). These oligosaccharide preparations were a generous gift by Glycomed, Inc., Alameda, CA. Cell monolayers were washed three times with ice-cold HBSS to remove unattached organisms and then solubilized with 10% SDS, and cell-associated radioactivity was determined by scintillation counting. Results represent triplicate assessments from three experiments + the standard error of the mean (S.E.).

**Infecitivity Inhibition Assay—**C. trachomatis infectivity was determined using confluent HeLa 229 cell monolayers prepared on 12-mm diameter sterile glass coverslips (Fisher) placed in wells of 24-well tissue culture plates (Corning). Cells were plated and grown for 24 h prior to infection by chlamydiae. C. trachomatis EBs (1–5 \( \times \) 105 IFU/ml) were added to triplicated HeLa cell monolayers with 100 \( \mu \)g/ml heparin, each of a panel of heparin disaccharides, 2–12 oligosaccharide units of heparin or modified species of heparin (CDNSNs and NDSNACs) and 0.2 ml of the mixture was added to duplicate monolayers and incubated at 4 °C for 1 h. One hour after inoculation, monolayers were washed three times with ice-cold HBSS and incubated in warm RPMI 1640 tissue culture medium supplemented with 10% fetal calf serum, 0.1 mg/ml streptomycin, 1 \( \mu \)g/ml cycloheximide and 10 \( \mu \)g/ml heparitinase for 48–72 h at 37 °C. Cell monolayers were fixed with 100% methanol for 5 min, and chlamydial inclusions were detected by staining with C. trachomatis species-specific murine monoclonal antibody 2C5. Inclusions were counted in 30 fields at \( 400 \times \) magnification, and the mean number of inclusions was calculated from the duplicate coverslips. If the number of inclusions was less than 30, the mean number of inclusions for each coverslip was determined. Results represent duplicate assessments from two experiments + S.E. The panel of heparin disaccharides (Sigma) tested included the following: I-A (\( \alpha \)-UA-GlcA), II-A (\( \alpha \)-UA-GlcN), III-A (\( \alpha \)-UA-GlcN), IV-A (\( \alpha \)-UA-GlC), V-A (\( \alpha \)-UA-GlC), I-B (\( \alpha \)-UA-GlC), II-B (\( \alpha \)-UA-GlC), III-B (\( \alpha \)-UA-GlC), IV-B (\( \alpha \)-UA-GlC), V-B (\( \alpha \)-UA-GlC).
ing \( ^3 \)H heparin was binding a specific surface acceptor molecule saturable within the concentration range used (Fig. 1), suggesting \( ^3 \)H heparin was binding a specific surface acceptor molecule saturable within the concentration range used (Fig. 1), suggesting \( ^3 \)H heparin was binding a specific surface acceptor molecule saturable within the concentration range used (Fig. 1), suggesting \( ^3 \)H heparin was binding a specific surface acceptor molecule saturable within the concentration range used (Fig. 1), suggesting \( ^3 \)H heparin was binding a specific surface acceptor molecule saturable within the concentration range used (Fig. 1), suggesting \( ^3 \)H heparin was binding a specific surface acceptor molecule saturable within the concentration range used (Fig. 1), suggesting \( ^3 \)H heparin was binding a specific surface acceptor molecule saturable within the concentration range used (Fig. 1), suggesting \( ^3 \)H heparin was binding a specific surface acceptor molecule saturable within the concentration range used (Fig. 1), suggesting \( ^3 \)H heparin was binding a specific surface acceptor molecule saturable within the concentration range used (Fig. 1), suggesting \( ^3 \)H heparin was binding a specific surface acceptor molecule saturable within the concentration range used (Fig. 1), suggesting \( ^3 \)H heparin was binding a specific surface acceptor molecule saturable within the concentration range used (Fig. 1), suggesting \( ^3 \)H heparin was binding a specific surface acceptor molecule saturable within the concentration range used (Fig. 1), suggesting \( ^3 \)H heparin was binding a specific surface acceptor molecule saturable within the concentration range used (Fig. 1), suggesting \( ^3 \)H heparin was binding a specific surface acceptor molecule saturable within the concentration range used (Fig. 1), suggesting \( ^3 \)H heparin was binding a specific surface acceptor molecule saturable within the concentration range used (Fig. 1), suggesting \( ^3 \)H heparin was binding a specific surface acceptor molecule saturable within the concentration range used (Fig. 1), suggesting \( ^3 \)H heparin was binding a specific surface acceptor molecule saturable within the concentration range used (Fig. 1), suggesting \( ^3 \)H heparin was binding a specific surface acceptor molecule saturable within the concentration range used. To determine if the enzyme preparation nonspecifically affected EB viability, aliquots of heparitinase and EB mixtures were incubated on ice for 70 min prior to infection. The measurement of infectivity or attachment following heparitinase digestion was determined as described above.

Attachment Restoration Assay—To assess the restoration of attachment of heparitinase-digested EBs, \( ^3 \)S-labeled EBs were digested with heparitinase at 30°C for 1 h and chilled on ice for 10 min. Twenty-five \( \mu \)g/ml of heparin, 2–12 monosaccharide units of heparin or modified derivatives of heparin (CDSNS or NDSNAc) were added to replicate aliquots of heparitinase-treated organisms and incubated at 4°C for 1 h. After incubation, the EBs were washed twice with ice-cold HBBS prior to inoculation of HeLa 229 cell monolayers. Infected monolayers were washed three times with ice-cold HBBS after 1 h, and attachment was determined as described above.

Infectivity Restoration Assay—To assess the restoration of infectivity of heparitinase-digested C. trachomatis, 1–5 \( \times 10^5 \) IFU/ml EBs were digested with heparitinase at 30°C for 1 h and chilled on ice for 10 min. Heparin, 2–12 monosaccharide units of heparin, sulfation-modified heparin derivatives (CDSNS or NDSNAc), or dextran sulfate were added separately (25 \( \mu \)g/ml) to aliquots of heparitinase-treated organisms and incubated at 4°C for 1 h. After incubation, the EBs were washed twice with ice-cold HBBS prior to inoculation of HeLa 229 cell monolayers. Infected monolayers were washed three times with ice-cold HBBS after 1-h infection, and then infectivity was determined as described above.

RESULTS

\( ^3 \)H Heparin Binding to Chlamydia—It has been shown that treatment of LGV or trachoma biovar EBs with heparitinase abolishes >90% of their infectivity (12); however, the effect of heparin treatment on EB attachment to host cells differs for the two biovars. LGV biovar attachment is inhibited by >90%, whereas trachoma biovar attachment is inhibited by 56% (18). As heparitinase-digested EBs treated with exogenous heparin can be rescued for both attachment and infectivity, these data suggest that heparin binds to EBs (12, 13). A direct binding assay was used to assess the amount of \( ^3 \)H heparin bound to C. trachomatis LGV and trachoma biovariants. \( ^3 \)H heparin was allowed to bind EBs and organism-bound heparin was determined by scintillation counting. For both LGV and trachoma biovariants, \( ^3 \)H heparin binding to chlamydial organisms was saturable within the concentration range used (Fig. 1), suggesting \( ^3 \)H heparin was binding a specific surface acceptor molecule. Using approximately the same number of chlamydial organisms, trachoma biovar (serovar B) EBs bound more heparin to their surface than LGV biovar (serovar L2) EBs at most \( ^3 \)H heparin concentrations.

To examine the contributions made by sulfation of heparin to bind EBs, two modified species of heparin, CDSNS and ND-SNAc, were tested for their ability to compete with \( ^3 \)H heparin for binding to C. trachomatis EBs. Unlike unlabeled heparin, the modified species of heparin did not competitively inhibit \( ^3 \)H heparin binding to C. trachomatis organisms (data not shown). The failure of these modified heparin derivatives to competitively inhibit the binding of \( ^3 \)H heparin to chlamydial organisms suggests the binding affinity of the sulfation-modified heparin derivatives to C. trachomatis was low.

Effect of the Length of Heparin Saccharide Chain on Inhibition of C. trachomatis Attachment and Infection—A panel of 11 chemically defined heparin disaccharides (see list under “Experimental Procedures”) was initially tested for inhibition of chlamydial infectivity but none of the disaccharides caused significant inhibition (data not shown); although the most highly sulfated disaccharide (I-S) had a small effect (~16% inhibition). To determine what chain length of heparin inhibited chlamydial attachment and infectivity, size-fractionated even-numbered heparin oligosaccharides, which ranged from 2 to 12 monosaccharide units (27), were used as competitors of chlamydial attachment and infection of host cells. Fig. 2 shows the results obtained using size-fractionated heparin oligosaccharides to compete for binding to host cells and inhibition of attachment.
chlamydial infection. At similar concentrations, the smaller the oligosaccharide chain length the less efficiently the oligosaccharide inhibited C. trachomatis attachment and infectivity, despite increased molar concentration. For the LGV biovar the minimum chain length that reduced attachment (Fig. 2A) and infectivity (Fig. 2B) was an octasaccharide, with the maximum inhibition achieved by the dodecasaccharide. For the trachoma biovar the minimum chain length that reduced attachment and infectivity was a decasaccharide (Fig. 2B). The limited inhibition of trachoma biovar attachment is consistent with previous studies that heparin-dependent attachment only accounts for about 56% of trachoma biovar binding to host cells (18). The level of reduction in attachment and infectivity with the dodecasaccharide was similar to the level with reduction with heparin. These assessments are presented using a weight-based, rather than molar, comparison because of the repetitive structure of heparin that following depolymerization results in the production of analogous numbers of binding equivalents. Thus, a dodecasaccharide was the minimum chain length to effectively compete for C. trachomatis attachment to the host cell GAG adhesin receptor.

Restoration of Chlamydial Attachment and Infectivity with Heparin Oligosaccharides of Heparitinase-digested EBs—Previously, it has been demonstrated that purified heparin, when added to heparitinase-digested LGV or trachoma biovar organisms prior to cell infection, restores the ability of heparitinase-digested organisms to attach to and infect host cells (12, 13). In contrast to heparin-mediated restoration, heparin oligosaccharides were less efficient in the restoration of chlamydial attachment (Fig. 3A) and infectivity (Fig. 3B) for both the LGV and trachoma biovar strains. By treating heparitinase-digested EBs with the oligosaccharide preparations, chlamydial attachment was maximally restored to half of the original level for the trachoma biovar with a dodecasaccharide and 62% of the original level for the LGV biovar with a decasaccharide (Fig. 3A). For the restoration of chlamydial infectivity, 68% of original activity for LGV biovar and 52% of original activity for trachoma biovar were achieved by a dodecasaccharide (Fig. 3B). As for attachment to host cells, the shorter the oligosaccharide chain length the less effective was the restoration of chlamydial attachment and infectivity. These data suggest that heparin oligosaccharides can specifically bind to EBs and rescue chlamydial attachment and infectivity for eukaryotic host cells, but the length of oligosaccharide to fully restore both chlamydial attachment and infectivity is greater than a dodecasaccharide.

Effect of Sulfation-modified Derivatives of Heparin in Attachment and Infectivity Inhibition—in order to assess the role of sulfation and sulfation density of heparin binding to host cell GAG receptors, sulfation-modified species of heparin were used to inhibit chlamydial attachment or infection. CDSNS had no effect on chlamydial attachment or infection (Fig. 4). In contrast, the O-sulfated derivative, NDSNAc, significantly reduced LGV biovar attachment and infectivity, suggesting a host cell receptor requirement for a moderately sulfated (predominantly O-sulfated) GAG ligand. Although the NDSNAc derivative inhibited trachoma biovar attachment at a level similar to heparin inhibition (35% versus 42% reduction; Fig. 4A), only an 18% reduction in trachoma biovar infectivity was observed (Fig. 4B). However, increasing the NDSNAc concentrations from 500 to 750 µg/ml or higher resulted in 85–98% reduction in trachoma biovar infectivity (data not shown).

Restoration of Chlamydial Attachment and Infectivity with Sulfation-modified Derivatives of Heparin for Heparitinase-digested EB—Although it was shown that the sulfation-modified heparin derivative NDSNAc could competitively inhibit EB attachment and infectivity (Fig. 4), it was also shown that the NDSNAc derivative could not competitively inhibit heparin binding to EBs (data not shown). Together these data suggest that the NDSNAc derivative was competitive with the natural ligand for binding the eukaryotic cell surface receptor but it could not effectively bind the chlamydial surface receptor. To test this conclusion, and to further investigate the importance of the sulfation group and sulfate density of heparin on restoring heparitinase-digested organisms, modified species of heparin were used in restoration assays of chlamydial attachment or infectivity. For the restoration of chlamydial attachment and infectivity of heparitinase-digested EBs, the modified species of heparin restored the LGV biovar attachment from 15 to 30% of original control activity (Fig. 5). A smaller effect was observed for the trachoma biovar (Fig. 5). NDSNAc, which had similar efficacy as unmodified heparin in inhibiting attachment and infectivity of LGV biovar, only restored chlamydial attachment and infectivity to a limited degree, yet slightly better than CDSNS. The limited restoration of attachment and infectivity of chlamydiae by sulfation-modified derivatives of heparin is likely due to poor binding to EBs.
Ability of Dextran Sulfate to Restore Chlamydial Infectivity—Kuo and Grayston (18) demonstrated that dextran sulfate, like heparin or heparan sulfate, can competitively inhibit chlamydial infectivity. Dextran sulfate is a glucose polymer that is highly 0-sulfated and structurally analogous to heparin and heparan sulfate (24). To evaluate the specificity of the chlamydial GAG ligand-acceptor interaction, dextran sulfate was incubated with heparitinase-digested LGV biovar organisms in an infectivity restoration assay. Interestingly, while dextran sulfate was an effective inhibitor of both attachment and infectivity, dextran sulfate restored only 46% of the original infectivity (Fig. 6). This finding suggests that dextran sulfate, like the NDSNAc derivative, is not a potent functional analog of chlamydial adhesin ligand. The limited ability of dextran sulfate and NDSNAc heparin to rescue infectivity indicates the selective specificity of the chlamydial acceptor molecule for binding heparin, heparan sulfate, and the natural chlamydial ligand.

DISCUSSION

A trimolecular working model of chlamydial attachment has been proposed in which a Chlamydia-synthesized heparan sulfate-like ligand is bound by chlamydial acceptors and host cell receptors, thereby mediating chlamydial attachment and infectivity (13). This model was tested by investigating the role of analogs of the heparan sulfate-like ligand for binding chlamydial EBs and mediating interactions with mammalian host cells. Heparin and heparin derivatives were used in direct binding assays to chlamydial organisms and in indirect competition assays for organism binding to host cells to independently probe the structural and functional requirements for binding the chlamydial GAG acceptor and the host cell receptor, respectively. In the direct binding assays, for EBs representing both C. trachomatis biovars, binding of heparin was saturable suggesting that heparin binding to EBs is specific. Although the kinetics of heparin binding to EBs was similar for the two biovars, trachoma biovar EBs bound twice the amount of heparin than LGV biovar EBs. These estimates could be confounded by the amount and avidity of bound natural ligand, and by estimates of the number of EBs used in these experiments. Nevertheless, these data suggest a significant difference in the native surface architecture for these biovariants. This conclusion is consistent with ion-exchange chromatography of native EBs demonstrating that the LGV biovar has a higher negative surface charge than trachoma biovar EBs (17). If the difference in surface charge is attributable to the presence of the native heparan sulfate-like ligand, then the LGV biovar may have quantitatively more or more highly sulfated native ligand on its surface than the trachoma biovar.

In order to begin elucidation of the structural requirements of the heparan sulfate-like ligand as an adhesin, chemically
modified species of heparin and homogeneously sized oligosaccharides obtained from depolymerized heparin were tested. To examine the length requirements of the heparan sulfate-like ligand for mediation of cell adhesion, oligosaccharides of defined length were used to compete with chlamydial organisms for binding host cells. Inhibition of attachment and neutralization of chlamydial infectivity were measured to estimate the ability of competing oligosaccharides to bind to the host cell receptor. As the size of oligosaccharide decreased, the ability to inhibit chlamydial attachment or infection also decreased, with heparin fragments smaller than octasaccharide having no effect on inhibition of attachment or infectivity. These results suggest the heparin molecules longer than decasaccharides were able to compete for binding to host cell receptors with the native chlamydial adhesin. The ability of these molecules to rescue chlamydial attachment and infectivity that was abolished by pretreatment of EBs with heparitinase, revealed that the decasaccharide or dodecasaccharide heparin oligosaccharides fall short of fully rescuing chlamydial infectivity despite increased molar concentrations of oligosaccharides. The lack of full restoration may indicate 1) the relatively poor binding of heparin oligosaccharides to the EB surface, 2) longer oligosaccharides are required to optimally bridge and interact simultaneously with two different proteins, or 3) other structural differences between the natural C. trachomatis heparan-sulfate-like ligand and heparin oligosaccharides.

Structurally heparin and heparan sulfate molecules consist of repeated disaccharide units that are sulfated differently. Each of these molecules can be subdivided into domains based on a combination of sulfation density and saccharide units. In general, heparin contains a higher proportion of sulfation and a higher 6-iduronic acid/6-glucuronic acid ratio than heparan sulfate; however, heparan sulfate shares some highly sulfated regions that are similar to heparin (28). It is possible that the different GAG-sulfation requirements observed for the host receptor and chlamydial acceptor derive from the polymeric nature of the adhesin ligand to which they bind. The adhesin bridge model (13) predicts that both the host cell receptor and chlamydial acceptor bind the GAG adhesin, but the receptor and acceptor may contact different domains of the adhesin molecule that could be substantially different with respect to sulfation location and density. In the experiments designed to investigate whether the sulfation of the chlamydial adhesin affects chlamydial infectivity, differentially sulfated forms of heparin were used in inhibition of attachment or infectivity neutralization assays. Although the N-sulfated heparin derivative (CDNSN) was unable to inhibit chlamydial attachment or infection, the O-sulfated heparin derivative (NDSNAc) showed nearly the same strong inhibition ability as native heparin for chlamydial neutralization. The limited restoration of chlamydial attachment and infection observed by incubating the sulfation-modified derivatives of heparin with heparitinase-digested EBs was due to the poor binding of sulfation-modified heparin derivatives to chlamydial organisms as these derivatives were weak competitors of heparin binding to EBs. These results indicate that the chlamydial adhesin acceptor has a sulfation requirement that differs from that required by the host cell receptor.

The effect of the O-sulfation or N-sulfation of the heparin molecule on the ability of heparin to bind to host cells or chlamydial organisms cannot be determined unequivocally because heparin molecules carrying the same high sulfate content with either O- or N-sulfate groups are not available. Nevertheless, the NDSNAc heparin derivative and dextran sulfate (both O-sulfated) inhibited chlamydial attachment and infectivity to the host cell but did not effectively rescue chlamydial infectivity. These data implicate a requirement for both O- and N-sulfation of GAG for binding the chlamydial acceptor and only a requirement for O-sulfation to effectively interact with the host cell receptor. It has been shown previously that chondroitin sulfate does not strongly inhibit chlamydial infection (12, 13). Chondroitin sulfate is moderately sulfated and contains only O-sulfated saccharide residues (23) suggesting that other structural features defined by the carbohydrate backbone contribute to the specificity of binding. Thus, while a relatively high sulfation density of GAG appears essential, it is evident that the position of sulfate groups and the composition and length of the carbohydrate backbone also play crucial roles in the interaction of heparan-sulfate-like ligand to its chlamydial acceptor and eukaryotic cell receptor.

The findings that the chlamydial acceptor and eukaryotic cell receptor have different requirements for binding heparin derivatives have significant implications for the design and use of sulfated compounds for prophylactic and therapeutic applications. While the use of a compound like heparan sulfate might be considered as an antimicrobial antagonist for a variety of pathogens that bind heparan sulfate proteoglycans, this may be contraindicated for chlamydiae as such compounds can enhance attachment and rescue infectivity over a broad range of concentrations. However, the NDSNAc derivative of heparin did not bind chlamydiae, could not rescue attachment or infectivity, yet was nearly as potent as heparin in its ability to competitively inhibit chlamydial interactions with eukaryotic cells. Thus understanding the structural requirements of sulfated compounds in relation to their functional interactions with both the eukaryotic host cell and microbial pathogen promises to permit the design of infectivity antagonists with targeted specificity and defined activity.
Heparin Binding to Chlamydia

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