

Adherence of Pilus⁻ Opa⁺ Gonococci to Epithelial Cells In Vitro Involves Heparan Sulfate

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Summary

Neisseria gonorrhoeae attaches to host epithelial cells via pili and opacity-associated (Opa) outer membrane proteins. Pilus⁻ gonococci (Gc) of strain MS11 adhere to both human and nonhuman cells, but only when particular Opa proteins are expressed; OpaA⁺ variants adhere best, OpaC⁺ variants are next best, and the seven other Opa⁺ variants adhere poorly or not at all. The adherence of OpaA⁺ Gc to Chinese hamster ovary (CHO) cells is inhibited by heparin or heparan sulfate (HS), but not by chondroitin sulfate. OpaA⁺ Gc do not adhere to CHO cells devoid of HS proteoglycans; low concentrations of heparin restore OpaA⁺ Gc adherence to these HS-deficient CHO cells and high concentrations inhibit it. ³H-heparin binding to whole Gc parallels their adherence abilities (OpaA⁺ > OpaC⁺ > OpaH⁺ >> OpaB, D, E, F, G, I = Opa⁻ = 0). Opa proteins separated by SDS-PAGE also bind ³H-heparin. These data suggest that adherence of pilus⁻, Opa⁺ Gc involves HS-proteoglycan of eukaryotic cells.

Many pathogenetic details of *Neisseria gonorrhoeae* infections are incompletely defined, including this bacterium's interactions with epithelial cells of its human hosts. Light microscopy studies describe gonococci (Gc)¹ adherent to, within, or between mucosal epithelial cells and clustered in submucosal sites of tissues from individuals with gonorrhea (1). Electron microscopy studies show Gc adhering to and partially embedded in host epithelial cells of urethral exudates (2, 3). Similar events occur in tissue culture cells and Fallopian tubal organ cultures (FTOC) exposed to Gc in vitro (4, 5). Gc attach to tissue culture cells and eventually abound in phagolysosomes (6–8); in FTOC, Gc attach to nonciliated cells, are engulfed into phagosomes, transit the host cells in the membrane-limited compartments, and are exocytosed on the cells' basal sides (9–13). Some reports even suggest that Gc attain true intracellular status, i.e., not intraphagosomal, but lying free in the cytoplasm (14). Gc that gain access to intracellular or submucosal sites have possible but unproven relevance to initiation or maintenance of local gonorrhoeal infections and to the dissemination of Gc.

Pili are the only known requisites for virulence of Gc in human males (15, 16), probably because these organelles mediate Gc attachment to human cells and thereby enhance mucosal colonization (17, 18). Although they attach well, pilus⁺ Gc are not taken up readily by human epithelial

cells in vitro (7). It is mainly pilus⁻ Gc expressing certain opacity-associated (Opa) outer membrane proteins that achieve intracellular location in epithelial cells of either human or nonhuman origin (7, 19) (Chen, T., R. J. Belland, and J. Swanson, unpublished observation). *Escherichia coli* that express recombinant Opa proteins may adhere to tissue culture cells (20) or FTOC cells (21) and may or may not be engulfed, depending on the tissue culture cell line, the *E. coli* strain, and the Opa protein expressed (22).

The studies of others indicate that Opa proteins influence Gc adherence to human cells, and our preliminary observations indicated that pilus⁻ Opa⁺ Gc also adhered to nonhuman cell lines, including Chinese hamster ovary (CHO), canine renal (MDCK), and monkey kidney (COS-7) cells. Opa⁺ Gc attach less avidly when grown on agar-containing medium "contaminated" with sulfated polysaccharides or when exogenous DNA is added (23). These observations suggest that adherence of pilus⁻ Opa⁺ Gc involves highly negatively charged surface molecules common to both human and nonhuman epithelial cells; a likely candidate is heparan sulfate (HS)-proteoglycan, which is used by several molecules and microbes in cell binding. This report explores the role of HS in Opa phenotype-dependent adherence of Gc to epithelial cells.

Materials and Methods

Reagents. Heparin, HS, chondroitin sulfate (CS), hyaluronic acid (HA), collagen types VI and VIII, and emetine were purchased

¹ Abbreviations used in this paper: CHO, Chinese hamster ovary; CS, chondroitin sulfate; FTOC, fallopian tube organ cultures; Gc, gonococci; HA, hyaluronic acid; HS, heparan sulfate; Opa, opacity-associated.

from Sigma Chemical Co. (St. Louis, MO). All tissue culture materials were obtained from GIBCO BRL (Gaithersburg, MD).

Bacterial Strains. Gc strain MS11_{mk} was cultured and maintained as previously described (23, 24). Only pilus⁻ Gc were used, and their characteristic opacities on agar-containing medium were useful for maintenance of their respective Opa phenotypes, which were confirmed periodically by SDS-PAGE. All Gc had identical LOS phenotype (LOS_b) defined with two mAbs (anti-LOS_a, anti-LOS_b) (25). Opa protein designations are those used previously (26), and their deduced sequences are known (20, 27). *Yersinia pseudotuberculosis* was obtained from Joe Hinnebusch (Rocky Mountain Laboratories, National Institutes of Health, Hamilton, MT).

CHO Cells. Wild-type CHO-K1 and isogenic mutants that have specific deficiencies in proteoglycan biosynthesis (28, 29) were kindly provided by Jeffrey D. Esko (Department of Biochemistry, University of Alabama in Birmingham) (30–33). The relevant properties of these mutant CHO cell strains 745, 618, 677, and 608, as summarized in reference 34, are as follows: 745 (*pgsA*, xylosyltransferase defect; expresses no HS nor CS); 618 (*pgsB*, galactosyltransferase deficit; expresses no HS nor CS); 677 (*pgsD*, defective *N*-acetylglucosaminyl and glucuronosyltransferases; produces no HS but 2–3× increased CS); and 606 (*N*-sulfotransferase deficiency, HS and CS, but reduced sulfation of HS). The human endometrial adenocarcinoma cell line HEC-1B was obtained from American Type Culture Collection (Rockville, MD; ATCC HTB 113).

Adherence Assays. CHO cells were cultured in RPMI 1640 medium (GIBCO BRL) with 10% FCS (Hyclone Laboratories, Inc., Logan, UT). For adherence assays, cells were grown to confluence (~2 × 10⁵ cells per well) in 24-well culture plates (Linbro; Flow Laboratories, Hamden, CT), and washed twice with RPMI. Gc grown on agarose-containing plates to minimize clumping of Opa⁺ variants (23) were suspended in PBS to OD₅₄₀ = 0.5; this was then diluted 1:10 with RPMI containing 5% FCS and 0.5 ml of these Gc suspensions was added to each well. The plates were incubated at 37°C with 5% CO₂ for 3 h. Experiments were terminated by washing four times, each with 1 ml serum-free RPMI for 2 min on an orbital shaker at 110 rpm. Adherent bacteria were enumerated by suspending the cells in PBS containing 0.5% saponin (Calbiochem Corp., San Diego, CA), plating dilutions on Gc-clear typing medium, and determining the CFUs associated with the host cell monolayer. The assays were performed in duplicate or triplicate. For adherence inhibition and restoration assays by soluble polysaccharides and glycosaminoglycans, the CHO cell monolayers were first treated with 2 μg/ml emetine for 30 min to inhibit new protein synthesis, and then bacteria were added as a suspension in RPMI containing 5% FCS, 2 μg/ml emetine, and desired concentrations of polysaccharide or glycosaminoglycan. Internalization assays were done by incubating OpaA⁺ Gc with cells in the presence of gentamicin at a concentration of 100 μg/ml for 1.5 h before washing, disruption, plating, and counting of CFUs the next day.

³H-Heparin Labeling. Gc grown on agarose-containing solid medium were suspended in PBS to OD₅₄₀ = 0.5 and diluted 1:5 with serum-free RPMI. 0.6 ml of the bacterial suspension was transferred to a 1.5-ml microfuge tube. 200 μl ³H-heparin (4 μCi/ml) (DuPont NEN, Boston, MA) was added to the bacterial suspensions, which were incubated at 37°C for 30 min. The bacteria were pelleted, washed once with RPMI, and mixed with fluor. The amount of ³H-heparin bound to Gc was determined in a liquid scintillation counter (model LS 6000LL; Beckman Instruments, Inc., Fullerton, CA).

³H-Heparin was also reacted with nitrocellulose filters (model HA; Millipore Corp., Bedford, MA) to which SDS-PAGE-sepa-

rated whole Gc lysates had been transferred, as described before (24). This filter was incubated with 1 ml 0.5% BSA in TSGAN buffer (8.77 g sodium chloride, 1.9 g EDTA 6.8 g Tris HCl, 0.85 g Tris base, 2.5 g gelatin, and 0.5 ml NP-40/liter) for 1 h, and then ³H-heparin (100 μl of 1 mCi/ml) was added to this solution and incubation was continued overnight. The blot was washed twice for 10 min with TSGAN buffer, and exposed to Hyperfilm ³H (RPN 535; Amersham Corp., Arlington Heights, IL). A second filter to which identical SDS-PAGE-separated Gc lysates had been transferred was probed with mAb 4B12, which recognizes all Opa proteins, and then with HRP-conjugated antibody and enhanced chemiluminescence detection reagents as prescribed by the supplier (Amersham Corp.).

Results

Opa⁺ Gc Adhere to and are Internalized by CHO-K1 Cells. Seven Opa⁺ (OpaA, B, C, D, F, H, and I) and Opa⁻ variants, all pilus⁻ and LOS_b phenotype, were incubated with CHO-K1 cells that synthesize highly sulfated HS proteoglycan and CS (30, 31). OpaA⁺ variants adhered best, followed by OpaC⁺ and OpaH⁺; OpaB⁺, D⁺, F⁺, and I⁺ Gc showed negligible adherence, as did Opa⁻ Gc (Fig. 1). Although the Opa⁺ Gc adhered to CHO-K1 cells and to human cell lines (Chang, Hec-1B, ME180, HeLa229, and Henle) at roughly comparable levels (data not shown), their apparent internalization by CHO-K1 cells was lower (19). Recovery of gentamicin-resistant OpaA⁺ Gc from CHO-K1 cells increased when lysosome acidification inhibitors (ammonium chloride and chloroquine) were present (Fig. 2).

OpaA⁺ Gc Do Not Adhere To HS-deficient CHO Cells. Adherences of Opa⁺ Gc to mutant CHO cells with defective HS-proteoglycan biosynthesis were also assessed (Fig. 3). OpaA⁺ Gc adhered at significantly reduced levels to CHO mutants 745, 618, and 677 that lack HS and to strain CHO 606 cells with undersulfated HS. Other Opa⁺ variants did not adhere to CHO mutants 745, 618, and 677 at measurable levels (data not shown). Both K1 and mutant CHO cells supported adherence of *Y. pseudotuberculosis*, which binds β1 integrin (Fig. 3) (35).

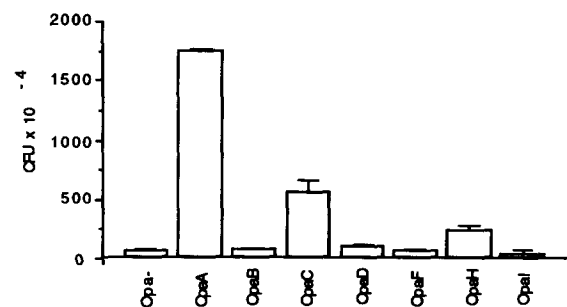


Figure 1. Opa⁻ Gc and Opa⁺ variants expressing individual Opa proteins (A, B, etc.) were incubated with CHO-K1 cell monolayers. OpaA⁺ Gc adhere best; OpaC⁺ and OpaH⁺ variants adhere at lower levels that exceed the nearly negligible levels of other Opa⁺ and Opa⁻ Gc.

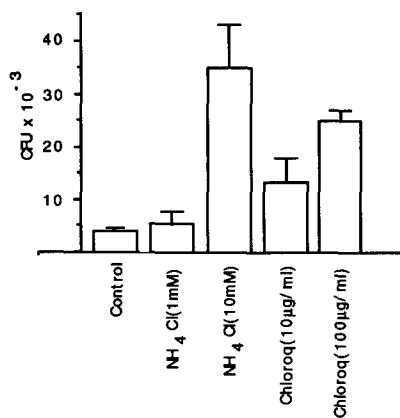


Figure 2. Increased numbers of “intracellular” OpaA⁺ Gc were recovered from CHO-K1 cells that had been treated with ammonium chloride (NH₄Cl) or chloroquine (Chloroq), incubated with gentamicin, and disrupted.

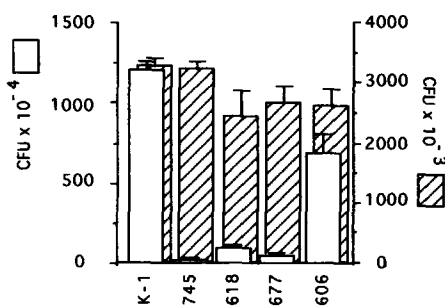


Figure 3. OpaA⁺ Gc (open bars) and *Y. pseudotuberculosis* (cross-hatched bars) were incubated with CHO-K1 cells that express HS proteoglycan and with CHO cell variants 745, 618, 677, and 606 that are defective in HS proteoglycan synthesis. OpaA⁺ Gc adhere best to CHO-K1 cells, less to variant 606 with undersulfated HS, and much less to variants 745, 618, and 677 that are devoid of HS. *Y. pseudotuberculosis* adheres well to all of the CHO cell variants.

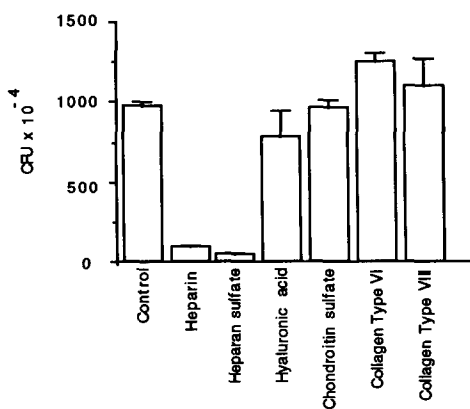


Figure 4. Heparin and HS virtually eliminate adherence of OpaA⁺ Gc to CHO-K1 cells; three other polysaccharides or glycosaminoglycans (HA, CS, and collagens) had no effect when added to the same final concentrations (50 µg/ml).

Exogenous Heparin Affects CHO Cell Adherence of OpaA⁺ Gc. Adherence of OpaA⁺ Gc to CHO-K1 cells was inhibited by exogenous heparin and HS, but not by HA, CS, or collagen (Fig. 4); inhibition by heparin was dose dependent (Fig. 5 A). OpaA⁺ Gc attachment to HS-deficient CHO mutant 745 was enhanced by heparin at low concentrations (0.8–6.4 µg/ml) and was progressively inhibited at higher concentrations (>6.4 µg/ml) (Fig. 5 B). CS neither restored nor inhibited adherence (data not shown). Adherent OpaA⁺ Gc were eluted from CHO-K1 cells by heparin, but not by other GAGs (Fig. 6).

³H-Heparin Binds to Opa⁺ Gc and to Denatured Opa Proteins. ³H-heparin bound best to whole OpaA⁺ cells, less to OpaC⁺ cells, and poorly or not at all to the other Opa⁺ or Opa⁻ Gc (Fig. 7). ³H-heparin binding was inhibited by nonradioactive heparin, but not by CS, HA, or collagen (Fig. 8). *Escherichia coli* that expressed the corresponding recombinant Gc opa genes displayed corresponding relative ³H-heparin binding (data not shown). Radiographically visible

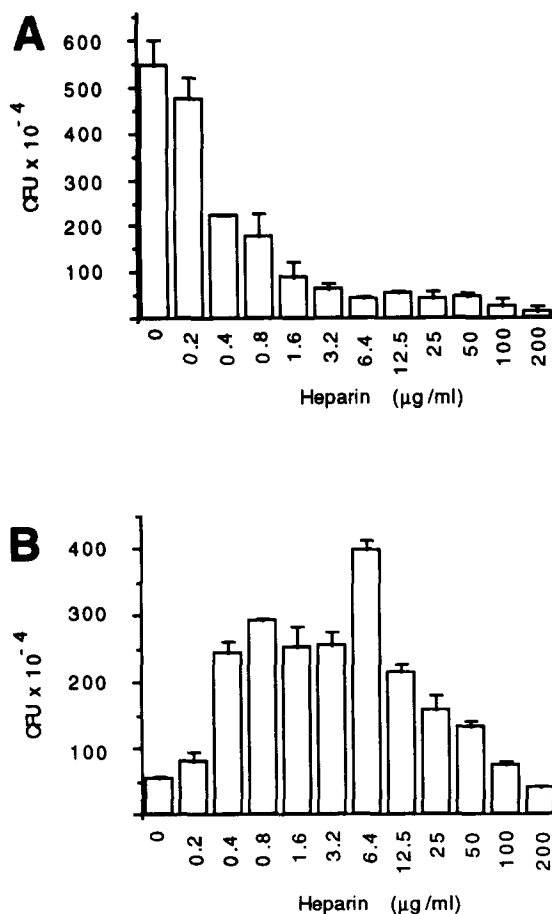


Figure 5. (A) Increasing amounts of heparin markedly adherence of OpaA⁺ Gc to CHO-K1 cells. (B) Addition of heparin to HS-defective CHO-745 cells enhances adherence of OpaA⁺ Gc up to concentrations of 6.4 µg/ml; higher heparin concentrations progressively diminish OpaA⁺ Gc adherence to CHO-745 cells.

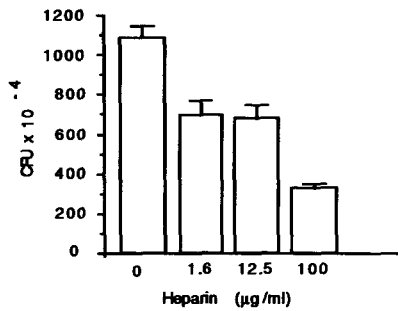


Figure 6. After allowing OpaA⁺ Gc to attach to CHO-K1 cells, increasing concentrations of heparin progressively reduced the number of organisms that remain attached to the monolayer.

amounts of ³H-heparin bound to all Opa proteins and to several other (i.e., non-Opa), unidentified Gc proteins after SDS-PAGE separation of whole Gc lysates; binding was better to Opa A and C proteins than to Opa B, D, F, H, or I proteins, which bound least in this format (Fig. 9).

Discussion

Both pili and Opa proteins influence Gc attachment to epithelial cells, but in different ways. Pilus⁺ Gc, regardless of Opa phenotype, adhere exclusively to human epithelial cells, with different pilin (Pile) and PilC constitutions conferring distinctive patterns and degrees of adherence (36). Receptors for Gc pili are undefined. Unless they express certain Opa proteins, pilus⁻ Gc adhere to neither human nor nonhuman epithelial cells.

Pilus⁻ OpaA⁺ Gc attach to both human and nonhuman cells at high levels in vitro, sometimes exceeding pilus⁺ Gc in adherence to human cells. Such Opa protein-mediated attachment appears to use HS-proteoglycans of the eukaryotic cells. In using heparin/HS to gain intimate association with epithelial cells, Opa⁺ Gc resemble several other microorganisms that possess HS-binding polypeptides: glycoproteins B and C of HSV type 1 (37, 38); fimbrial hemagglutinin of *Bordetella pertussis* (39); and two high molecular weight

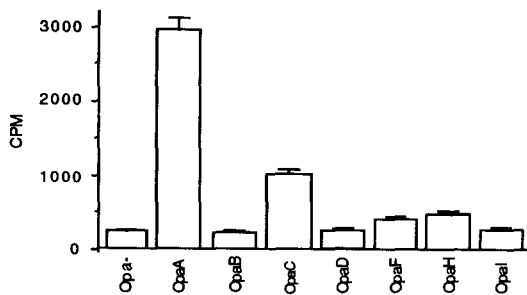


Figure 7. Identical amounts of ³H-heparin were incubated with equivalent numbers of Gc that express no Opa (Opa⁻) or one Opa (OpaA⁺, OpaB⁺, etc.) protein. ³H-heparin bound to these variants as follows: OpaA⁺ >> OpaC⁺ > OpaH⁺ > OpaF⁺ > OpaB⁺, D⁺, I⁺ = Opa⁻.

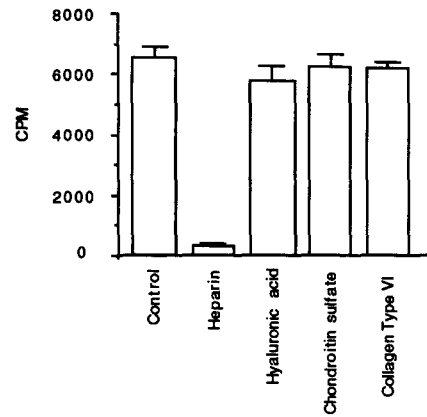


Figure 8. Binding of ³H-heparin to whole OpaA⁺ Gc was assessed in the presence of 50 µg/ml each heparin, HA, CS, and collagen type VI. Only heparin interfered with ³H-heparin binding.

proteins of *Haemophilus influenzae* (40). HS-proteoglycan binding is also an essential step in the cellular uptake and biological activity of several growth factors, hormones, and other soluble polypeptides (41).

The interactions of microbes or proteins with heparin/HS can depend on levels and patterns of sulfation as well as the size of the glycosaminoglycans. Heparin denotes molecules with relatively high sulfation levels, whereas HS denotes less-sulfated molecules (42). Most proteins bind heparin more avidly than HS, apparently because of the higher charge density of the former (43, 44). Molecules collectively designated as heparin or HS differ in size, sulfation levels, and patterns of sulfation. Mammalian cells can differ in the composition, sulfation level, and expression of their GAGs. *H. influenzae* and bFGF appear to “discriminate” among subtly differing species of HS/heparin molecules (28, 29, 40). Whether Gc

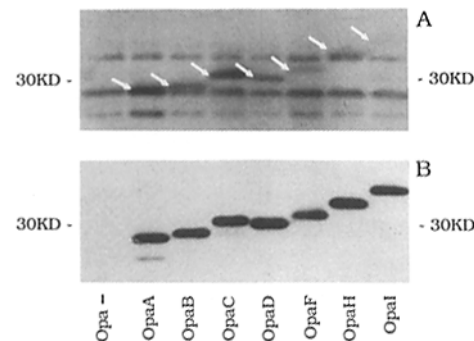


Figure 9. ³H-Heparin was incubated with nitrocellulose to which SDS-PAGE separated whole Gc lysates had been transferred. (A) Several Gc components bind ³H-heparin including Opa proteins (arrows) and unidentified, common moieties of 32, 28.5, and 26.5 kD. Of the Opa proteins, OpaA and OpaC bind most ³H-heparin, Opa B, D, and F bind less, and OpaI binds little or none; OpaH binding is hard to assess because of radiolabel binding to a common 32-kD moiety. (B) Opa phenotypes of these organisms were verified by immunoblotting.

distinguish among HS molecules of differing size and sulfation patterns/levels is unknown.

HS/heparin-protein interactions depend on electrostatic interactions between basic, cationic amino acids (arginine, lysine, and possibly histidine) of the protein and the highly acidic, anionic sulfate groups of HS/heparin. Heparin-binding proteins typically possess abundant basic amino acids, sometimes scattered throughout the protein, but often clustered in particular regions (45–48). Particular topographic arrangements of the basic amino acids may constitute heparin binding domains (44), but heparin-binding consensus motifs may be difficult to define because of the overall abundance of positively charged residues (49) and because heparin-binding domains may be constituted by distant portions of a folded polypeptide (50). Secondary structure predictions suggest that two large “hypervariable” (HV1, HV2) and two small regions of Opa proteins are surface exposed (51). Both HV1 and HV2

of all Opa proteins in strain MS11, except OpaE HV1, contain net excesses of basic residues; HV1 of OpaA contains the largest surfeit of basic residues (27).

The functional and pathobiological relevance of HS-mediated adherence of pathogenic microbes to host cells is regularly implied, infrequently proven, and can be more complex than it seems initially. Even when their heparin-binding regions are defined with monoclonal antibodies, site-specific mutants, and oligopeptides, polypeptides and microbes may have another “specific” eukaryotic cell-binding sites. For example, heparin-inhibitible adsorption of HSV clearly involves two viral components (gB and gC) (37, 38); but productive uptake of this virus depends on interaction of yet another viral glycoprotein (gD) with a non-HS host cell receptor (52, 53). Whether Gc have an additional non-Opa molecule that promotes uptake by host cells is unknown.

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