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Gonococci Possessing Only Certain P.II Outer Membrane Proteins Interact with Human Neutrophils

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We investigated the role of the protein II (P.II) family of gonococcal outer membrane proteins in the interaction of seven single P.II variants of *Neisseria gonorrhoeae* FA1090 with human neutrophils in vitro. The abilities of nonpiliated gonococci to adhere to and be killed by neutrophils and to stimulate luminol-dependent chemiluminescence (CL) depended on the possession of at least one P.II. Gonococci lacking P.II (i.e., P.II$^-$) adhered poorly to and were not killed by neutrophils and induced only minimal CL. Although most P.II-containing (i.e., P.II$^+$) variants adhered to, stimulated, and were readily killed by neutrophils, one variant, containing P.IIa, possessed none of these characteristics; it acted just like a P.II$^-$ variant. No correlation was found between the colony opacity phenotype and the interaction of gonococci with neutrophils. Data from CL experiments suggest that the stimulatory effect of P.II was dominant over that of pili; i.e., piliated P.II$^+$ gonococci were much more stimulatory than piliated P.II$^-$ gonococci. The results indicate that most but not all P.II proteins mediate, in part or in full, the interaction of *N. gonorrhoeae* with human neutrophils, including adherence, stimulation of the neutrophil respiratory burst, and phagocytic killing.

Strains of *Neisseria gonorrhoeae* produce colonies that vary in their ability to deflect transillumination; colonies are thus transparent or, to various degrees, opaque. Opacity is accompanied by the appearance of extra outer membrane proteins, termed opacity-associated proteins (19, 28, 29, 36). Opacity-associated proteins are a subset of protein IIs (P.IIs), since gonococci can possess certain P.IIs and yet not form opaque colonies. P.IIs are about 30 kilodaltons in size and are heat modifiable when analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (19, 20, 28, 29, 36). Individual strains of gonococci possess the capacity to express at least six to eight different P.IIs, and switch from P.II positive (P.II$^+$) to P.II negative (P.II$^-$) or from one P.II type to another at a rate of about 10^{-3} per cell per generation (1, 21, 27; T. D. Connell, W. J. Black, T. H. Kawula, D. S. Barritt, J. F. Dempsey, K. Kverland, Jr., A. Stephenson, B. S. Schepart, G. L. Murphy, and J. G. Cannon, Mol. Microbiol., in press).

P.IIs are associated with increased adherence of gonococci to human buccal epithelial cells, conjunctival cells, and human neutrophils (2, 20, 34, 35, 37). Swanson and colleagues described a specific P.II that is not associated with opacity, termed leukocyte association protein (16, 17, 31, 32). Apparently only certain P.IIs, defined by their electrophoretic mobility in polyacrylamide gels, are associated with increased leukocyte or epithelial cell association (37). It is unclear, however, whether opacity and leukocyte association are related (2, 16, 17, 30) and whether possession of all P.IIs leads to increased leukocyte association (35, 37).

Some strains of opaque gonococci are phagocytized and killed by human neutrophils and monocytes, regardless of the presence of fresh serum opsonins (22, 24). Virji and Heckels have indicated that all P.II$^+$ gonococci of strain P9 stimulate human neutrophil chemiluminescence (CL) and are phagocytically killed by such neutrophils (35). Rest et al. showed that a monoclonal antibody to a P.II of strain FA1090 specifically inhibits the stimulation of neutrophil CL by the homologous P.II$^+$ gonococcal variant (25), whereas Virji and Heckels found inhibition of such function with Fab fragments of anti-P.II monoclonal antibody (35).

Clinically, opaque variants are recovered more often from localized symptomatic gonococcal infections, from infected male urethras, and from women during midcycle, whereas transparent (but not shown necessarily to be P.II$^-$) variants are isolated more often from asymptomatic or disseminated infections and from infected women during menstruation (12). Anti-P.II antibody is made during gonococcal infection (38). Thus, P.IIs appear to be heterogeneous functionally, as well as structurally, and may play a role in pathogenesis. Neutrophils are the primary phagocytes found in gonorrhea (11). Although a good deal of attention has been focused on the role of P.IIs in the interaction of gonococci with human leukocytes, the picture remains unclear as to the generality of such observations. We have quantitated gonococcal adherence to neutrophils and have identified a P.II$^+$ gonococcal variant that formed highly opaque colonies, yet it did not stimulate or adhere to neutrophils.

MATERIALS AND METHODS

Bacteria. *N. gonorrhoeae* FA1090 was obtained from Janne Cannon, Department of Microbiology, University of North Carolina School of Medicine, Chapel Hill. Seven P.II variants were used for this study; one contained no P.II, and six contained one P.II each and were designated P.IIa through P.IIf. The presumptive P.II contents of the variants were determined daily by colony morphology, since each of the variants produced colonies of slightly different color and opacity (Table 1). The P.II content was verified by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (18) (Fig. 1) or transfer to nitrocellulose paper with subsequent Western (immunoblot) analysis with monoclonal antibodies specific for the various P.IIs (data not shown). Variant P.IIf always had a major and a minor heat-modifiable protein. We could not differentiate at this time whether these proteins are

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TABLE 1. Colony morphologies of P.II variants[a]

Variant	Colony opacity	Colony color
P.II$^-$	Transparent	Blue
P.IIa	Very opaque	Yellow-gold
P.IIb	Transparent	Snowy blue
P.IIc	Medium opaque	Blue-white
P.IId	Very opaque	White-yellow
P.IIe	Medium opaque	White
P.IIf	Medium opaque	Snowy blue-white

[a] Well-isolated, 16- to 20-h colonies of strain FA1090 were identified by the method of Swanson (28). All colonies had smooth or buttery consistencies.

two different P.IIs or whether they are two similar forms of the same P.II that are always coexpressed. Hybridoma culture supernatants containing monoclonal antibodies specific for FA1090 P.IIa, P.IIb or P.IId, P.IIc, P.IIe, and P.IIf were generous gifts of J. Cannon. These variants and P.II monoclonal antibodies have been described elsewhere (1, 3; T. D. Connel et al., in press). We believe that the P.II variants discussed below are similar to the P.II variants described by J. Cannon and her colleagues, as determined by reaction on Western blots with monoclonal antibody; however, in our hands, the mobilities of the P.IIs in polyacrylamide gels were slightly different from that previously reported (3). Piliated P.II$^+$ variants of strain F62, originally gifts from P. F. Sparling, University of North Carolina School of Medicine, were used for one series of experiments. The P.II content of this strain has been discussed elsewhere (26). For the experiments comparing phagocytoses of mixed populations of P.II$^+$ and P.II$^-$ gonococci, strain P9 was used. Strain P9 was a gift from John Heckels, University of Southampton Medical School, Southampton General Hospital, Southampton, United Kingdom.

Various colony phenotypes were selectively transferred daily on GC medium base (Difco Laboratories, Detroit, Mich.) with added supplements and incubated in a humidified incubator at 36°C in 6% CO_2 in air, as described previously (23, 26). Representative colonies of the variant(s) to be used for each experiment were suspended in 100 μl of GC broth containing 20% glycerol and frozen for later evaluation and confirmation of P.II content. Colony types were identified by the criteria of Kellogg et al. (13, 14) and Swanson (28, 29). For daily use, suspensions of plate-grown gonococci were diluted and grown in GC broth containing supplements to mid-log phase (Klett reading with green filter, 90 to 110) in a rotary water bath shaker at 36°C (26). Gonococci were washed once (5,000 × *g*, 10 min) and suspended in Hanks balanced salt solution containing 0.1% gelatin (pH 7.4) (HBSG) to an optical density at 550 nm of 0.3 (Spectronic 20 spectrometer; Bausch & Lomb, Inc., Rochester, N.Y.). This suspension contained 2 × 10^8 CFU/ml.

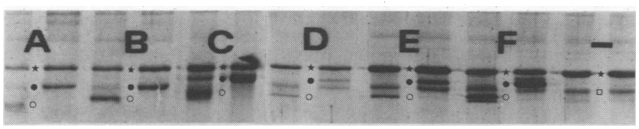

FIG. 1. Coomassie blue-stained sodium dodecyl sulfate-polyacrylamide gel electrophoretogram of outer membrane preparations of *N. gonorrhoeae* FA1090 P.II variants (P.IIa through P.IIf [lanes A through F] and P.II$^-$ [lane −]). The left sample of each pair was boiled for 5 min before being subjected to electrophoresis; the right sample was maintained at 37°C. Symbols: (★), protein I; (●), boiled P.II; (○), unboiled P.II; (□), protein III.

Neutrophils. Peripheral venous blood was obtained from consenting, apparently healthy adult volunteers. Neutrophils were purified in one step from whole blood through a Ficoll-Hypaque mixture, as described by Ferrante and Thong (10). In some experiments, contaminating erythrocytes were hypotonically lysed; however, erythrocytes did not interfere with the adherence, phagocytic, or CL assay when nonpiliated gonococci were used (data not shown). Leukocyte suspensions contained ≥95% neutrophils (with eosinophils as the major contaminating cell type) and were ≥98% viable as determined by exclusion of 0.2% trypan blue in HBSG. Purified neutrophils were suspended in HBSG to 1 × 10^7 to 5 × 10^7/ml.

Phagocytic killing. Phagocytosis was performed in rotating polypropylene tubes (12 by 75 mm; Becton Dickinson and Co., Cockeysville, Md.) at 37°C (24). Phagocytosis mixtures contained 1 × 10^7 each of neutrophils and gonococci. No serum was present in the assays. At various times, 10-μl samples were removed, appropriately diluted in GC broth or HBSG, plated on GC medium base, and incubated. Gonococcal viability was determined by quantitating CFU at 18 to 36 h. This assay measures viable, nonphagocytized gonococci (24); significant gonococcal clumping of most variants was not observed, as determined by phase-contrast microscopy. When clumping did occur, a given variant did not change its clumping characteristics over time. This was indicated by the fact that in the absence of neutrophils all variants retained their viability throughout the assay period. Had clumping significantly increased or decreased, CFU would have decreased or increased, respectively. Results are expressed as percent viable gonococci and were determined by the following formula: percent viable gonococci = 100 × (CFU at time *N*)/(CFU at time 0), where *N* is 45, 90, or 135 min.

CL. Luminol-enhanced CL was measured according to Trush et al. (33). Assays were performed in 10-ml polyethylene scintillation vials containing the following per ml: 17.8 μg of luminol (10^{-5} M), 10^6 neutrophils, and 1 × 10^6 to 50 × 10^6 CFU of gonococci in a final HBSG volume of 5 ml. Some of the later assays were run in a final HBSG volume of 1 ml, with no differences observed in results. No serum was present in the assays. Vials were counted in various scintillation counters, at ambient temperature, in the in-coincidence mode, and with tritium-preset windows. Each vial was counted for 6 to 12 s, and the counting cycle was repeated without further mixing of the vial contents. Results are expressed as counts per minute.

Adherence of gonococci to neutrophils. Unless indicated otherwise, gonococci (250 × 10^5 to 500 × 10^5) and neutrophils (5 × 10^5) were suspended in 1.0 ml of HBSG and were tumbled end over end at 37°C at 12 rpm in 4-ml plastic tubes. Alternatively, the tumbling-tube assay was performed with 125 × 10^5 to 250 × 10^5 gonococci and 2.5 × 10^5 neutrophils in 500 μl of HBSG in 1.5-ml conical, snap-cap plastic centrifuge tubes. To prevent phagocytosis, neutrophils were treated for 15 min at room temperature with cytochalasin b at a final concentration of 5 μg/ml before they were added to adherence assay tubes. Preliminary experiments showed that cytochalasin b did not affect neutrophil viability as measured by 0.2% trypan blue exclusion. At 30 min, 150 μl of the adherence assay suspension was removed, centrifuged onto glass slides with a cytocentrifuge (Shandon Southern Corp., Sewickley, Pa.), and stained with Wright's stain. One to two hundred consecutive, stained neutrophils were counted (oil immersion light microscopy, magnification, ×1,000), and results are reported, in histogram form, as the

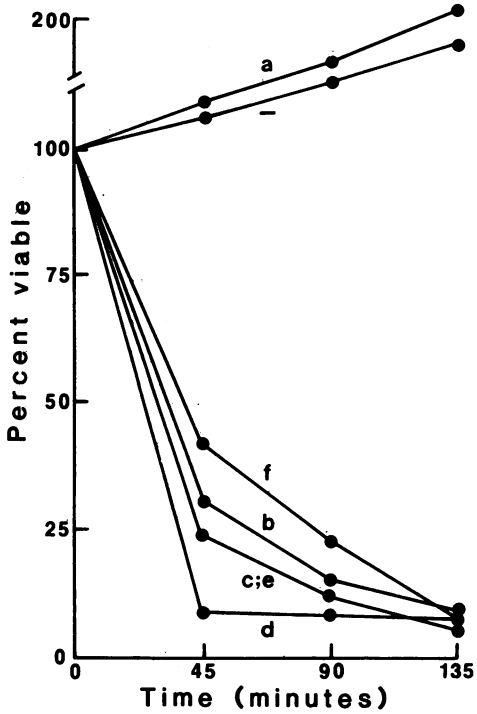

FIG. 2. Phagocytic killing of nonpiliated *N. gonorrhoeae* FA1090 P.II variants by human neutrophils in the absence of serum. The data are representative of three similar experiments. –, P.II$^-$; a through f, P.IIa through P.IIf, respectively.

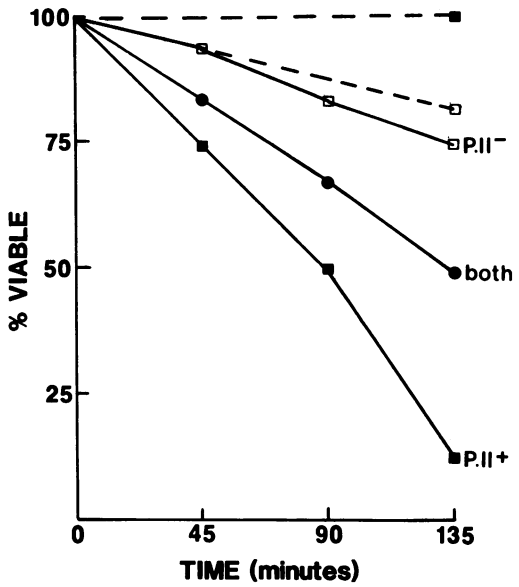

FIG. 3. Phagocytic killing of nonpiliated *N. gonorrhoeae* P9-1 (P.II$^-$; transparent) and P9-6 (P.II$^+$; opaque) by human neutrophils in the absence of serum. ———, Gonococcal viability in the presence of neutrophils; -----, gonococcal viability in the absence of neutrophils. The data are representative of three similar experiments.

percentage of neutrophils with zero, one to two, three to five, six to eight, or more than eight adherent diplococci. By presenting data in this manner, both quantitative and qualitative observations can be made.

RESULTS

Phagocytic killing. All but one of the strain FA1090 P.II variants were killed by neutrophils in the absence of serum (Fig. 2). P.IIa, a variant that produced deep yellow, butyrous colonies, was not phagocytically killed; it acted just like it had no P.II. The possession of more than one P.II did not lead to increased killing above that observed for gonococci possessing only one P.II (data not shown). In experiments in which no killing occurred, i.e., with the P.IIa or P.II$^-$ variants, we often saw increases in CFU above control numbers at time 0.

Whereas FA1090 P.IIa was not phagocytically killed, it formed very opaque colonies. FA1090 P.IId was another variant that formed very opaque colonies, yet it was fully stimulatory. On the other hand, other fully stimulatory variants, such as P.IIb, formed transparent colonies. These observations suggest there is no correlation between colony opacity and the ability of gonococci to interact with neutrophils.

Only about 95% of input gonococci were killed by neutrophils (Fig. 2). We noted that the survivors yielded transparent colonies and were probably P.II$^-$. This observation presented us with an opportunity to investigate whether extracellular killing of gonococci occurred during active phagocytosis or whether there might be soluble gonococcal factors that could stimulate neutrophils to phagocytize P.II$^-$ organisms in the vicinity. Equal numbers of variant P9-6, which contains a P.II (19, 35, 37), and P9-1, which is P.II$^-$, were incubated with neutrophils in the phagocytic killing assay, and the viability and colony morphology of the survivors were observed over time. We could differentiate between killing of the two variants since P9-6 forms opaque colonies whereas P9-1 forms transparent colonies; only the opaque variant was killed (Fig. 3). (The remaining viable transparent colonies were P.II$^-$ as determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis.) The presence of the transparent variant neither inhibited nor augmented the killing of the opaque variant. This observation supports and extends previous observations that phagocytosis is needed for killing of gonococci and that killing of extracellular (P.II$^-$) gonococci does not occur, even during active phagocytosis and killing of neighboring P.II$^+$ gonococci. These experiments have been repeated with strains FA1090 and F62, with similar results (data not shown).

CL. The ability of the P.II$^+$ variants to stimulate CL by neutrophils paralleled their ability to be phagocytically killed. All but one of the P.II$^+$ variants stimulated active CL; the P.IIa and P.II$^-$ variants did not (Fig. 4). The low amount of CL induced by P.IIa or P.II$^-$ variants at later assay times was probably due to P.II$^+$ variants arising from P.II$^-$ or P.IIa variants. We have shown previously that both the degree and time course of neutrophil CL induced by opaque gonococci are dose dependent (24). Thus, even the few P.II$^+$ variants that are present in a large population of P.II$^-$ organisms are enough to stimulate late and low neutrophil CL.

To investigate the relative roles of pili and P.II in the stimulation of neutrophil CL, one set of experiments was done with opaque, piliated (type 1 or type 2) variants of strain F62. The results of such experiments were very similar to those presented above for nonpiliated variants. Transparent (P.II$^-$) type 1 or type 2 organisms were only minimally stimulatory, whereas opaque (P.II$^+$) type 1 or type 2 organisms were highly stimulatory (Fig. 5). Thus the

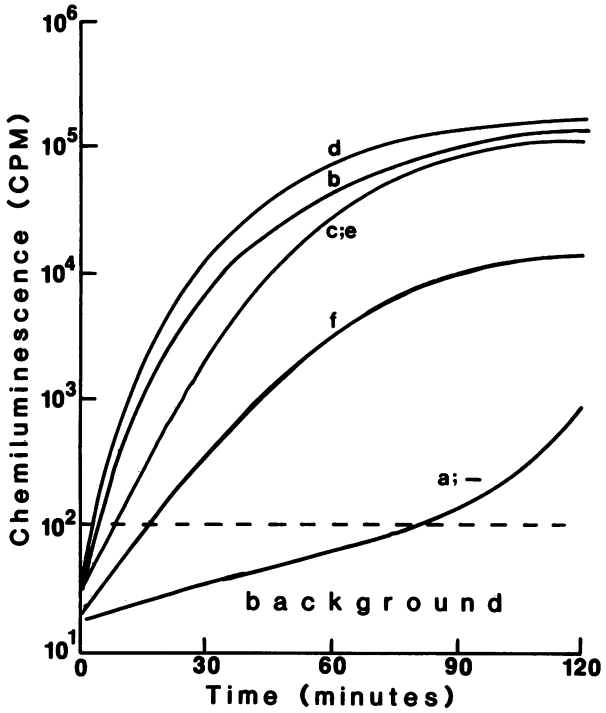

FIG. 4. Neutrophil CL induced by nonpiliated *N. gonorrhoeae* FA1090 P.II variants in the absence of serum. The area below the dashed line represents background CL produced by neutrophils in the absence of gonococci. Gonococci by themselves produced no CL above the background. The data are representative of three similar experiments. –, P.II$^-$; a through f, P.IIa through P.IIf, respectively.

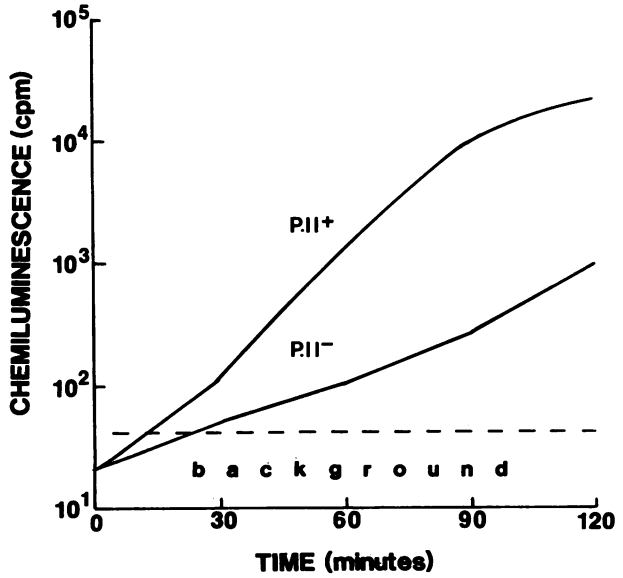

FIG. 5. Neutrophil CL induced by opaque (P.II$^+$) or transparent (P.II$^-$) type 2, piliated *N. gonorrhoeae* F62 in the absence of serum. The area below the dashed line represents background CL produced by neutrophils in the absence of gonococci. The data are representative of three similar experiments.

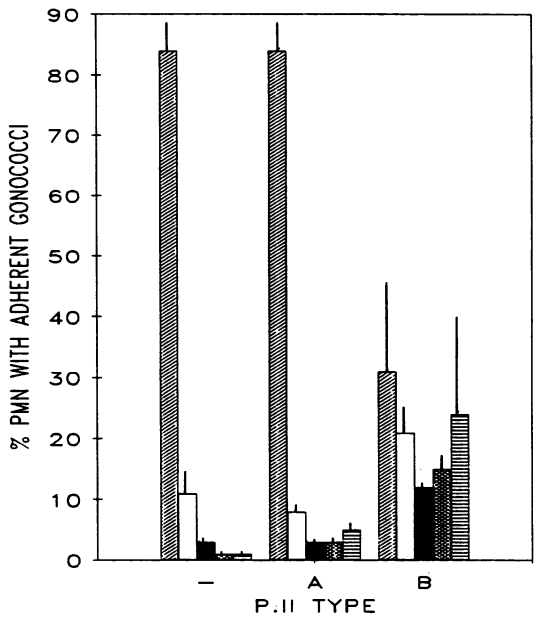

FIG. 6. Adherence of strain FA1090 P.II$^-$, P.IIa, and P.IIb variants to human neutrophils. Results are expressed as the percentages of neutrophils with zero (▨), one to two (▭), three to five (■), six to eight (▩), or more than eight (▤) adherent gonococci per polymorphonuclear neutrophil (PMN). Numbers are averages. Bars represent one sample standard deviation. For P.II$^-$, there were nine replicates over 5 days; for P.IIa, there were five replicates over 3 days; and for P.IIb, there were six replicates over 3 days.

effects of P.IIs appear to be stronger than those of pili in inducing neutrophil CL and are not masked by pili. In addition, piliated, P.II$^-$ gonococci appeared to be only weakly stimulatory. Pili type was not determined.

Adherence. We next asked whether the lack of phagocytic killing and the lack of induction of neutrophil CL by P.IIa and P.II$^-$ variants were due to the lack of adherence of the variants to neutrophils or whether such gonococci did adhere to neutrophils but lacked a stimulatory, but as yet unidentified, prophagocytic component. The P.II$^-$ and P.IIa variants did not adhere to neutrophils, whereas all other P.II variants tested did (Fig. 6; data for variants P.IIc through P.IIf not shown). The adherence assay depended on input numbers of gonococci (Fig. 7) and on temperature (data not shown). At 3°C, gonococci did not stick to neutrophils, and adherence increased with increasing temperature. The results suggest that P.II is responsible both for adherence to and stimulation of neutrophils.

DISCUSSION

Gonococcal outer membrane proteins have been implicated in mediating adherence to host cells (35, 37) and in mediating association with, stimulation of, and phagocytic killing by human neutrophils and monocytes (16, 17, 22, 24, 25, 35). The P.II family of outer membrane proteins may be responsible for some or all of the above effects (19, 20, 27, 28). In the present report, we show that most but not all P.IIs conferred upon gonococci the ability to adhere to, stimulate, and be phagocytized by human neutrophils. Gonococci that did not have P.II lacked all of these properties. Most of the present studies were performed with nonpiliated gonococci, not because pili are unimportant in gonococcal pathogenesis

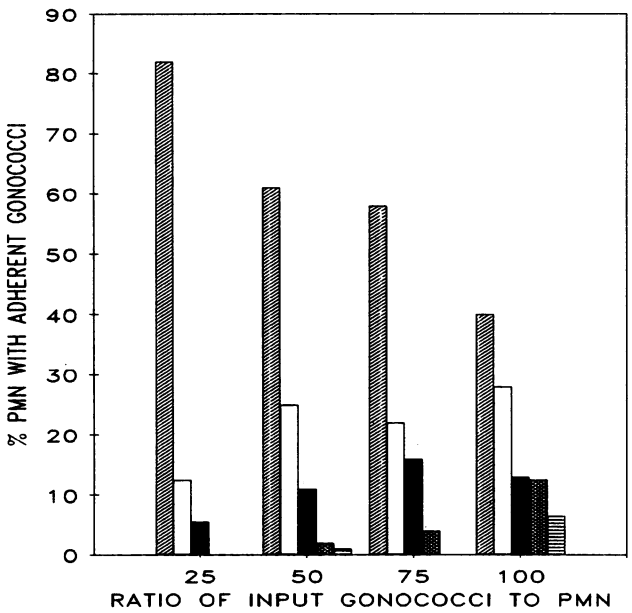

FIG. 7. Adherence of increasing input numbers of strain FA1090 P.IIb to human neutrophils. Results are expressed as described in the legend to Fig. 6. Numbers are the averages of duplicate samples. The data are representative of three similar experiments. Symbols and abbreviations are as described in the legend to Fig. 6.

but, rather, because we wanted to investigate P.II-mediated gonococcus-neutrophil interactions in the absence of gonococcal components that might be alternative mediators of such interactions.

Fancioli et al. (9) reported that *Trichomonas vaginalis* phagocytically kills type 3 (opaque, most likely P.II$^+$), but not type 4 (transparent, possibly P.II$^-$), gonococci in the absence of serum. Thus the prophagocytic activity of P.II might be functional with eucaryotic cells other than human neutrophils and monocytes. Blackwell and colleagues have suggested that lipooligosaccharide type is involved in the association of piliated gonococci with neutrophils, with localized strains adhering to a greater extent than disseminated strains do, and that such interactions might be mediated by neutrophil surface lectins (4, 15). Since we used both serum-sensitive (localized) and serum-resistant (disseminated) strains and since the adherence of both strains depended on the possession of at least one P.II, it appears that P.IIs are more important than lipooligosaccharide in the interaction of gonococci with neutrophils. Blackwell et al. did not comment on the presence of P.IIs in their studies.

We have repeated phagocytic, bactericidal, and CL assays with FA1090 P.IIa (the nonstimulatory variant) many times over a period of several years, always with the same results. This consistency of results suggests that if there is a gonococcal surface component other than P.II that is responsible for neutrophil interactions, its lack of expression must be tightly linked with P.IIa expression.

Virji and Heckels reported that all strain P9 P.II variants they tested are killed effectively by human neutrophils in the absence of serum and that all variants stimulate neutrophil luminol-enhanced CL, although the kinetics of induction of CL differs for the different P.II variants (35). It is puzzling that no differences were found amongst the P.II variants, since Lambden et al. and Watt and Ward observed that some of the same variants adhered to neutrophils quite differently (20, 37). Thus, whereas strain P9-16 (containing P.IIb) actually adhered less well to neutrophils than did strain P9-1 (P.II$^-$), Virji and Heckels (35) observed excellent killing and rapid induction of CL by strain P9-16 and no killing or induction of CL by P9-1. The reasons for such differences remain to be determined.

The experiments that investigated neutrophil killing of a population of gonococci composed of a P.II$^+$ variant and a P.II$^-$ variant (Fig. 3) suggest that P.IIs act locally, on the surface of the gonococcus, as opposed to being released into the environment to act upon the neutrophil in an extragonococcal form. If the P.IIs were being released and were randomly stimulating the neutrophils to increased phagocytic activity, we would expect that the transparent variants would also be phagocytized and killed; however, they were not. In addition, toxic oxygen intermediates and lysosomal components are continuously released in the tumbling-tube assays, which leads to continuous exposure of extracellular gonococci to these products (7). Since the transparent gonococci (P.II$^-$) survived the phagocytic killing assays, the concentration of extracellular antibacterial components must have been at subbactericidal levels. These results support our previous observations and those of Densen and Mandell that phagocytosis is necessary for killing of gonococci in vitro (8, 24).

Our results do not support the data or conclusions of Smith and his colleagues who have shown that gonococci survive and multiply within human neutrophils (5). Although there probably are unlimited reasons for these discrepancies, the most likely reasons are that Smith and his colleagues used a phagocytic bactericidal assay and gonococcal strains and growth conditions that differed significantly from those used in our laboratory. Neither do these results support, nor necessarily contradict, the recently proffered hypothesis by Cohen and Cooney that gonococci, especially in the presence of serum, might reduce neutrophil killing by depleting local concentrations of oxygen (6). Several explanations exist. (i) Our reactions did not contain serum; (ii) our experiments were well aerated and included a low number of gonococci; and perhaps of greatest importance, (iii) oxygen is not needed for killing of gonococci by neutrophils, since neutrophils from patients with chronic granulomatous disease, which are incapable of mounting a respiratory burst, readily kill gonococci (24).

Several mechanisms could account for the ability of P.II$^+$ gonococci to stimulate neutrophils. Two mechanisms that seem most reasonable are (i) that P.II itself is stimulatory and binds to a specific neutrophil receptor and (ii) that P.II acts in conjunction with another gonococcal surface component to bind to and stimulate neutrophils. Further characterization of gonococcus-neutrophil interactions and investigation of the molecular differences between the inactive P.IIa and active P.IIs should lead to a better understanding of how P.IIs mediate adherence to human neutrophils.

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