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+ >> Opas B, 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.

Materials and Methods

**Reagents.** Heparin, HS, chondroitin sulfate (CS), hyaluronic acid (HA), collagen types VI and VIII, and emetine were purchased...
from Sigma Chemical Co. (St. Louis, MO). All tissue culture materials were obtained from GIBCO BRL (Gaithersburg, MD).

Bacterial Strains. Gc strain M511ab 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 (LOSb) defined with two mAbs (anti-LOS, anti-LOSb) (25). Opa protein designations are those used previously (26), and their deduced sequences are known (20, 27).

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 LOSb phenotype, were incubated with CHO-K1 cells that synthesize highly sulfated HS proteoglycan and CS (30, 31). Opa+ 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-I-B, 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.

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 ops genes displayed corresponding relative 3H-heparin binding (data not shown). Radiographically visible

Figure 3. OpaA+ Gc (open bar) and Y. pseudotuberculosis (cross-hatched bar) 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).

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.
amounts of \(^3\text{H}\)-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. Pili\(^+\) 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.

Pili\(^-\) 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 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
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 surfact 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-inhibitable 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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