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Natural Proteoglycan Receptor Analogs Determine the Dynamics of Opa Adhesin-Mediated Gonococcal Infection of Chang Epithelial Cells

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Many bacterial pathogens possess a complex machinery for the induction and/or secretion of factors that promote their uptake by mammalian cells. We searched for the molecular basis of the 60- to 90-min lag time in the interaction of *Neisseria gonorrhoeae* carrying the heparin-binding Opa adhesin with Chang epithelial cells. Infection assays in the presence of chloramphenicol demonstrated that the Opa-mediated gonococcal infection of Chang cells required bacterial protein synthesis when the microorganisms were derived from GC agar but not when grown in liquid media. Further analysis indicated that contact with agar ingredients rather than the growth state of the microorganisms determined the infection dynamics. DEAE chromatography of GC agar extracts and sodium dodecyl sulfate-polyacrylamide gel electrophoresis analyses and testing of collected fractions in infection assays identified negatively charged high-molecular-weight polysaccharides in the agar as inhibitors of the cellular infection. Electron microscopy showed that agar-grown gonococci were surrounded by a coat of alcian blue-positive material, probably representing accreted polysaccharides. Similar antiphagocytic material was isolated from bovine serum, indicating that in biological fluids gonococci producing the heparin-binding Opa adhesin may become covered with externally derived polysaccharides as well. Binding assays with gonococci and epithelial proteoglycan receptors revealed that polysaccharides derived from agar or serum compete with the proteoglycans for binding of the heparin-binding Opa adhesin and thus act as receptor analogs. Growth of gonococci in a polysaccharide-free environment resulted in optimal proteoglycan receptor binding and rapid bacterial entry into Chang cells. The recognition that gonococci with certain phenotypes can recruit surface polysaccharides that determine in vitro infection dynamics adds a different dimension to the well-recognized biological significance of genetic variation for this pathogen.

Bacterial attachment to and penetration of the human mucosa are thought to play a pivotal role in the establishment of a variety of mucosal infections. One bacterial pathogen that colonizes the human mucosa is *Neisseria gonorrhoeae*, the causative agent of gonorrhea. Histological examination of tissues from cervical biopsies and purulent exudates from acute stages of gonococcal infection generally shows large numbers of gonococci that are often deeply embedded in the epithelial layer and that are variably located inside both epithelial cells and leukocytes (1, 15, 44). In the past decade, considerable progress to unravel the molecular events that underlie the anchoring to and penetration of the mucosal tissue by gonococci has been made (for reviews, see references 27, 29, and 41). Key gonococcal adhesins that confer binding to mammalian cells include pili and the pilus tip-located PilC protein (31) and certain members of the opacity (Opa) outer membrane protein family (3, 26, 33, 45). This family may consist of up to 11 isoforms that are variably produced in a single gonococcal strain depending on the number of pentameric nucleotide repeats in the corresponding genes (28, 34). The variant Opa proteins are structurally relatively conserved but contain variable surface-exposed regions, leading to antigenic variation (2, 9) and the recognition of different types of host receptor molecules (4, 7, 24, 41). Prime receptors recognized by distinct Opa proteins include epithelial heparan sulfate proteoglycans (8, 40) and various members of the CD66 (CEA [carcinoembryonic antigen]) receptor family that are present on leukocytes and certain epithelia (4–6, 43). Thus, Opa protein variation may contribute to the tissue tropism displayed by gonococci.

The molecular mechanisms behind the internalization of Opa-producing gonococci by mammalian cells are much less defined. It has been demonstrated that both proteoglycan and CD66 receptor-mediated adherence are often followed by uptake of the microorganisms in the eukaryotic cells. For gonococci producing the Opa with specificity for proteoglycan receptors (designated heparin-binding Opa), this event involves a phagocytosis-like process with a local transient recruitment of F-actin at the site of bacterial entry (19). Production of the corresponding Opa in *Escherichia coli*, however, does not appear to induce a rearrangement of the host cell actin cytoskeleton, and these recombinants are unable to enter the epithelial cells (19), suggesting that additional bacterial factors may be needed for efficient gonococcal entry. In support of this, screening of a mutant library of gonococcal strain MS11 for bacterial entry-deficient clones yielded several mutants. At this time, the relationship between the genetic defects in these mutants (which produced the heparin-binding Opa protein) and their inability to enter epithelial cells, however, remains elusive (23).

A typical characteristic of the Opa/proteoglycan-mediated entry of gonococci into Chang epithelial cells is that bacterial adherence and uptake take several hours (45), which is a rather long time in comparison to the very short ingestion period reported for several other gram-negative pathogens. *Yersinia* spp. producing the invasin protein, for example, are rapidly

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(within minutes) taken up into the cells through a β1 integrin-dependent mechanism (46). *Salmonella* spp. are also rapidly ingested by host cells (16), but this occurs only when the bacteria are in an entry-competent state, which is defined as a narrow window during the bacterial growth phase during which the microbes are capable of efficiently entering mammalian cells (14, 25, 32). Once in a competent state, no bacterial protein synthesis is required for *Salmonella* entry into cultured cells (18, 25). Identification of an entry-competent state for *Salmonella* spp. has greatly facilitated studies on the molecular mechanisms of host cell entry allowing synchronization of bacterial uptake, which is important in defining the cell signalling events that drive the entry process.

In the present study, we sought to define the molecular basis for the delay in the Opa/proteoglycan-mediated infection of Chang epithelial cells. Initial experiments suggested that bacterial protein synthesis and a specific bacterial growth state were required for efficient infection of Chang cells. A more thorough investigation, however, provided evidence that the interaction of gonococci with Chang cells actually is a very rapid process but that agar- or serum-derived polysaccharides block the interaction of the heparin-binding Opa adhesin with cell surface proteoglycan receptors by acting as receptor analogs. Growth of gonococci in a polysaccharide-free environment is required to produce unoccupied Opa adhesin and to reach the entry-competent state.

MATERIALS AND METHODS

Organisms and culture conditions. The characteristics of *N. gonorrhoeae* MS11 and VP1 and of *E. coli* DH5α carrying the *inv* gene of *Yersinia pseudotuberculosis* (*E. coli*^{inv}) have been described previously (19, 26, 38). All gonococcal variants used were nonpiliated, carried lacto-*N*-neotetraose on their lipopolysaccharide (LPS), and produced heparin-binding Opa proteins (MS11-OpaA and VP1-Opa27.5) (40), unless indicated otherwise. *E. coli*^{inv} was generously provided by T. F. Meyer (MPI für Biologie, Tübingen, Germany). All strains were routinely grown on conventional GC agar plates (composition per liter, 3.75 g of Trypticase peptone [BBL, Becton-Dickinson, Cockeysville, Md.], 7.5 g of Thiotone E [BBL], 4 g of KH_2PO_4, 1 g of KH_2PO_4, 5 g of NaCl, 1 g of soluble starch [BBL], 1% of Bacto Agar [Difco, Detroit, Mich.], and 1% IsoVitaleX [BBL; pH 7.4]) at 37°C in a 5% CO_2 atmosphere. When appropriate, gonococci were grown in a liquid medium (1 to 8 h, 37°C, 125 rpm on a gyratory shaker) in 50-ml Erlenmeyer flasks containing 10 ml of (i) GC broth (composition same as that of GC agar plates but without agar), (ii) HEPES-RPMI (RPMI 1640 [Gibco-BRL, Gaithersburg, Md.] containing 20 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid [HEPES; pH 7.4]), or (iii) HEPES medium (10 mM HEPES, 140 mM NaCl, 2.5 mM KCl, 5 mM glucose, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 1.5% proteose peptone no. 3 [Difco; pH 7.4]). Supplementation of these media with additional vitamins (1% IsoVitaleX) did not influence growth of strains VP1 and MS11 and had no effect on the bacterium-host cell kinetics. In some experiments, gonococci were grown on sterile dialysis tubing (Thomas, Philadelphia, Pa.; molecular weight cutoff, 12,000) layered onto GC agar plates to prevent direct contact of the bacteria with the agar. This procedure had no measurable effect on bacterial growth.

Cell culture and infection experiments. Chang human conjunctiva epithelial cells (ATCC CCL20.2) were maintained in 5 ml of RPMI 1640 tissue culture medium supplemented with 5% fetal bovine serum (FBS; Gibco-BRL) in 25-cm^2 plastic tissue culture flasks (Corning Glass Works, Corning, N.Y.). For infection experiments, cells were seeded onto 12-mm-diameter circular glass coverslips in a 24-well tissue culture plate (1 ml of medium per well) and grown to near confluence in 48 h. Infection experiments were routinely carried out in tissue culture medium (without FBS) or in HEPES buffer (HEPES medium without proteose peptone no. 3 but with 5 mM phosphate added), unless indicated otherwise. Chloramphenicol, FBS, or DEAE-purified polysaccharides were added as indicated. Bacteria swabbed from plates or collected from broth cultures by centrifugation (2,000 × *g*, 6 min, 24°C) were suspended in HEPES buffer and added to the cells at a bacterium/host cell ratio of 10:1 (6-h infections in RPMI 1640–5% FBS) or 100:1 (2-h infections in HEPES buffer), unless indicated otherwise. At various times, infection was stopped by rinsing the cells three times with HEPES buffer followed by fixation in 0.1% glutaradehyde–1% paraformaldehyde in phosphate-buffered saline (PBS; pH 7.4) for at least 30 min at 24°C. Bacterial adherence and entry were scored as described previously (39, 40), and values are given as the mean number of adherent and intracellular bacteria per epithelial cell.

Radioactivity assays. The effect of chloramphenicol on bacterial protein synthesis was estimated from the incorporation of [^{35}S]methionine in the absence and presence of the antibiotic (10 to 100 μg/ml). A total of 5×10^7 gonococci maintained in a 24-well plate in 0.4 ml of methionine-free RPMI 1640 supplemented with 1% FBS and 5 μCi of [^{35}S]methionine (Amersham, Arlington Heights, Ill.) were incubated at 37°C in a 5% CO_2 environment. Wells containing a 1,000-fold excess of unlabelled methionine served as a control. At regular intervals, bacteria were transferred to 1-ml tubes and unbound label was removed by centrifugation (12,000 × *g*, 3 min). The pellets were washed twice with PBS, and incorporated label was quantitated with a Beckman liquid scintillation counter (model 6000L). When bacterial protein synthesis was monitored during infection of Chang cells, label was added 30 min before the end of the infection assay. Incorporation of label into eukaryotic proteins was inhibited by the addition of 100 μg of cycloheximide (Sigma, St. Louis, Mo.) per ml. Infected cells were collected, and lysates were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and autoradiography (40). Measurement of eukaryotic protein synthesis was performed as described previously (19). It was determined that chloramphenicol concentrations between 30 and 100 μg/ml blocked bacterial protein synthesis by more than 90% without affecting host cell protein synthesis. The labelling and isolation of Chang cell surface proteoglycan receptor fragments and the binding of this material to gonococci (receptor assay) have been described (40).

DEAE chromatography of agar plate washes and FBS. Infection-inhibitory activity in agar plates was extracted by incubating two GC agar plates or pure agar (Bacto Agar) plates with 2 ml of 50 mM Tris-HCl (pH 8.0; Tris buffer) for 15 min at 37°C. Extracts were loaded onto 1.5 ml of DEAE-Sephacel (Pharmacia, Uppsala, Sweden) packed in a 1- by 9-cm column preequilibrated with 50 mM Tris (pH 8.0). After 10 1-ml washes with Tris buffer, bound material was eluted 10 times with 1 ml of 50 mM Na acetate buffer (pH 4.5) and the same buffer containing 0.2, 0.4, and 1 M NaCl, respectively. One-milliliter fractions were collected, either ethanol precipitated (80% ethanol, 16 h, −20°C) or desalted and concentrated (about 10 times) by Centricon C10 ultrafiltration (Amicon Inc., Beverly, Mass.) as described in the manufacturer's instructions, and stored in HEPES buffer at −20°C. All fractions (final dilutions, 1:10 to 1:100) were tested in the infection assay and proteoglycan receptor binding assay, and active fractions were analyzed by SDS-PAGE. Basically, the same procedure was followed for the isolation of the inhibitory factor(s) in FBS. One milliliter of DEAE-Sephacel effectively removed all infection-inhibitory activity from 15 ml of FBS. DEAE-purified material was analyzed by 4 to 15% Tris-borate gradient gels (40). Gels were fixed in 50% ethanol, and polysaccharides were visualized with azure A (0.008% in 10% ethanol, 10 min; Sigma); proteins were stained with Coomassie brilliant blue G250 (11).

Biotinylation and digestion of serum proteins. Biotinylation of DEAE-purified serum proteins was achieved by incubating approximately 50 μg of protein in 50 μl of HEPES buffer containing 8 μg of normal human serum-LC-biotin (Pierce) for 30 min at room temperature. Seven hundred fifty microliters of Tris buffer was then added, and residual free biotin was removed by ultrafiltration (Centricon C10 filter; Amicon). Binding of biotinylated proteins to gonococci was assayed by incubating 5×10^7 HEPES medium-grown bacteria with approximately 5 μg of proteins (15 min, 24°C) and then removing unbound proteins and washing the gonococci three times with 1 ml of HEPES buffer. Bacteria were collected by centrifugation (12,000 × *g*, 3 min, 24°C), lysed, and stored at −20°C. Bound biotinylated proteins were detected by SDS-PAGE and Western blotting of whole-cell lysates (45). Biotin was detected in a chemiluminescence assay (Pierce) in combination with horseradish peroxidase (HRP)-streptavidin (Amersham; 1/1,500). Biotinylation did not affect the infection-inhibitory activity (data not shown). DEAE-purified serum extract (250 μl) was also digested with proteinase K (100 μg, 16 h, 37°C; Boehringer Mannheim) to evaluate the contribution of serum proteins to the effect of serum on the infection. Digested material was removed by Centricon C30 ultrafiltration.

Transmission electron microscopy. For detection of bound polysaccharides, gonococci propagated on GC agar plates were fixed in situ by flooding the plates with 3 ml of alcian blue solution (0.5% alcian blue 8GX and 3% glutaraldehyde in 0.1 M cacodylate buffer [pH 7.2]). After 15 min, colonies were gently loosened with a Pasteur pipette, transferred to microcentrifuge tubes, pelleted (12,000 × *g*, 3 min), washed twice with 0.1 M cacodylate buffer (pH 7.2), and placed in this buffer at 4°C. Similarly, HEPES broth-grown gonococci were collected by centrifugation (2,000 × *g*, 6 min), fixed in alcian blue solution (15 min, 24°C), and washed and stored in 0.1 M cacodylate buffer. Samples then were washed twice for 15 min in 0.1 M sodium phosphate buffer, fixed in 0.5% osmium tetroxide, rinsed with tap water (three times, 15 min), and poststained (16 h, 4°C) in 1% aqueous uranyl acetate (pH 3.9). After dehydration in a graded series of ethanol, samples were embedded in Spurr's resin, placed in BEEM capsules, and polymerized (70%C, 16 h). Silver-gold sections were cut, poststained with 1% $KMnO_4$ in water (20 min), and rinsed with 0.25% sodium citrate (30 s) and water. Specimens were viewed in a Hitachi HU-11E-1 electron microscope.

RESULTS

Requirement of bacterial protein synthesis for gonococcal entry into Chang epithelial cells. Infection of Chang epithelial cells maintained in RPMI 1640–5% FBS with GC agar plate-grown gonococci (strain VP1) expressing the entry-promoting Opa27.5 resulted in a slow but steady uptake of bacteria over

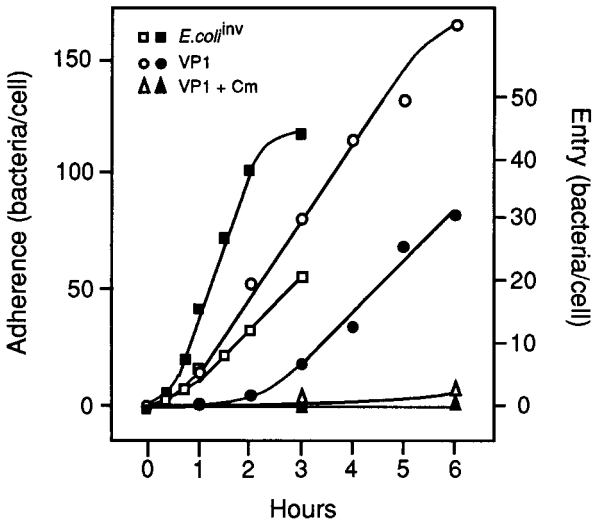


FIG. 1. Time course of the adherence (open symbols) and entry (closed symbols) into Chang epithelial cells in the absence and presence of 30 μg of chloramphenicol (Cm) per ml of *N. gonorrhoeae* VP1-Opa27.5 and of *E. coli*^{inv} propagated on GC agar base. Note the difference in the rate of internalization (closed symbols) between *E. coli*^{inv} and the gonococcus strain. Experiments were performed in RPMI 1640–5% FBS. Values are from one of six experiments, all of which yielded similar results.

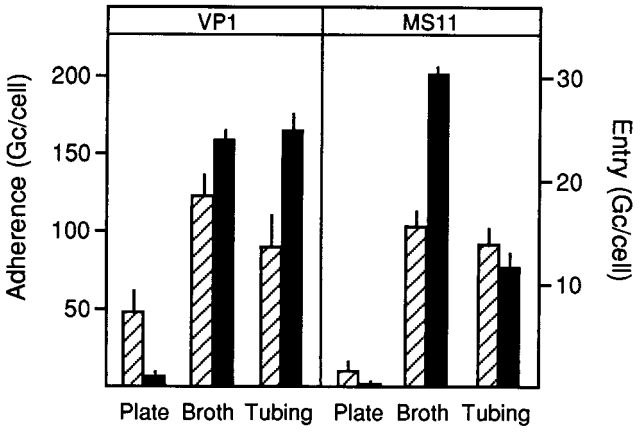


FIG. 2. Effect of bacterial growth conditions on the adherence to (hatched bars) and entry into (closed bars) Chang cells of gonococcus strains VP1-Opa27.5 and MS11-OpaA. Gonococci (Gc) were cultured either on GC agar plates for 16 h (Plate), in GC broth for 2 h (Broth), or on dialysis tubing layered onto GC agar medium for 16 h (Tubing) prior to infection of Chang cells. Infection was in RPMI 1640–5% FBS for 2 h. Values are the means ± standard errors of the means of at least six experiments.

the 6-h infection period after an initial 60- to 90-min lag time (Fig. 1). To seek the cause for this delayed interaction, which was not observed for *E. coli* producing the invasin of *Y. pseudotuberculosis* (Fig. 1), we investigated the requirement of bacterial protein synthesis for gonococcal entry by use of the inhibitor chloramphenicol (30 μg/ml). This drug strongly reduced gonococcal adherence and totally blocked bacterial entry into Chang cells over the 6-h infection period (Fig. 1). An increase in the inoculum size to compensate for the multiplication of bacteria that occurred in the absence of the antibiotic gave similar values (data not shown). To ascertain that the gonococci maintained their intrinsic ability to infect the cells during chloramphenicol treatment, we recovered bacteria from supernatants of 3-h-infected cells by centrifugation and used them as an inoculum in a second infection experiment without antibiotic. This resulted in levels of adherent and intracellular gonococci similar to those obtained for untreated bacteria (data not shown). Together, these results strongly suggested that bacterial protein synthesis was required to establish an adhesion- and entry-competent gonococcal phenotype.

Effect of bacterial growth conditions on the gonococcus-host cell interaction. Several strategies were employed to define which proteins the gonococci produced during the infection period that enabled interaction with the epithelial cells. Extensive SDS-PAGE analyses, including autoradiography of [^{35}S]methionine-labelled gonococci collected at times during the infection process, revealed no reproducible differences in protein profiles when these gonococci were compared with bacteria in the inoculum (data not shown). Attempts to reduce the lag phase for gonococcal entry by increasing the size of the inoculum and thus perhaps the number of entry-competent bacteria in the population unexpectedly resulted in a dramatic reduction in gonococcal adherence and entry during the 6-h infection period (7 versus 38 intracellular gonococci per cell at a multiplicity of infection of 500 and 10, respectively). We interpreted this finding as possibly resulting from the presence at high cell densities of increased concentrations of inhibitory factors such as blebs or DNA, which has been shown to interfere with gonococcal adherence (36).

A much more efficient gonococcus-host cell interaction was obtained when we grew the microorganisms in liquid media to preadapt them to the conditions of the infection assay (Fig. 2). This effect was observed after growth for at least 2 h in GC broth, in HEPES-RPMI, and in rather nutrient-poor media such as phosphate- or HEPES-buffered salt solution enriched with proteose peptone and did not occur when chloramphenicol was included in the medium. Growth of the gonococci on agar plates composed of HEPES-RPMI, however, did not promote bacterial entry, suggesting that either the growth state of the gonococci or the agar itself influenced the interaction. To discriminate between these possibilities, we grew the bacteria on dialysis tubing layered on a GC agar plate, which prevents diffusion of medium-derived molecules and avoids direct contact of the organism with the agar. This resulted in efficient gonococcal adherence and entry into Chang cells (Fig. 2). Thus, contact with a high-molecular-weight compound in the agar plates rather than a growth phase element in gonococci determined the infection kinetics.

Purification of an inhibitory factor from GC agar plates. The inhibitory factor was isolated from agar plates by extraction with HEPES buffer followed by anion-exchange chromatography and testing of the eluted fractions for their inhibitory activity in the infection assay. The inhibitory material bound to DEAE-Sephacel and eluted with buffer containing 0.5 M NaCl. The effect of crude GC agar extracts and the purified material on the adherence and entry into Chang cells of gonococcus strain VP1-Opa27.5 is shown in Fig. 3A. Similar results were obtained with strain MS11-OpaA (data not shown). Further experiments in which either the gonococci or the epithelial cells were preincubated with the active fraction prior to the infection assay showed that the inhibition was mediated through an effect on the bacteria (data not shown).

Analysis of the active fraction by SDS-PAGE revealed no visible protein bands. However, staining of the gel with the cationic dye azure A demonstrated large amounts of polysaccharides ranging in molecular weight from about 90,000 to 200,000 (Fig. 4, lanes 1 and 4). The possibility that gonococci expressing heparin-binding Opa proteins bound plate-derived polysaccharides was further supported by electron microscopy on GC agar plate- and GC broth-derived VP1-Opa27.5 stained

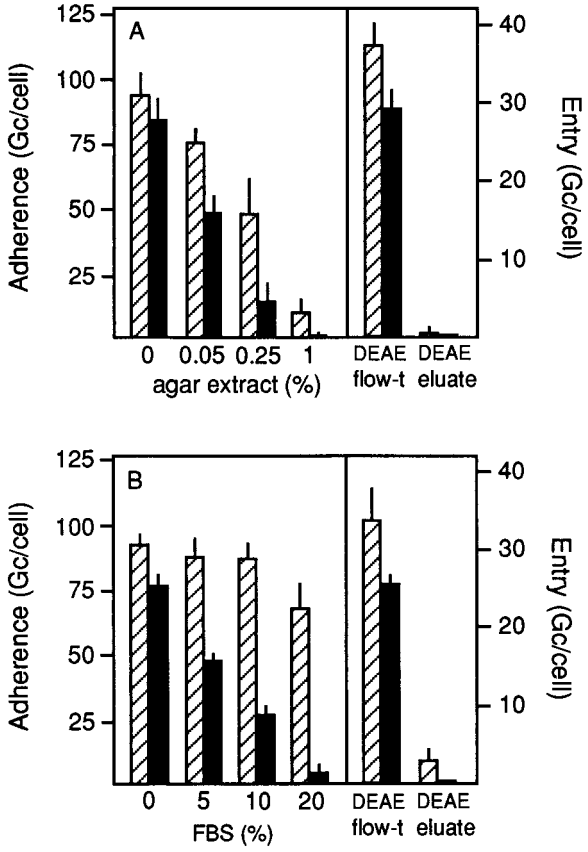


FIG. 3. (A) Infection experiment demonstrating the inhibitory effect of crude GC agar extract and DEAE-fractionated extract on the adherence to (hatched bars) and entry into (closed bars) Chang cells of gonococcus strain MS11-OpaA. Gonococci (Gc) were grown in HEPES medium for 2 h prior to infection. Infection (2 h, 37°C) was carried out in HEPES buffer. Crude GC agar extract was added at final concentrations of 0.05, 0.25, and 1%. DEAE-purified inhibitor (0.2 M DEAE eluate) and the noninhibitory flowthrough of the DEAE column (DEAE flow-t) were present at a final concentration of 1%. Values are the means ± standard errors of the means of at least four experiments. (B) Infection experiment demonstrating the inhibitory effect of FBS and DEAE-fractionated FBS on the adherence to (hatched bars) and entry into (closed bars) Chang cells of gonococcus strain MS11-OpaA. Infection conditions were identical to those described for panel A. FBS was present during the infection assay at final concentrations of 5, 10, and 20%; the DEAE flowthrough (DEAE flow-t) fraction was present at a concentration of 25%, and the inhibitory DEAE eluate (DEAE eluate) was present at 1%. Similar results were obtained for gonococcus strain VP1. Values are the means ± standard errors of the means of at least four experiments.

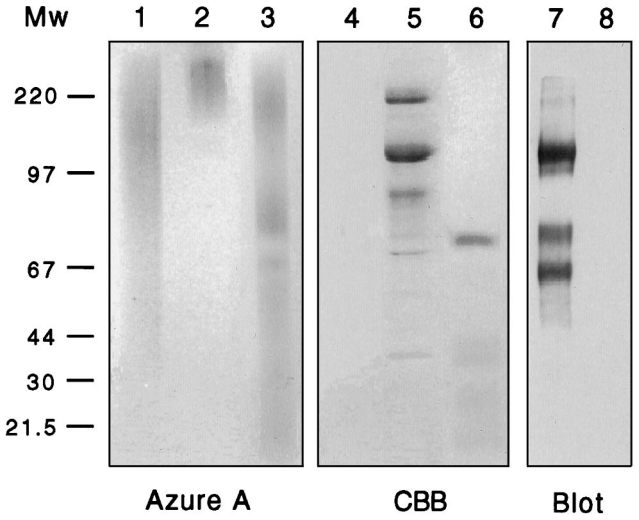


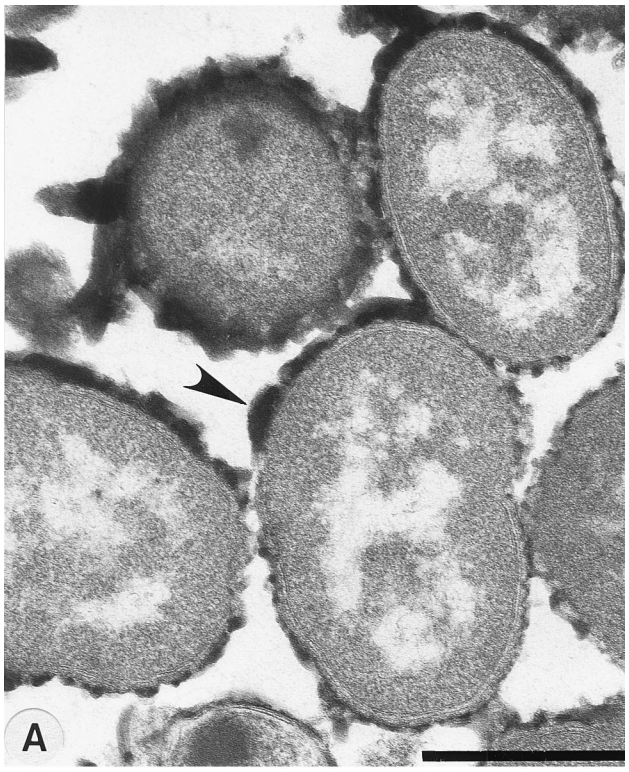
FIG. 4. Composition of the infection-inhibitory fractions obtained by DEAE purification from GC agar extract (lanes 1 and 4) and from FBS before and after digestion with proteinase K (lanes 2, 3, 5, and 6) as analyzed by 4 to 15% Tris-borate gradient gel electrophoresis and staining with azure A (lanes 1 to 3) or Coomassie brilliant blue G250 (lanes 4 to 6; CBB). Biotinylated proteins from the active FBS fraction were analyzed by Western blotting (Blot) with streptavidin-HRP (lane 7). Lane 8 represents a Western blot of whole-cell lysates of gonococcus strain MS11 after 15 min of incubation with the biotinylated serum fraction and staining with streptavidin-HRP. One-microliter aliquots were loaded onto all lanes of the gel, except for lane 8, which 20 μl was loaded. Mw, molecular size in kilodaltons.

with the polyanionic stain alcian blue. Gonococci propagated on agar-containing media were covered with a thick layer of alcian blue-positive material, probably representing accreted polysaccharides (Fig. 5A). This layer was absent from broth-cultured microorganisms (Fig. 5B). Further analysis of the various components of the agar plates revealed that the inhibitory polysaccharides were contained in the agar and probably represent agaropectin (13).

FBS contains a natural inhibitor of gonococcal entry into Chang cells. Upon evaluation of the infection assay conditions, we noticed that the kinetics of gonococcal entry into Chang cells were also affected by the presence of FBS. FBS caused a dose-dependent decrease in the number of cell-associated gonococci, with nearly complete inhibition of gonococcal entry occurring at 20% FBS (Fig. 3B). Gonococcal adherence was much less affected but was further reduced at higher serum concentrations, with 90% inhibition at 50% FBS. A further search for the inhibitor in the serum demonstrated that it was not the sialyl donor CMP-NeuNAc, which has been demonstrated to inhibit the gonococcal entry process by enabling sialylation of LPS (38). This was inferred from the observations that entry of a LPS variant of strain VP1-Opa27.5 lacking the acceptor site for sialic acid (38) and that of a heparin-binding Opa-producing F-62 mutant lacking sialyltransferase activity (17) were still blocked by FBS (data not shown).

Fractionation of serum by anion-exchange chromatography and analyses of the eluates by SDS-PAGE and infection assays similar to the approach followed above related the inhibitory activity to a polysaccharide-rich fraction containing several serum proteins (Fig. 4, lanes 2 and 5). Again, the inhibition of bacterial adherence and entry could be established by preincubation of the gonococci but not the epithelial cells with the active fraction (data not shown). To further unravel the nature of the inhibitory activity, we first treated the active fraction with proteinase K. This resulted in almost complete digestion of the isolated serum proteins and in a more heterogeneous migration of azure A-stained polysaccharides (Fig. 4, lanes 3 and 6). However, it did not prevent effective inhibition of gonococcal entry into Chang cells (data not shown), suggesting that negatively charged polysaccharides rather than serum proteins were the prime infection-inhibitory factor in FBS. To further substantiate this conclusion, we biotinylated the proteins in the active serum fraction (Fig. 4, lane 7) and measured their binding to gonococci through SDS-PAGE analysis of whole-cell lysates and Western blotting with streptavidin-peroxidase. As shown in Fig. 4 (lane 8), no binding of biotinylated serum proteins was detected.

Kinetics of the gonococcus-host cell interaction. To ascertain that the identified inhibitory factors in agar and serum contributed to the previously observed lag time (Fig. 1 and 6) in the Opa/proteoglycan-mediated interaction of gonococci



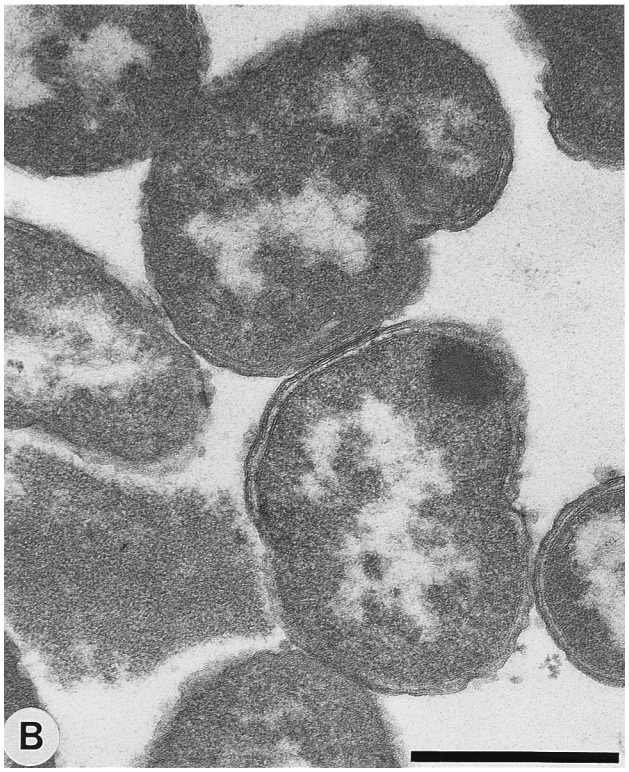


FIG. 5. Transmission electron micrographs of gonococci (VP1-Opa27.5) grown on GC agar base (A) and in HEPES medium (B). Colonies were stained and fixed in situ with the cationic dye stain alcian blue 8GX. Note the heavy electron-dense coat that surrounds plate-grown bacteria (arrowhead) but that is absent from broth-grown organisms. Bars, 0.5 μm.

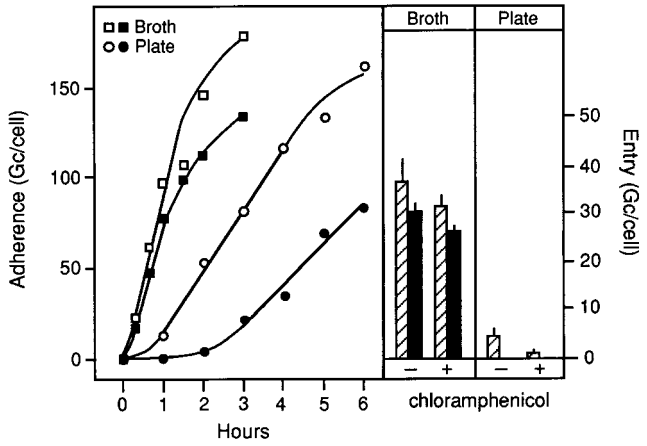


FIG. 6. Time course (left panel) and protein synthesis dependence (right panels) of the adherence (open symbols and hatched bars) and entry (closed symbols and closed bars) into Chang epithelial cells of gonococcus strain VP1-Opa27.5 grown in HEPES medium (Broth). All experiments were performed in HEPES buffer. For measurements of protein synthesis dependence, chloramphenicol was added at a final concentration of 30 μg/ml and infection was stopped at 1 h. The infection kinetics and protein synthesis dependence of microorganisms grown on GC agar base (Plate) are shown for comparison. Values graphed in the left panel are from one of six experiments; values graphed in the right panels are the means ± standard errors of the means of five experiments. Gc, gonococci.

and Chang cells, we repeated the time course studies with HEPES medium- instead of agar-grown organisms and with FBS omitted from the infection assay. This modification resulted in immediate attachment and rapid gonococcal entry into the host. The first intracellular bacteria were observed after 15 min, and at 1 h of infection, cells had taken up to 30 bacteria per cell (Fig. 6). These results were independent of the inoculum size in a range of bacterium/host cell ratios of 10 to 10,000 (data not shown). Moreover, experiments performed in the presence of chloramphenicol (30 μg/ml) showed nearly identical infection rates (Fig. 6), suggesting that the previously observed requirement of bacterial protein synthesis (Fig. 1) merely reflected a need for bacterial growth resulting in a dilution of externally derived inhibitory factors rather than the induction of distinct entry-promoting bacterial factors.

Mechanism behind the inhibition of gonococcal uptake. The identification of negatively charged polysaccharides as inhibitors of gonococcal infection of Chang cells combined with the capsule-like appearance around agar plate-grown microorganisms in the electron microscope suggested that this material may exert its effect by shielding critical gonococcal surface components. One key factor for entry into Chang cells is the Opa adhesin, which has specificity for glycosaminoglycan moieties of epithelial cell proteoglycan receptors (40). Considering this, we isolated the extracellular domain of $^{35}SO_4$-labelled heparan sulfate receptors from Chang cells (40) and evaluated its ability to bind to gonococci producing the heparin-binding Opa after growth on GC agar plates, on GC agar plates that had been covered with dialysis tubing, or in GC broth both in the absence and presence of DEAE-purified polysaccharides from agar and the proteinase K-treated active serum fraction. These experiments unequivocally demonstrated that the purified polysaccharides competed with the radiolabelled receptor for binding of the Opa protein (Fig. 7). These data strongly suggest that the molecular basis for the lag time in proteoglycan-mediated entry of gonococci into epithelial cells is a masking of the required heparin-binding Opa adhesin (and perhaps

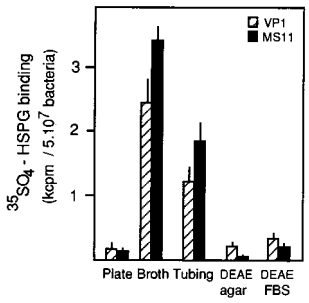


FIG. 7. Effect of bacterial growth conditions and infection-inhibitory polysaccharides derived from agar (DEAE agar) and FBS (DEAE FBS, proteinase K treated) on the binding of isolated $^{35}SO_4$-labelled proteoglycan receptor ($^{35}SO_4$-HSPG) by gonococcus strain VP1-Opa27.5. Gonococci were grown on GC agar plates for 16 h (Plate), in HEPES medium (Broth) for 2 h, or on dialysis tubing layered onto GC agar plates for 16 h (Tubing). Binding experiments were carried out in HEPES-buffer for 10 min on ice. DEAE-purified material was present at a final concentration of 1% (DEAE agar) and 5% (DEAE FBS) and was added to gonococcus strain VP1 grown in HEPES medium. Values are the means ± standard errors of the means of at least four experiments.

additional antigens), preventing effective interaction with the host cells.

DISCUSSION

In the present study, we resolved the molecular basis for the delayed adherence and entry of Opa-producing gonococci into Chang epithelial cells. Our data indicate that the observed lag time in cellular infection results from the acquisition by gonococci of polysaccharides that are present in agar as well as in serum. These externally derived polysaccharides compete with epithelial cell proteoglycan receptors for binding to the heparin-binding Opa adhesin; i.e., they act as receptor analogs. Bacterial growth for several generations in medium lacking these polysaccharides is required to produce unoccupied Opa proteins and to establish an entry-competent gonococcal phenotype. Gonococci grown in the absence of these inhibitory polysaccharides are immediately ingested by Chang epithelial cells even in the presence of chloramphenicol.

A key step in the identification of the inhibitory polysaccharides was that an entry-competent gonococcal phenotype could be established both by growing gonococci in liquid media and by propagation on conventional GC agar plates that had been covered with dialysis tubing. The latter indicated that contact with agar plate ingredients rather than the growth state of the organism was the critical parameter to reach the entry-competent state in contrast to the situation with *Salmonella* spp. (14, 25, 32). Further evidence that agar-derived polysaccharides interfered with the Opa/proteoglycan-mediated infection of Chang cells was provided by the dose-dependent inhibition of gonococcal adherence, entry, and proteoglycan receptor binding in the presence of DEAE-purified agar polysaccharides. Our findings also provide a basis for the previously observed reduced adherence to HEC-1B epithelial cells of gonococci (producing the heparin-binding Opa) propagated on GC agar-containing media compared with that of agarose-grown microorganisms (36). Based on measurements of the gonococcal surface charge, this effect was suggested to result from the accretion of polyanions from the medium (35, 37). Our data confirm this hypothesis. In agreement with these results, we were unable to isolate inhibitory polysaccharides from GC agarose plates (date not shown).

The rapid internalization of gonococci when grown in the absence of inhibitory polysaccharides and even with chloramphenicol present during the infection assay implies that de novo protein synthesis is not required for proteoglycan-mediated uptake of gonococci. This conclusion seems at variance with an earlier report that synthesis of host cell-induced bacterial factors may be required for gonococcal infection of epithelial cells (5). However, it should be emphasized that gonococci can exploit multiple strategies to enter mammalian cells (41) and that other Opa-independent uptake mechanisms may require induction of previously unexpressed proteins. With respect to Chang cells, the observed requirement for protein synthesis seems only to serve the need for bacterial growth, i.e., the production of unoccupied Opa proteins. At this point, our findings may be instrumental in the further classification of gonococcal mutants that are unable to enter Chang cells (23). On the basis of our results, it is likely that any mutation that reduces bacterial growth rate will impair the uptake of GC agar-grown gonococci by Chang cells. Propagation of the isolated mutants on media lacking inhibitory polysaccharides may be a valuable tool to determine whether the Opa adhesin is sufficient to mediate gonococcal entry into Chang cells or that additional bacterial factors are required.

The antiphagocytic effect of the DEAE-purified polysaccharides appears to be based on their physicochemical similarities to cell surface proteoglycans. The fact that agar-derived negatively charged polysaccharides compete with proteoglycan receptors for binding of the heparin-binding Opa suggests that structural homology and/or charge are important factors in Opa recognition. Opa proteins may carry highly positively charged surface-exposed loops (37), and these may confer binding to negatively charged molecules such as glycosaminoglycans and agaropectin. Functional mimicry of glycosaminoglycans and other negatively charged sulfated polysaccharides has also been reported for other biological systems, including the interactions of cytomegalovirus, *Chlamydia* spp., and *Plasmodium* spp. with mammalian cells (10, 30, 47). While the molecular basis for this mimicry remains to be defined, it raises the possibility that in a natural infection, gonococci carrying heparin-binding Opa proteins acquire similar polysaccharides from the environment. Our finding that serum-derived polysaccharides may compete with proteoglycans for Opa binding supports this concept and suggests that such polysaccharides are available in biological fluids. Gonococci isolated from natural infection sites have been reported to carry a polysaccharide surface coat, which has been interpreted as evidence for a gonococcal capsule (20, 21). In the light of current knowledge, this capsule may represent agar polysaccharides.

The acquisition by gonococci of polysaccharides from the environment adds a novel chapter to the gonococcal repertoire of surface variation that goes beyond the well-recognized genetic mechanisms of phase and antigen variation. Whether this feature has implications for gonococcal pathogenesis beyond those of the Opa-mediated binding to proteoglycan receptors has yet to be investigated. Gonococci supposedly do not produce a capsule, but a pseudocapsule consisting of accreted polysaccharides (Fig. 5) may have a similar function. Our data indicate that this capsule interferes with the binding of the heparin-binding Opa proteins to proteoglycan receptors, thereby blocking entry via this pathway into epithelial cells. Similar effects have been attributed to the inherently produced meningococcal capsule (12, 42). On the basis of this resemblance, it can be envisioned that the polysaccharides recruited by gonococci also have other capsule-related effects such as the shielding of other surface antigens and evasion of killing by antibodies and complement (22). Given these considerations, it is currently impossible to foresee whether the identified polysaccharides have potential prophylactic or therapeutic value as receptor analogs or may have detrimental effects on the course of infection.

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