INFECTION AND IMMUNITY, Dec. 1991, p. 4628–4635
0019-9567/91/124628-08$02.00/0

Vol. 59, No. 12

Interactions of *Neisseria gonorrhoeae* with Human Neutrophils: Studies with Purified PII (Opa) Outer Membrane Proteins and Synthetic Opa Peptides

FREDRICK L. NAIDS, BARBARA BELISLE, NICOLE LEE, AND RICHARD F. REST*

Department of Microbiology and Immunology, Hahnemann University School of Medicine, Broad and Vine Streets, Philadelphia, Pennsylvania 19102-1192

Received 10 June 1991/Accepted 23 September 1991

We investigated the role of gonococcal outer membrane protein PII (also called Opa protein) in nonopsonic adherence to human neutrophils. Gonococcal outer membranes, purified Opa in detergent (Opa), purified Opa in liposomes (Opa^+ lips), and peptides composing the second hypervariable (HV_2) region of OpaB (strain FA1090) in liposomes ($pepHV_2$ lips) were tested for their abilities to inhibit subsequent gonococcal adherence to human neutrophils. Outer membranes from gonococci possessing adherent Opa, liposomes containing adherent Opa, purified adherent Opa, and two of three liposome preparations ($pepHV_2$ lips) containing peptides from the HV_2 region of an adherent Opa inhibited subsequent adherence to neutrophils of homologous Opa^+ gonococci. On the other hand, outer membranes from Opa^- gonococci, outer membranes containing a nonadherent Opa (OpaA from strain FA1090), purified OpaA, and OpaA lips had little or no inhibitory effect. Outer membranes containing adherent Opas, purified adherent Opas, and liposomes containing such Opas all bound to neutrophils, whereas preparations containing OpaA or no Opa protein did not. The results indicate that (i) Opa proteins can bind to neutrophils in a partially purified or purified form and (ii) the HV_2 region of Opa appears to at least partially mediate Opa's biological role.

Adherence of *Neisseria gonorrhoeae* to host cells is facilitated by pili (30, 33, 46, 50) and possibly by a 36,000-Da adhesin molecule that binds to glycolipid receptors on mammalian epithelial cells (31). Certain carbohydrate structures may act as receptors for gonococci and other *Neisseria* species (29, 41). In addition, the presence of outer membrane protein PII, or Opa protein (Opa), on the gonococcal surface has been correlated with attachment of *N. gonorrhoeae* to many cell types, including urogenital cells, HeLa cells, conjunctival cells, and buccal epithelial cells (3, 13, 21, 30, 42). Opa appears to be the primary mediator of the interactions of gonococci with human neutrophils (10, 18, 19, 39, 44, 45, 49).

Opas undergo antigenic and phase variation at a frequency of 0.2×10^{-3} to 4.0×10^{-3} per cell per generation (27, 28, 40, 48, 53). This variation leads to the addition or deletion of one or more Opas upon daily clonal subculture, which in turns leads to the formation of colonies of various opacities and antigenicities. Swanson and colleagues originally described a specific Opa, termed leukocyte association protein, that was not associated with opacity (19, 44, 45). Apparently, only certain Opas, defined by their electrophoretic mobility in polyacrylamide gels and antibody reactivity, are associated with increased leukocyte or epithelial cell association (49, 51). Fischer and Rest described a gonococcal Opa variant (FA1090 OpaA), expressing a very opaque phenotype, that neither adheres to nor stimulates neutrophils (13). Thus, gonococcal colony opacity per se cannot be directly related to neutrophil adherence.

Investigation of the role of Opa proteins in gonococcus-neutrophil interactions has been hampered by the insolubility of purified Opa in the absence of detergents (11, 18); therefore, no studies have been performed with purified Opa. However, indirect techniques, such as (i) use of anti-Opa monoclonal antibodies to inhibit gonococcal adherence to human epithelial cells or neutrophils, or to inhibit stimulation by gonococci of human neutrophil oxidative metabolism (2, 10, 42, 49); (ii) use of carbohydrates and glycosidases to block gonococcal stimulation of neutrophils (36); and (iii) use of proteases to inhibit gonococcal adherence to neutrophils (9), have been used.

To better understand the molecular mechanisms of Opa-mediated gonococcal interactions with neutrophils, we investigated the abilities of gonococcal outer membranes, purified Opa in detergent, purified Opa inserted into liposomes (Opa^+ lips), and synthetic peptides (composing the second hypervariable [HV_2] region of OpaB from strain FA1090) associated with liposomes ($pepHV_2$ lips) to adhere to neutrophils. Outer membranes, Opa, Opa^+ lips, and $pepHV_2$ lips were also tested for their abilities to inhibit subsequent gonococcal adherence to neutrophils. On the basis of the results of these studies, we conclude that purified Opa can be used as a tool to investigate its mechanism of action and that the HV_2 region mediates at least some of the biological activity of Opa proteins.

MATERIALS AND METHODS

Bacteria. *N. gonorrhoeae* F62 and FA1090 were maintained by daily passage on GC agar base (Difco Laboratories, Detroit, Mich.) with added growth supplements, as previously described (34, 37). Colony type was identified by criteria established by Kellogg et al. (17) and Swanson (43). Only nonpiliated gonococci were used in these studies. The Opa content of the variants was checked regularly by electrophoresis through polyacrylamide gels (see below) and reactivity with anti-Opa antisera or monoclonal antibodies (MAbs) (10). Table 1 lists the Opa variants used in these studies and their abilities to adhere to neutrophils. For daily

* Corresponding author.

TABLE 1. Gonococcal variants used in these studies

Strain	Opa variant[a]	Adherence to neutrophils	Reference
FA1090	A	No	14
	B	Yes	14
	—	No	14
F62	4	Yes	11
	—	No	12

[a] —, no Opa detected by SDS-PAGE analysis or antibody reactivity.

use, gonococci were grown to mid-log phase in GC broth (GC base without agar) with added supplements at 37°C for 3 to 4 h in a shaking water bath, washed once in phosphate-buffered saline (PBS) containing 0.1% gelatin (PBSG; pH 7.4), resuspended to 2×10^8 to 4×10^8 CFU/ml, and held at room temperature (37).

SDS-PAGE. Samples for sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) were incubated in equal volumes of 2× SDS-prep buffer at 100°C for 5 min or 37°C for 60 min, as indicated in the text, and run on 12% gels by the method of Laemmli (20). All material for SDS-PAGE was purchased from Bio-Rad (Richmond, Va.). Gels were consecutively stained with silver by the method of Tsai and Frasch (47) and Coomassie brilliant blue by methods described in the Bio-Rad electrophoresis instruction manual.

Monitoring LOS phenotype. Throughout these studies, gonococcal lipooligosaccharide (LOS) phenotype was monitored by running whole-cell preparations, treated with proteinase K, on Tricine-SDS-polyacrylamide gels as described by Lesse et al. (23). Strains F62 and FA1090 had different LOS phenotypes, whereas FA1090 OpaA and OpaB had the same LOS phenotype. Most importantly, LOS phenotypes of the different variants did not change during the course of the studies.

Isolation of gonococcal outer membranes. Outer membranes were prepared by the $LiCl_2$ extraction procedure described by Leith and Morse (22). Outer membranes were washed once in water and resuspended in water by Dounce homogenization to a protein concentration of 1 to 3 mg/ml (24). Aliquots of outer membranes were stored at −20°C and used fresh daily. The Opa content of the outer membranes was checked by SDS-PAGE.

Purification of Opa outer membrane proteins. Opas were purified by chromatofocusing according to the method of Newhall et al. (28). Opas were precipitated from residual Triton X-100 with ethanol by adding washed Opa dropwise to cold 100% ethanol (The Warner-Graham Co., Cockeysville, Md.) at a ratio of 1:9 and kept in an ethanol-dry ice bath for at least 1 h. Opas were then pelleted (40,000 × *g*, 4°C, 1 h) and resuspended in 1% octylglucoside in water to a protein concentration of 0.5 to 1.0 mg/ml (26). Total protein was determined by the BCA method (Pierce, Rockford, Ill.) with bovine serum albumin (BSA) as a standard. Opas were identified by Western immunoblotting of outer membranes and of purified Opas, as described by Bszewczyki and Koloft (5). Opa-specific MAbs were detected by using a protein A-alkaline phosphatase conjugate (Sigma Chemical Co., St. Louis, Mo.) with the substrate described by Blake et al. (4). For the purpose of this report, purified $OpaB_{FA1090}$, $OpaA_{FA1090}$, and $Opa4_{F62}$ will be referred to as OpaB, OpaA, and Opa4, respectively.

Liposomes. Lipid stock solutions (10 mM in chloroform) were pipetted into 25-ml pear-shaped flasks in molar ratios of 2:1.5:0.22, phosphatidylcholine-cholesterol-dicetyl phosphate. The chloroform was rotoevaporated at 30°C (32, 38), and 1.0 ml of liposome buffer (0.14 M NaCl in 10 Mm Tris HCl buffer, pH 7.4) and several borosilicate glass beads (precoated with 1% BSA) were added. The contents were then vortexed to remove all phospholipid from the flask walls. Purified Opa (100 μg) and liposome buffer were then added to bring the total volume of the suspension to 2.0 ml. Subsequently, the mixture was sonicated at room temperature for 2 to 4 min to form a very slightly turbid suspension of small, unilamellar liposomes (15, 16). A typical liposome preparation used a total of 2.5 ml of lipids in chloroform, 200 μl of purified Opa, and 1.8 ml of liposome buffer. Liposomes were made fresh daily and kept at room temperature until use. All lipids were purchased from Avanti Polar Lipids, Birmingham, Ala.

Peptides. Three 15-amino-acid peptides were synthesized by Jan Pohl, Microchemical Facility, Emory University School of Medicine, Atlanta, Ga., by standard t-Boc chemistry. Purity was confirmed by high-performance liquid chromatography performed at the time of synthesis. The peptides were sent as lyophilized powders and stored desiccated at 4°C. The peptides, designated $pepHV_2$-A, $pepHV_2$-B, and $pepHV_2$-C, were overlapping and encompass the entire HV_2 region of FA1090 OpaB as described by Connell et al. (6). $pepHV_2$-A consists of amino acids 149 to 163 (SIDSTKKITGTLTAY), $pepHV_2$-B consists of amino acids 161 to 175 (TAYPSDADAAVTVYP), and $pepHV_2$-C consists of amino acids 174 to 188 (YPDGHPQKNTYQKSN).

Neutrophils. Human neutrophils were purified from freshly heparinized venous blood by a single centrifugation through Ficoll-Hypaque, as described by Ferrante and Thong (12). Erythrocytes were lysed once by exposure to cold water for 15 s. Neutrophil suspensions were ≥94% pure as determined by Wright's stain and ≥98% viable as determined by exclusion of 0.25% trypan blue in saline at room temperature. Purified neutrophils were suspended to 10^7/ml in PBSG and kept on ice until use.

Gonococcal adherence to neutrophils. Adherence assays were performed in 1.5-ml snap-cap tubes with 5×10^5 neutrophils and 2×10^7 gonococci in PBSG to a final volume of 1 ml. Incubations were at 37°C unless indicated otherwise. Neutrophils were pretreated with 5 μg of cytochalasin B per ml (5-mg/ml stock in dimethyl sulfoxide [DMSO]) for 5 min to prevent phagocytosis and 10 to 20 ng of phorbol myristate acetate (PMA; 100-μg/ml stock in DMSO) for 5 min to increase the number of Opa receptors on the neutrophil surface (11). To test the effect of outer membranes, purified Opa, Opa^+ lips, or $pepHV_2$ lips on the ability of gonococci to adhere to neutrophils, they were preincubated with neutrophils for 20 min before the addition of gonococci. After addition of gonococci, mixtures were tumbled end over end (10 to 20 rpm) for an additional 20 to 30 min, at which time 150 μl was withdrawn and deposited on a microscope slide with a cytocentrifuge (Shandon Southern Corp., Sewickley, Pa.). Samples were air dried and stained with Wright's stain, and adherence was quantitated by light microscopy (100×, oil immersion) by counting the number of adherent gonococci per 100 neutrophils in contiguous fields. Results are expressed as the average number of gonococci per neutrophil or as percentage of control adherence (untreated neutrophils and untreated gonococci).

Binding of purified Opa proteins to neutrophils. Neutrophils were pretreated with 5 μg of cytochalasin B per ml (5-mg/ml stock in DMSO) for 5 min to prevent phagocytosis and 10 to 20 ng of PMA (100-μg/ml stock in DMSO) for 5 min

to increase the number of Opa receptors on the neutrophil surface (11). To test the ability of purified Opa proteins to bind to neutrophils, 3 μg of purified OpaA or OpaB was added to various amounts of neutrophils (total volume of 1 ml in PBSG) for 20 min. To decrease nonspecific binding of the proteins to the sides of the tubes, all tubes were first rinsed with 6% BSA (in PBSG) and then washed twice with PBSG. The suspension was then centrifuged at 6,000 × *g* for 6 min at room temperature. The supernatants were carefully aspirated and placed in a clean microcentrifuge tube, while the pellets were resuspended to 1 ml in PBSG. An aliquot from each tube was then removed and mixed with 2× SDS-sample buffer and a SDS–12% polyacrylamide gel was run. Proteins were then electroblotted to nitrocellulose, and the blot was probed with either Opa-specific MAbs or anti-Opa rabbit antiserum (PII4-1 [see below]). The blots were developed by using enhanced chemiluminescence (ECL protein detection kit; Amersham, Arlington Heights, Ill.).

MAbs and serum. MAb 53C4 was provided by Charles E. Wilde, Indiana University School of Medicine, Indianapolis, as ascitic fluid (28). MAb 10.1 was supplied by Janne Cannon, University of North Carolina School of Medicine, Chapel Hill, as hybridoma culture supernatant (42). Both are immunoglobulin G2a and react with Opa4 and OpaB gonococci on Western blots and in a whole-cell binding assay (2, 10) and inhibit Opa4 and OpaB gonococci from adhering to and stimulating neutrophil CL (10, 36). Antiserum PII4-1 was raised by conventional techniques in rabbits against partially purified PII4 (Opa4) from strain F62. Antiserum PII4-1 reacts with Opa4 and OpaB on colony, dot, and Western blots and also recognizes OpaA (the nonadherent Opa of strain FA1090) on Western blots.

Statistical analysis. Where appropriate, experimental data were compared to appropriate controls by using the Student *t* test for paired samples.

RESULTS

Purification of Opa proteins and outer membranes. Purification of Opa proteins proceeded as described by Newhall et al. (28). The individual gonococcal Opa variants used in these studies contained only one Opa protein, as did their respective outer membranes. Opa4 was purified to apparent homogeneity as observed on Coomassie blue-silver double-stained SDS-polyacrylamide gels (Fig. 1). Western blots, with Opa-specific MAbs or antiserum PII4-1, were done to confirm which Opa proteins were being used (data not shown). No LOS was detected in the Opa preparations as determined by Tricine-SDS-PAGE and subsequent silver staining (data not shown).

Role of Opa in adherence of gonococci to neutrophils. To investigate whether Opa proteins could bind to neutrophils in either a semipurified or purified state, we examined the ability of gonococcal outer membranes or purified Opas to inhibit subsequent binding of viable Opa$^+$ gonococci to neutrophils. Outer membranes from OpaB-containing gonococci, in a dose-responsive manner, significantly inhibited the subsequent binding of OpaB gonococci to neutrophils (Fig. 2). Although at higher concentrations outer membranes from strain FA1090 containing no Opa proteins (Opa neg) or outer membranes from OpaA-containing gonococci also inhibited adherence of OpaB gonococci to neutrophils, they did so to a significantly lower degree than did outer membranes containing OpaB (Fig. 2). OpaA is the only Opa protein so far investigated that does not mediate adherence

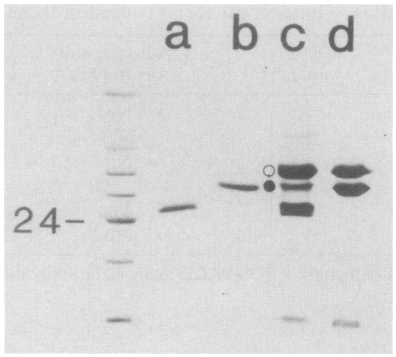

FIG. 1. Coomassie blue- and silver-stained SDS-polyacrylamide gels of purified Opa4 and of outer membranes from Opa4-containing gonococci. Lane a, purified Opa4 (4 μg of protein), 37°C, 1 h; lane b, purified Opa4 (4 μg of protein), 100°C, 5 min; lane c, Opa4 outer membrane preparation (25 μg of protein), 37°C, 1 h; lane d, Opa4 outer membrane preparation (25 μg of protein), 100°C, 5 min. Note that Opa4 is heat modifiable; upon boiling in SDS under reducing conditions it migrates at an apparently higher molecular weight. Note also the presence of protein I (○) and protein III (●) in the outer membrane preparations in lanes c and d.

of gonococci to neutrophils (13). Further analysis demonstrated that all outer membrane preparations, regardless of Opa content, were increasingly toxic to neutrophils at a concentration of ≥25 μg/ml (Fig. 3). This possibly explains the inhibitory action of Opa neg and OpaA outer membranes at higher concentrations. We conclude from these set of experiments that (i) Opa proteins remain biologically active in outer membranes, (ii) the ability of outer membranes to inhibit subsequent gonococcal binding to neutrophils is directly related to the presence of Opa, and (iii) gonococcal viability is not required for Opa function.

Since the above-described experiments showed that outer

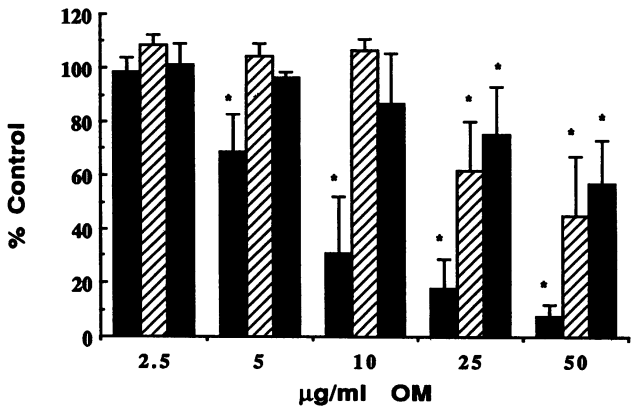

FIG. 2. Inhibition of adherence of FA1090 OpaB gonococci to neutrophils by gonococcal outer membranes. Neutrophils (5 × 10^5/ml, pretreated with cytochalasin B and PMA) were incubated with increasing concentrations of outer membranes from OpaB (▨)- or OpaA (▨)-containing gonococci or with outer membranes from gonococci possessing no Opa proteins (Opa neg) (▩) for 20 min. OpaB-containing gonococci were then added (ratio of 40:1, gonococci/neutrophil), and the suspension was tumbled for 20 min before removal of aliquots for quantitation. Results are expressed as the mean percentage of controls ± standard deviation ($n \geq 4$). Control neutrophils were preincubated with no outer membranes. *, $P < 0.05$ versus control neutrophils with no outer membranes.

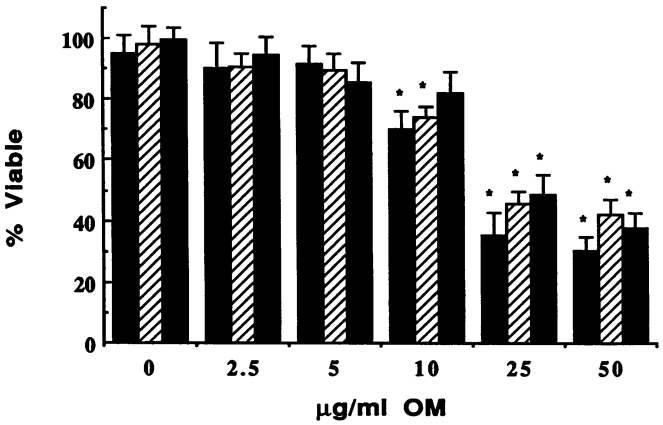

FIG. 3. Effects of gonococcal outer membranes on neutrophil viability. Neutrophils (5×10^5/ml, pretreated with cytochalasin B and PMA) were incubated with increasing concentrations of outer membranes from either OpaB (■)- or OpaA (▨)-containing gonococci or with outer membranes from gonococci possessing no Opa proteins (Opa-neg) (▩) for 20 min. An aliquot was then mixed with an equal volume of 0.16% trypan blue in PBS. Viability was quantitated as the number of neutrophils that excluded the dye divided by the total number of neutrophils counted. Results are expressed as the mean percent viable neutrophils relative to controls ± standard deviation ($n = 3$). Control neutrophils were preincubated with no outer membranes. *, $P < 0.05$ versus control neutrophils with no outer membranes.

membranes were increasingly toxic to neutrophils at a concentration of ≥25 μg/ml, we carried out the same type of inhibition experiments but used purified Opa proteins instead of outer membranes. Neutrophils were first preincubated with purified Opa4, OpaB, or OpaA before addition of gonococci. Purified Opa4, in a dose-responsive manner, inhibited Opa4 gonococci from adhering to neutrophils by a

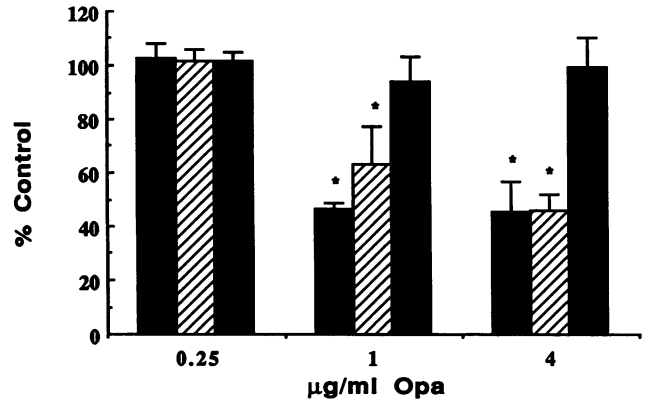

FIG. 4. Inhibition by purified Opa proteins of gonococcal binding to neutrophils. Neutrophils (5×10^5/ml, pretreated with cytochalasin B and PMA) were incubated with increasing concentrations of different Opas for 20 min, gonococci were added (ratio of 40:1, gonococci/neutrophil), and the suspension was tumbled for 20 min more. Opa4-containing gonococci were added to neutrophils preincubated with purified Opa4 (■), and OpaB gonococci were added to neutrophils preincubated with either purified OpaB (▨) or purified OpaA (▩). Results are expressed as the mean percentage of controls ± standard deviation ($n \geq 4$). Control neutrophils were preincubated with no Opa proteins. *, $P < 0.05$ versus control neutrophils with no Opa proteins.

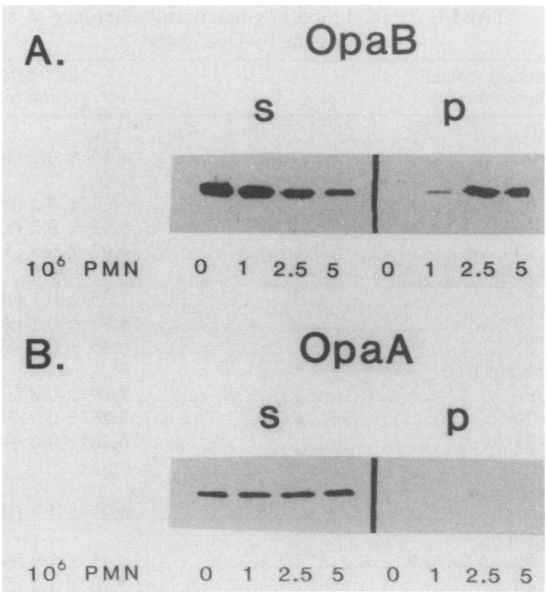

FIG. 5. Adherence of purified Opa proteins to neutrophils. Purified OpaB or OpaA (3 μg of each) was added to increasing numbers of cytochalasin B- and PMA-treated neutrophils. Suspensions were incubated for 20 min and then centrifuged (6,000 × g, 6 min, room temperature). Aliquots of supernatants and pellets were subjected to SDS–12% PAGE before being electroblotted to nitrocellulose. Blots were probed with either anti-Opa MAb 53C4 for OpaB or anti-Opa rabbit antiserum PII4-1 for OpaA and were developed by using enhanced chemiluminescence. (A) Results of a representative experiment using purified OpaB; (B) results of a representative experiment using purified OpaA.

maximum of about 50% (Fig. 4). Similarly, OpaB inhibited OpaB gonococci from adhering to neutrophils. On the other hand, OpaA had no effect on adherence of either strain FA1090 OpaB (Fig. 4) or strain F62 Opa4 (data not shown). Of importance is the fact that none of the Opa proteins were toxic for neutrophils at the concentrations used. For reasons that remain unclear, we never saw more than 55% inhibition of adherence by purified Opas.

Interestingly, purified OpaB also inhibited subsequent adherence to neutrophils of Opa4 gonococci (data not shown). This supports the idea that there is a shared binding epitope(s) between certain Opa variants of different gonococcal strains, as described by Elkins and Rest (10), who showed that anti-Opa MAb 53C4 recognizes both Opa4 and OpaB gonococci on Western blots, inhibits Opa4- and OpaB-mediated gonococcal binding to neutrophils, and inhibits Opa4- and OpaB-mediated gonococcal stimulation of neutrophil CL. These results also support the idea of a shared receptor for Opa proteins on the human neutrophil surface (11).

To further define the biological activity of purified Opa proteins, we investigated their ability to bind directly to neutrophils. By using a simple centrifugation assay, described in Materials and Methods, we observed that purified OpaB (3 μg) bound, in a dose-responsive manner, to increasing numbers of neutrophils (Fig. 5). Conversely, OpaA, which does not mediate adherence of OpaA gonococci to neutrophils, did not bind to neutrophils (Fig. 5). These data suggest that purification of Opa proteins does not dramatically alter their binding capabilities for neutrophils and support our contention that purified Opa proteins retain at

TABLE 2. Inhibition of gonococcal adherence to neutrophils by Opa$^+$ lips[a]

Gonococcal variant and liposome (μl)	GC/PMN (% control)
OpaB	
None	7.15 ± 0.1 (100)
OpaB	
5[b]	6.7 ± 0.2 (94)[c,d]
10	5.95 ± 0.2 (83)[c,d,e]
25	4.02 ± 0.5 (56)[c,d,e]
OpaA	
5	7.35 ± 0.1 (103)
10	6.87 ± 0.2 (96)[c,e]
25	6.35 ± 0.1 (89)[c]
Protein free	
5	7.29 ± 0.8 (102)
10	7.26 ± 0.5 (102)
25	6.30 ± 0.0 (88)[c]
Opa4	
None	6.95 ± 1.3 (100)
Opa4	
5	4.45 ± 1.9 (64)[c,e]
10	3.70 ± 1.3 (53)[c,e]
25	2.45 ± 0.9 (35)[c,e]
Protein free	
5	7.98 ± 0.4 (115)
10	7.03 ± 0.2 (101)
25	5.35 ± 0.5 (77)[c]

[a] Neutrophils (5×10^5/ml, pretreated with cytochalasin B and PMA as described in Materials nd Methods) were incubated with liposomes for 20 min. Gonococci were then added (ratio of 40:1, gonococci/neutrophil), and the suspension was tumbled for an additional 20 min. Opa4 gonococci were added to neutrophils preincubated with Opa4 lips, while OpaB gonococci were added to neutrophils preincubated with OpaB lips or OpaA lips. Results are expressed as the average number of gonococci per neutrophil (GC/PMN) ± standard deviation ($n \geq 4$).

[b] 5 μl of Opa$^+$ lips contains 0.25 μg of purified Opa.

[c] $P < 0.05$ versus no liposomes.

[d] $P < 0.05$ versus OpaA lips at same concentration.

[e] $P < 0.05$ versus protein-free liposomes at same concentration.

least partial biological activity. If indeed a partial loss of activity is suffered during purification, this might explain why purified Opa4 or OpaB yielded only 55% inhibition of gonococcal binding to neutrophils.

Effect of Opa$^+$ lips on gonococcal adherence to neutrophils. Since we were able to inhibit gonococcal adherence by only about 55% with purified Opa in detergent, we tested the ability of various Opa proteins incorporated into liposomes (Opa$^+$ lips) to inhibit gonococcal adherence to neutrophils, reasoning that in a lipid environment Opa might more closely resemble its native conformation. In experiments similar to those described above for purified Opas, OpaB lips and Opa4 lips inhibited subsequent gonococcal adherence to neutrophils in a dose-responsive manner compared with protein-free or OpaA lips (Table 2). Protein-free and OpaA lips at 25 μl/ml (representing a final concentration of 1.25 μg of Opa per ml) had some marginal inhibitory effects. We do not know what component of the liposome preparations was responsible for this inhibition. Regardless, the data clearly indicate that Opa4 and OpaB specifically inhibit adherence of gonococci to neutrophils. However, as with purified Opa, we did not see complete inhibition of gonococcal binding by Opa$^+$ lips. These data indicate that incorporating purified Opa into the liposomes used in these studies did not increase Opa's ability to inhibit subsequent binding of gonococci to neutrophils.

TABLE 3. Inhibition of gonococcal adherence to neutrophils by pepHV$_2$ lips[a]

Treatment (μl)	GC/PMN (% control)
None	6.90 ± 0.14 (100)
pepHV$_2$-A lips	
5	4.82 ± 1.13 (70)[b,c]
25	3.05 ± 0.21 (44)[b,c]
pepHV$_2$-B lips	
5	6.35 ± 0.35 (92)[b]
25	5.55 ± 0.51 (80)[b]
pepHV$_2$-C lips	
5	4.40 ± 0.42 (64)[b,c]
25	2.75 ± 0.07 (40)[b,c]
Peptide free	
5	6.45 ± 0.50 (94)
25	5.65 ± 0.35 (82)[b]

[a] Neutrophils (5×10^5/ml, pretreated with cytochalasin B and PMA) were incubated with liposomes for 20 min. OpaB gonococci were then added (ratio of 40:1, gonococci/neutrophil), the suspension was tumbled for 20 min, and aliquots were removed for quantitation. Results are expressed as the average number of gonococci per neutrophil (GC/PMN) ± standard deviation ($n \geq 4$).

[b] $P < 0.05$ versus no liposomes.

[c] $P < 0.05$ versus peptide-free liposomes at same concentration.

To indicate that Opa was indeed associated with liposomes, 1-ml suspensions of Opa$^+$ lips were centrifuged at 170,000 × *g* for 2 h, the supernatants were saved, and the resultant pellets were resuspended in 1 ml of water. Samples of the starting liposome suspension, the resuspended liposome pellets, and the liposome-free supernatant were then run on SDS-PAGE. Opa was found in the Opa$^+$ lips suspension and the resuspended pellets but not in the supernatants, indicating that Opa was indeed associated with the liposomes (data not shown). Purified Opa proteins in 1% octylglucoside are not pelleted under these conditions.

Effect of OpaB peptides on gonococcal adherence to neutrophils. To begin characterizing the domain(s) on the Opa molecule involved in gonococcal adherence to neutrophils, we tested the ability of three 15-amino-acid peptides composing the HV$_2$ region of OpaB to inhibit gonococcal adherence to neutrophils. The peptides were designated pepHV$_2$-A, -B, and -C, as described in Materials and Methods. This approach was chosen because it appears that a major structural difference between adherent OpaB and nonadherent OpaA resides in or around the peptide sequence of the HV$_2$ region (1, 6, 7, 13). Initial attempts to use pure peptides yielded inconsistent and uninterpretable results. In an attempt to overcome these problems, we associated the peptides with liposomes (pepHV$_2$ lips) as described above with purified Opa. We then performed experiments similar to those described above for Opa$^+$ lips. Neutrophils were preincubated with pepHV$_2$ lips before the addition of gonococci. The results show that liposomes containing pepHV$_2$-A or pepHV$_2$-C, the peptides composing the amino and carboxyl ends, respectively, of the HV$_2$ region of OpaB, inhibited OpaB-mediated gonococcal adherence to neutrophils (Table 3). On the other hand, liposomes containing HV$_2$-B (which composes the middle of the HV$_2$ region of OpaB) or peptide-free liposomes did not (Table 3). When pepHV$_2$-A and pepHV$_2$-C lips were mixed together at relatively low volumes (<25 μl each), additive inhibition was seen. At higher volumes (>25 μl each) the liposomes were toxic to neutrophils (data not shown).

We conclude from these experiments that there may be multiple epitopes involved in Opa-mediated gonococcal binding to neutrophils and that more than one of these

domains may be necessary for intact, viable gonococci to adhere to neutrophils. This may explain why we never saw 100% inhibition of gonococcal binding to neutrophils by a single pepHV$_2$ lip or Opa lip, since it is possible that purification of Opa proteins results in the loss or alteration of one or more critical binding epitopes. Further investigation of the Opa protein and Opa peptides should lead to the characterization of the Opa binding domain(s) responsible for gonococcal binding to neutrophils.

DISCUSSION

In this study we used well-characterized Opa variants of gonococcal strains F62 and FA1090. Each of these variants possesses only a single heat-modifiable Opa outer membrane protein. The presence of the Opa protein in the gonococcal outer membrane allows the gonococcus to adhere to and induce an oxidative burst in human neutrophils (13, 19, 35, 36, 45, 49). By using Opa$^+$ outer membranes, Opa$^+$ lips, and peptides from the HV$_2$ region of OpaB (from strain FA1090), we were able to study the function of the Opa protein as well as begin to identify the specific region(s) on the Opa molecule which acts as the gonococcal ligand for the neutrophil Opa receptor(s). To reach this goal, we investigated Opa-mediated gonococcal adherence to human neutrophils in the presence of cytochalasin B, which inhibits the ability of neutrophils to phagocytose adherent gonococci (8, 35) but does not affect the ability of gonococci to adhere to neutrophils (11).

The studies presented here show that Opa-containing outer membranes, purified Opa, and Opa$^+$ lips inhibited subsequent adherence of gonococci to neutrophils. This was true for all Opa proteins tested except for OpaA$_{FA1090}$, which does not mediate adherence of gonococci to neutrophils. It is reasonable to assume from the data that the Opa protein is, in fact, the only gonococcal surface component responsible for gonococcal adherence to neutrophils (13).

Similar to Opa proteins and pili, gonococcal LOS undergoes frequent antigenic variation (25). We do not think that LOS is involved with the observations presented in this paper, however, since we monitored LOS phenotype throughout the studies. Although the LOS phenotypes of strains FA1090 and F62 were different (as would be expected with the high degree of LOS variation), the LOS phenotypes of FA1090 OpaA and OpaB variants were the same. Most importantly, the LOS phenotypes of the different Opa variants remained constant throughout the studies. This does not preclude, however, that LOS may be involved in modulating Opa-mediated adherence to neutrophils. Such controlled studies remain to be performed.

Farrell and Rest showed that human peripheral blood neutrophils bind substantially more Opa$^+$ gonococci after incubation with any of a number of potent neutrophil-stimulating agents, including the chemoattractant peptide formylmethionylleucylphenylalanine, the calcium ionophore A23187, and the cocarcinogen PMA (11). It was concluded from these observations that Opa receptors (as yet unidentified) were located within the membranes of specific granules and were being brought to the surface of the activated neutrophils (11). All of the experiments presented in the current work were performed with such stimulated neutrophils. Exudate neutrophils, e.g., neutrophils found in the urethra or cervix during gonorrhea, are naturally activated in that, during diapedesis and chemotaxis, they are induced to degranulate (release) some or all of their specific granule contents (52). Therefore, our use of activated neutrophils, as opposed to resting peripheral blood neutrophils, probably more accurately reflects gonococcus-neutrophil interactions occurring during gonorrhea.

Although we performed no formal studies as to the orientation of the Opa protein within or on the prepared liposomes, the data seem to preclude any conclusion other than that at least some of the Opa molecules were in a functionally active orientation. If they were not, the Opa$^+$ lips would have been expected to function in a manner similar to the protein-free liposomes. In fact, the mere presence of the Opa protein in or on liposomes was not responsible for their ability to inhibit subsequent gonococcal binding to neutrophils, since liposomes containing the nonadherent Opa protein, OpaA, acted similarly to protein-free liposomes.

Opa proteins, which actually compose a group of related basic proteins with apparent molecular masses of 24 to 30 kDa as determined by SDS-PAGE, were first described by Swanson as opacity-associated proteins (43). DNA sequencing of Opa genes by Stern et al. (40), later confirmed by Connell et al. (6, 7), has shown that Opa proteins exhibit near homology except at three areas: the semivariable region near the N terminus and two hypervariable regions termed HV$_1$ and HV$_2$. On the basis of amino acid sequences and immunologic detection, the two hypervariable regions are thought to be surface exposed (40). MAb 10.1, which is directed against an epitope on the OpaB protein (1), inhibits OpaB-containing gonococci from binding to and stimulating human neutrophils (9). Interestingly, MAb 10.1 also inhibits Opa4-containing gonococci from binding to and stimulating human neutrophils (9). F62 is unrelated to strain FA1090, and this evidence suggests shared epitopes between Opa proteins of different gonococcal strains. Supporting this notion, Elkins and Rest showed that MAb 53C4, which was derived from mice immunized against intact strain F62 gonococci, also inhibits adherence to and stimulation of human neutrophils by both Opa4- and OpaB-containing gonococci (10). These data strongly suggest that Opa4 and OpaB share the same neutrophil binding epitope(s) or that they are, in fact, the same Opa protein.

Characterization of MAb 10.1 suggests that it binds to an area proximal to the carboxyl terminus of the HV$_2$ region of OpaB (1, 7). On the basis of this information, we synthesized peptides which encompassed the entire HV$_2$ region of OpaB as well as a few adjacent amino acids. The peptides were 15 amino acids long and arbitrarily designated pepHV$_2$-A, pepHV$_2$-B, and pepHV$_2$-C. Although none of the peptides in solution consistently inhibited subsequent gonococcal adherence to neutrophils, liposomes that individually incorporated each of the peptides proved to be useful tools. While both pepHV$_2$-A lips and pepHV$_2$-C lips inhibited subsequent gonococcal adherence to neutrophils, pepHV$_2$-B lips had no inhibitory effect. According to the two-dimensional model of the gonococcal Opa protein described by Stern et al. (40), there appear to be at least two surface-exposed loops and one possible intramembrane loop of the Opa protein when it is in the gonococcal membrane. Our own hydrophobicity plots of the HV$_2$ region of OpaB suggest that the HV$_2$ region also has two surface-exposed loops and one intramembrane loop. pepHV$_2$-A contains the amino acids that make up the first surface-exposed loop, pepHV$_2$-B contains the amino acids which make up the intramembrane loop, and pepHV$_2$-C contains the amino acids that make up the second surface-exposed loop. Therefore, our results suggest that both surface-exposed areas may be involved in adherence of *N. gonorrhoeae* to human neutrophils but that both are not required.

While the results obtained by using MAb 10.1 suggest that there is a single binding epitope, it is possible that there exists a multivalent epitope consisting of both surface-exposed regions. Another possibility is that MAb 10.1 recognizes a single linear epitope, which may be located on one of the surface-exposed areas, and it simply sterically hinders other areas involved in binding. Further work at both the protein and molecular levels should elucidate the exact area(s) within the HV_2 region that is ultimately responsible for adherence of *N. gonorrhoeae* to human neutrophils.

ACKNOWLEDGMENTS

This work was supported in part by grant R01 AI20897 from the National Institute of Allergy and Infectious Diseases. F.L.N. was supported in part by a graduate stipend from Hahnemann University.

REFERENCES

1. **Barritt, D. S., R. S. Schwalbe, D. G. Klapper, and J. G. Cannon.** 1987. Antigenic and structural differences among six proteins II expressed by a single strain of *Neisseria gonorrhoeae*. Infect. Immun. **55**:2026–2031.
2. **Black, W. S., R. S. Schwalbe, I. Nachamkin, and J. G. Cannon.** 1984. Characteristics of *Neisseria gonorrhoeae* PII phase variation by use of monoclonal antibodies. Infect. Immun. **45**:453–457.
3. **Blake, M. S., and E. C. Gotschlich.** 1983. Gonococcal membrane proteins: speculations on their role in pathogenesis. Prog. Allergy **33**:298–313.
4. **Blake, M. S., K. H. Johnston, G. J. Russell-Jones, and E.F. Gotschlich.** 1984. A rapid, sensitive method for detection of alkaline phosphatase-conjugated anti-antibody on Western blots. Anal. Biochem. **136**:175–179.
5. **Bszewczyki, A., and L. M. Koloft.** 1985. A method for efficient blotting of basic proteins from SDS-PAGE to nitrocellulose. Anal. Biochem. **150**:403–407.
6. **Connell, T. D., W. J. Black, T. H. Kawula, et al.** 1988. Recombination among protein II genes of *Neisseria gonorrhoeae* generates new coding sequences and increases structural variability in the protein II family. Mol. Microbiol. **2**:227–236.
7. **Connell, T. D., D. Shaffer, and J. G. Cannon.** 1990. Characterization of the repertoire of hypervariable regions in the protein II (opa) gene family of *Neisseria gonorrhoeae*. Mol. Microbiol. **4**:439–449.
8. **Davis, A. T., R. Estensen, and P. G. Quie.** 1971. Cytochalasin B. III. Inhibition of human polymorphonuclear leukocyte phagocytosis. Proc. Soc. Exp. Biol. Med. **137**:161–164.
9. **Elkins, C., and R. F. Rest.** Unpublished data.
10. **Elkins, C., and R. F. Rest.** 1990. Monoclonal antibodies to outer membrane protein PII block interactions of *Neisseria gonorrhoeae* with human neutrophils. Infect. Immun. **58**:1078–1084.
11. **Farrell, C. F., and R. F. Rest.** 1990. Up-regulation of human neutrophil receptors for *Neisseria gonorrhoeae* expressing PII outer membrane proteins. Infect. Immun. **58**:2777–2784.
12. **Ferrante, A., and Y. H. Thong.** 1980. Optimal conditions for simultaneous purification of monocytes and polymorphonuclear leukocytes from human blood by Ficoll-Hypaque methods. J. Immunol. Methods **36**:109–118.
13. **Fischer, S. H., and R. F. Rest.** 1988. Gonococci possessing only certain PII outer membrane proteins interact with human neutrophils. Infect. Immun. **56**:1574–1579.
14. **Forslin, I., and D. Danielsson.** 1980. In vitro studies of the adherence of *Neisseria gonorrhoeae* and other urogenital bacteria to vaginal and uroepithelial cells, with special regard to the menstrual cycle. Gynecol. Obstet. Invest. **11**:327–340.
15. **Helenius, A., E. Fries, and J. Kartenbeck.** 1977. Reconstitution of Semliki forest virus membrane. J. Cell Biol. **75**:866–873.
16. **Hope, M. J., M. B. Gally, L. D. Mayer, A. S. Janoff, and P. R. Cullis.** 1987. Generation of multilamellar and unilamellar phospholipid vesicles. Chem. Phys. Lipids **40**:89–107.
17. **Kellogg, D. F., Jr., I. R. Cohen, L. C. Norins, A. L. Schroeter, and G. Reising.** 1968. *Neisseria gonorrhoeae*. II. Colonial variation and pathogenicity during 35 months in vitro. J. Bacteriol. **96**:596–605.
18. **King, G. J., J. F. James, and J. Swanson.** 1978. Studies on gonococcus infection. II. Comparison of in vivo and in vitro association of *Neisseria gonorrhoeae* with human neutrophils. J. Infect. Dis. **137**:38–43.
19. **King, G. J., and J. Swanson.** 1978. Studies on gonococcus infection. XV. Identification of surface proteins of *Neisseria gonorrhoeae* correlated with leukocyte association. Infect. Immun. **21**:575–583.
20. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227**:680–685.
21. **Lambden, P. R., and J. E. Heckels.** 1979. Outer membrane protein composition and colonial morphology of *Neisseria gonorrhoeae* strain P9. FEMS Microbiol. Lett. **5**:263–265.
22. **Leith, D. K., and S. A. Morse.** 1980. Effect of dissolved oxygen on outer membrane protein composition of *Neisseria gonorrhoeae* grown in continuous culture. FEMS Microbiol. Lett. **7**:191–194.
23. **Lesse, A. J., A. A. Campagnari, W. E. Bittner, and M. A. Apicella.** 1990. Increased resolution of lipopolysaccharides and lipooligosaccharides utilizing tricine-sodium dodecyl sulfate-polyacrylamide gel electrophoresis. J. Immunol. Methods **126**:109–117.
24. **Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall.** 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. **193**:265–275.
25. **Mandrell, R., H. Schneider, M. Apicella, W. Zollinger, P. A. Rice, and J. M. Griffiss.** 1986. Antigenic and physical diversity of *Neisseria gonorrhoeae* lipooligosaccharide. Infect. Immun. **54**:63–72.
26. **Mimms, L. T., G. Zampighi, Y. Nozaki, C. Tanford, and J. A. Reynolds.** 1981. Phospholipid vesicle formation and transmembrane protein incorporation using octylglucoside. Biochemistry **20**:833–840.
27. **Murphy, G. L., T. D. Connell, D. S. Barritt, M. Koomey, and J. G. Cannon.** 1989. Phase variation of gonococcal protein II: regulation of gene expression by slipped-strand mispairing of a repetitive DNA sequence. Cell **56**:539–547.
28. **Newhall, W. J., V, L. B. Mail, C. E. Wilde III, and R. B. Jones.** 1985. Purification and antigenic relatedness of proteins II of *Neisseria gonorrhoeae*. Infect. Immun. **49**:576–580.
29. **Nyberg, G., N. Strömberg, A. Jonsson, K.-A. Karlsson, and S. Normark.** 1990. Erythrocyte gangliosides act as receptors for *Neisseria subflava*: identification of the Sia-1 adhesin. Infect. Immun. **58**:2555–2563.
30. **Ofek, I., E. H. Beachey, and A. L. Bisno.** 1974. Resistance of *Neisseria gonorrhoeae* to phagocytosis: relationship to colonial morphology and surface pili. J. Infect. Dis. **129**:310–316.
31. **Paruchuri, D. K., H. S. Seifert, T. S. Ajioka, K.-A. Karlsson, and M. So.** 1990. Identification and characterization of a *Neisseria gonorrhoeae* gene encoding a glycolipid-binding adhesion. Proc. Natl. Acad. Sci. USA **87**:333–337.
32. **Porat, R., M. A. Johns, and W. R. McCabe.** 1987. Selective pressures and lipopolysaccharide subunits as determinants of resistance of clinical isolates of gram-negative bacilli to human serum. Infect. Immun. **55**:320–328.
33. **Punsalang, A. P., and W. D. Sawyer.** 1973. Role of pili in the virulence of *Neisseria gonorrhoeae*. Infect. Immun. **8**:255–263.
34. **Rest, R. F.** 1979. Killing of *Neisseria gonorrhoeae* by human neutrophil granule extracts. Infect. Immun. **25**:574–579.
35. **Rest, R. F., S. H. Fisher, Z. Z. Ingham, and J. F. Jones.** 1982. Interactions of *Neisseria gonorrhoeae* with human neutrophils: effects of serum and gonococcal opacity on phagocytic killing and chemiluminescence. Infect. Immun. **36**:737–744.
36. **Rest, R. F., N. Lee, and C. Bowden.** 1985. Stimulation of human leukocytes by protein II^+ gonococci is mediated by lectin-like gonococcal components. Infect. Immun. **50**:116–122.
37. **Rest, R. F., and E. Pretzer.** 1981. Degradation of gonococcal outer membrane proteins by human neutrophil lysosomal proteases. Infect. Immun. **34**:62–68.

38. **Rice, P. (Boston University).** 1990. Personal communication.
39. **Shafer, W. M., and R. F. Rest.** 1989. Interactions of gonococci with phagocytic cells. Annu. Rev. Microbiol. **43**:121–145.
40. **Stern, A., M. Brown, P. Nickel, and T. F. Meyer.** 1986. Opacity genes in *Neisseria gonorrhoeae*: control of phase and antigenic variation. Cell **47**:61–71.
41. **Stromberg, N., C. Deal, G. Nyberg, S. Normark, M. So, and K.-A. Karlsson.** 1988. Identification of carbohydrate structures that are possible receptors of *Neisseria gonorrhoeae*. Proc. Natl. Acad. Sci. USA **85**:4902–4906.
42. **Sugasawara, R. J., J. G. Cannon, W. J. Black, I. Nachamkin, R. L. Sweet, and G. F. Brooks.** 1983. Inhibition of *Neisseria gonorrhoeae* attachment to HeLa cells with monoclonal antibody directed against a protein II. Infect. Immun. **42**:980–985.
43. **Swanson, J.** 1978. Studies on gonococcus infection. XII. Colony color and opacity variants of gonococci. Infect. Immun. **19**:320–331.
44. **Swanson, J., E. Sparks, B. Zeligs, M. A. Siam, and C. Parrott.** 1974. Studies on gonococcus infection. V. Observations on in vitro interactions of gonococci and human neutrophils. Infect. Immun. **10**:633–644.
45. **Swanson, J., E. Sparks, D. Young, and G. King.** 1975. Studies on gonococcus infection. X. Pili and leukocyte association factor as mediators of interactions between gonococci and eukaryotic cells in vitro. Infect. Immun. **11**:1352–1361.
46. **Thomas, D. W., J. C. Hill, and F. J. Tyeryar, Jr.** 1973. Interactions of gonococci with phagocytic leukocytes from men and mice. Infect. Immun. **8**:98–104.
47. **Tsai, C., and C. E. Frasch.** 1982. A sensitive silver stain for detecting lipopolysaccharides in polyacrylamide gels. Anal. Biochem. **119**:115–119.
48. **van der Ley, P.** 1988. Three copies of a single protein II-encoding sequence in the genome of *Neisseria gonorrhoeae* JS3: evidence for gene conversion and gene duplication. Mol. Microbiol. **2**:797–806.
49. **Virji, M., and J. E. Heckels.** 1986. The effect of protein II and pili on the interaction of *Neisseria gonorrhoeae* with human polymorphonuclear leukocytes. J. Gen. Microbiol. **132**:503–512.
50. **Ward, M. E., and P. J. Watt.** 1972. Adherence of *Neisseria gonorrhoeae* to urethral mucosal cells. An electron microscopic study of human gonorrhea. J. Infect. Dis. **126**:601–604.
51. **Watt, P. J., and M. E. Ward.** 1980. Adherence of *Neisseria gonorrhoeae* and other neisseria species to mammalian cells, p. 251–288. *In* E. H. Beachey (ed.), Bacterial adherence. Chapman and Hall, Ltd., London.
52. **Wright, D. G., and J. J. Gallin.** 1979. Secretory responses of human neutrophils: exocytosis of specific (secondary) granules by human neutrophils during adherence *in vitro* and during exudation *in vivo*. J. Immunol. **123**:285–294.
53. **Zak, K., J. L. Diaz, D. Jackson, and J. E. Heckels.** 1984. Antigenic variation during infection with *Neisseria gonorrhoeae*: detection of antibodies to surface proteins in sera of patients with gonorrhea. J. Infect. Dis. **149**:166–174.