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Anaerobic Growth of Gonococci Does Not Alter Their Opa-Mediated Interactions with Human Neutrophils

JOSEPH V. FRANGIPANE* AND RICHARD F. REST

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

Received 23 December 1991/Accepted 5 February 1992

Gonococci grown anaerobically (anaerobic gonococci) in the presence of nitrite induce the expression of at least three novel outer membrane proteins (PANs 1 to 3). Although PAN 1 is expressed by gonococci during gonorrhea, the function of the PAN proteins remains unknown. In the absence of serum, gonococci possessing opacity-associated (Opa, formerly PII) outer membrane proteins adhere to, stimulate, and are phagocytically killed by human neutrophils. Gonococci lacking Opa proteins demonstrate none of these activities. We investigated whether the PAN proteins, or any other characteristics of anaerobic gonococci, altered the ability of nonpiliated, Opa$^+$ or Opa$^-$ gonococci to adhere to, stimulate, or be phagocytically killed by neutrophils. The expression of Opa4 by strain F62, as determined by its relative mobility on sodium dodecyl sulfate-polyacrylamide gels, appeared to be unaltered by anaerobic growth, as seen previously (V. L. Clark, L. A. Campbell, D. A. Palermo, T. M. Evans, and K. W. Klimpel, Infect Immun. 55:1359–1364, 1987). Anaerobic and aerobic Opa$^+$ gonococci adhered to and stimulated neutrophils to the same extent. Similarly, anaerobic and aerobic Opa$^-$ gonococci adhered to and stimulated neutrophils equally poorly. Finally, anaerobic and aerobic Opa$^+$ gonococci were equally sensitive to phagocytic killing by neutrophils, while anaerobic and aerobic Opa$^-$ gonococci were equally resistant to killing. Thus, the role of Opa proteins in mediating the interactions of gonococci with human neutrophils appears unaltered by anaerobic growth, and the PAN proteins, or other cryptic properties of anaerobic gonococci, do not seem to modulate or mediate these phenomena.

Neisseria gonorrhoeae, the causative agent of gonorrhea, can be isolated from the genitourinary tract (38), and from individuals suffering from pelvic inflammatory disease (4, 8), in the presence of obligate anaerobes. These primary isolates can survive and grow for long periods without oxygen (20, 37). However, upon passage in vitro, gonococci grow anaerobically only if provided with millimolar concentrations of nitrite, which they use as a terminal electron acceptor (24). Nitrite is readily available in vivo from biological fluids (48) and from the metabolism of normal anaerobic flora (16).

Anaerobically grown gonococci (anaerobic gonococci) express at least three novel outer membrane proteins (PANs 1 to 3) and repress at least five proteins (POXs 1 to 5) that are expressed only under aerobic conditions (9). Such an induction of new outer membrane proteins could be crucial to colonization or pathogenesis at anaerobic sites of infection, but the function, if any, of these proteins remains unknown. Sera from patients recovering from gonococcal infections contain antibody that reacts strongly with PAN 1 on Western blots (immunoblots), while sera from healthy individuals do not, indicating that gonococci express PAN 1 at some point during infection, probably as a result of anaerobic growth in vivo (10). This suggests that anaerobic cultivation of gonococci may more closely resemble clinical infection conditions than the routine aerobic incubation performed in most laboratories.

Gonococci possess antigenically variable outer membrane proteins, termed opacity-associated (Opa, formerly PII) proteins, which mediate gonococcal adherence to and stimulation of human polymorphonuclear leukocytes (neutrophils), as well as nonopsonic phagocytosis of gonococci by neutrophils (14, 18, 21, 26, 33, 36, 40, 43, 45). In addition, some Opa proteins mediate adherence to and invasion of various human epithelial cell lines (17, 17a, 29, 37a, 46, 47). Given the vital role that Opa proteins play in the interaction of gonococci with neutrophils, we were interested in discovering whether anaerobic growth alters these Opa-mediated interactions. We report here that neither PAN nor POX proteins seem to function in the adherence to or stimulation of neutrophils, nor do they modulate the ability of gonococci to be phagocytically killed by neutrophils. Thus, the role of Opa proteins in mediating these phenomena remains unaltered by anaerobic growth.

MATERIALS AND METHODS

Gonococcal strains and growth conditions. Nonpiliated Opa4 and Opa$^-$ variants of gonococcal strain F62 were transferred daily on GC medium base (Difco Laboratories) with supplements (32, 34). Proper Opa content and lack of piliation were determined by colony morphology, using the criteria established by Kellogg et al. (19) and Swanson (39), and were verified by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blots of outer membrane preparations (see below).

Agar plates were streaked confluently with a suspension of gonococci at ~6 × 10^8/ml, and the plates were incubated aerobically for 15 to 16 h at 37°C in 5% CO_2. Anaerobic gonococci were also plate grown, (9, 24) by using a centrally placed 0.5-in (ca. 1.3-cm) filter disk saturated with 45 μl of 16% sodium nitrite (0.10 mmol on the filter disk). Incubation in the absence of oxygen for 15 to 16 h at 37°C yielded a ring of growth extending 2 to 3 cm from the center of the filter disk. Anaerobic incubation was conducted inside an anaerobic jar containing a BBL GasPak Plus system or inside an

* Corresponding author.

anaerobic chamber. The chamber was filled and purged with an anaerobic gas mixture (5% CO_2, 10% H_2, 85% N_2) and contained a palladium-coated catalyst to remove residual oxygen. Strict anaerobiosis was maintained in the chamber, as determined with methylene blue anaerobic indicator strips (BBL) and routine growth of obligate anaerobes (*Clostridium* spp.). All liquid and agar media were prereduced in the chamber for 24 h before being used in experiments.

For all assays, gonococci were swabbed from agar plates and resuspended to 2×10^8 CFU/ml (optical density at 550 nm, 0.18; Spectronic 20) in Dulbecco's phosphate-buffered saline with 0.1% (wt/vol) gelatin (PBSG) and 0.01% (wt/vol) each $CaCl_2$ and $MgCl_2$. Gonococci were maintained at room temperature for no longer than 15 min prior to use.

Outer membrane preparations, SDS-PAGE, and Western blotting. Outer membranes were prepared from plate-grown anaerobic and aerobic gonococci by using 1.0 M LiCl, as described by Leith and Morse (28). Outer membrane preparations were solubilized in Laemmli sample buffer, incubated at either 37°C for 30 min or 100°C for 5 min, and separated by 12% acrylamide SDS-PAGE. Electrophoresis was conducted for approximately 80 min at 150 V, using the Laemmli buffer system (25), in a Bio-Rad Mini Protean II apparatus, and the gel was fixed overnight in a 50% methanol solution. Proteins were visualized by using a modification of the silver stain method of Tsai and Frasch (44), followed by staining with 0.3% Coomassie brilliant blue in 45% isopropanol–10% acetic acid, and destaining with 25% isopropanol–10% acetic acid. Western blots were done as described by Bszewczyki and Koloft (2), as modified by Elkins and Rest (11).

Neutrophil purification. Human neutrophils were purified from freshly heparinized venous blood by a single centrifugation through Ficoll-Hypaque, as described by Ferrante and Thong (13). Cells were washed once in ice-cold PBSG, depleted of erythrocytes by hypotonic lysis, and washed again. Then, neutrophils were resuspended to 10^7/ml in PBSG and maintained on ice until use. This procedure typically yields neutrophils that are ≥94% pure as determined with Wright's stain and ≥99% viable as measured by exclusion of 0.25% trypan blue in saline at room temperature. For anaerobic assays, neutrophils were prereduced in an anaerobic chamber on ice for 1 h prior to use.

Adherence of gonococci to neutrophils. Adherence assays were performed in either an aerobic environment or an anaerobic chamber (see above). Opa receptors were upregulated to the surface of 5×10^5 neutrophils in 1.0 ml of PBSG plus Ca^{2+}-Mg^{2+} by preincubating neutrophils with 5 μg of cytochalasin B and then with 10 ng of phorbol myristate acetate (PMA), each for 5 min at 37°C (12). Cytochalasin B inhibits actin polymerization, thereby enhancing neutrophil degranulation and preventing phagocytosis (1). Upregulation of Opa receptors promotes maximal adherence of Opa^+ gonococci to neutrophils (12). Gonococci (2×10^7) were added to the upregulated neutrophils, and the mixture was tumbled for 30 min at 37°C. Neutrophils and adherent gonococci were deposited onto glass slides by using a Shandon cytocentrifuge and stained with Wright's stain. Results were determined by counting gonococci adherent to 100 contiguous neutrophils and are expressed as the average number of gonococci per neutrophil.

Neutrophil LDCL. For determination of neutrophil luminol-dependent chemiluminescence (LDCL), 5×10^5 unstimulated neutrophils in 1.0 ml of PBSG plus Ca^{2+}-Mg^{2+} were mixed with 10 μl of 10^{-3} M luminol in dimethyl sulfoxide. Then, 4×10^7 gonococci were added to the mixture, and LDCL was measured continuously at 37°C with an LKB-Wallac 1251 luminometer until a peak was observed (~90 min). Results are expressed as the percentages of control peak LDCL stimulated by aerobic gonococci.

Phagocytic killing of gonococci by neutrophils. Phagocytic killing assays were performed by a modification of the method of Fisher and Rest (14). Unstimulated neutrophils (10^7) were preincubated in buffer alone (1.0 ml of sterile PBSG plus Ca^{2+}-Mg^{2+} and 0.1% glucose) or in buffer containing 5 μg of cytochalasin B for 5 min at 37°C. Cytochalasin B-treated neutrophils are incapable of phagocytosis and were used in negative control assays. Gonococci (10^7 in the buffer described above) were added to the neutrophils, and reaction tubes were rotated end over end at 37°C. At specific times, 10-μl samples were appropriately diluted in sterile buffer and plated on GC medium base for overnight incubation. After quantitation of colonies, values from duplicate plates were averaged. Results are expressed as percent viable gonococci, determined as follows: 100 × (CFU exposed to untreated neutrophils at time *T*/CFU exposed to cytochalasin B-inhibited neutrophils at time *T*), where *T* is 45, 90, or 135 min. Because assays under anaerobic and aerobic conditions were conducted about 30 min apart, 15-h-old plate cultures of anaerobic and aerobic gonococci were inoculated 30 min apart so that gonococci of both phenotypes were harvested at the same point in their growth phase. Assays performed under anaerobic conditions used prereduced buffers containing 0.01% sodium nitrite (1.4 mM) to maintain gonococcal viability, as described by Casey et al. (5, 6).

RESULTS

Anaerobic growth of gonococci in the presence of nitrite. Anaerobic or aerobic gonococci, both incubated in the presence of nitrite, grew at the same rate as aerobic gonococci grown without nitrite. Bacteria obtained by swabbing an equivalent area of growth from each culture plate yielded similar amounts of growth, as measured by turbidimetric methods, by viability (CFU), and by number of bacteria per milliliter (Petroff-Hauser counting chamber). Anaerobic growth of gonococci did not cause clumping of these nonpiliated organisms, nor did it change the average size of individual gonococcal cells, as observed by light microscopy (data not shown).

Several steps were taken in order to establish that gonococci were in fact growing under anaerobic conditions. Prereduction of agar medium and the use of methylene blue anaerobic indicator strips (BBL) assured an environment with a redox potential of at least −200 mV. Also, outer membranes were stripped from gonococci, solubilized in SDS, separated by 12% acrylamide SDS-PAGE and stained with silver and Coomassie blue (Fig. 1). Bands corresponding in relative mobility to PAN 1 were observed in preparations from anaerobic gonococci (lanes A and C) but not in preparations from aerobic gonococci grown without nitrite (lanes B and D) or with nitrite (data not shown). These results confirmed that we had grown gonococci under the anaerobic conditions described by Clark et al. and Knapp and Clark (9, 24). PAN 2, although not seen in Fig. 1, was detected in SDS-PAGE gels containing other outer membrane preparations (data not shown). PAN 3 is only observable in two-dimensional SDS-PAGE and was not visualized.

We also examined the appearance of Opa and other outer membrane proteins from anaerobic gonococci by SDS-PAGE (Fig. 1). Outer membrane preparations from anaero-

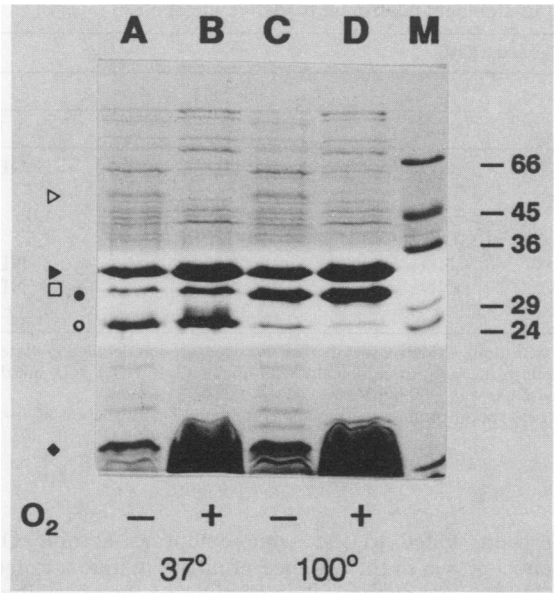

FIG. 1. Silver-Coomassie blue double-stained 12% acrylamide SDS-PAGE gel of gonococcal outer membrane proteins. Outer membranes were prepared from anaerobic F62 Opa4 gonococci (lanes A and C) and aerobic F62 Opa4 gonococci (lanes B and D) and incubated at 37°C (lanes A and B) or 100°C (lanes C and D). Outer membrane components of interest are PAN 1 (△), PI (▲), PIII (□), Opa4 at 100°C (●), Opa4 at 37°C (○), and LOS (◆). To better visualize PAN 1 and preserve the resolution of Opa4, PI, and PIII, the upper and lower regions of the gel were developed more, and the center region was developed less, during photography. Molecular weight markers are also pictured (lane M), and their masses in kilodaltons are shown on the right.

bic F62 Opa4 gonococci contained PI and PIII, as well as the Opa4 protein, which demonstrated a characteristic shift in relative mobility when boiled at 100°C (compare lanes A and C). The relative mobilities of these proteins appeared to be identical to those of their counterparts expressed by aerobic gonococci (lanes B and D). These results support similar data obtained by Clark et al. (9).

Our SDS-PAGE gels also revealed that the region containing lipooligosaccharide (LOS) molecules from anaerobic gonococci possessed considerably less silver-staining material than did that from aerobic gonococci (Fig. 1, compare lanes A and C to lanes B and D). It is not known whether the differences between the LOS regions of anaerobic and aerobic gonococci indicate a qualitative or quantitative change in LOS phenotype or in other silver-staining components. Further studies are in progress.

Adherence of anaerobic and aerobic gonococci to neutrophils. We wanted to determine the differences, if any, between unopsonized anaerobic and aerobic gonococci in their ability to adhere to human neutrophils. Neutrophils were preincubated with 5 μg of cytochalasin B per ml and 10 ng of PMA per ml, to prevent phagocytosis and upregulate Opa receptors. Then, gonococci were added, and adherence assays were performed in both anaerobic and aerobic environments (see Materials and Methods). We used Opa4 and Opa$^-$ gonococci of strain F62, which were grown aerobically, anaerobically in the presence of nitrite, or aerobically in the presence of nitrite to control for nitrite-specific effects.

In adherence assays performed in air, anaerobic and aerobic F62 Opa4 gonococci adhered equally well to neutrophils (an average of ~5.2 gonococci per neutrophil) (Table 1). Growth in nitrite did not alter the adherence of aerobic gonococci, thus excluding the possibility of nitrite effects. Regardless of anaerobic or aerobic phenotype, F62 Opa$^-$ gonococci adhered equally poorly to neutrophils (an average of ~0.4 gonococcus per neutrophil). We also performed two experiments entirely in an anaerobic chamber (Table 1). Similar results were obtained under these circumstances and eliminate the possibility that anaerobic gonococci had reverted to an aerobic phenotype during the 45 min it took to perform the assays. These results suggest that the PAN (and, conversely, the POX) proteins do not modulate the adherence of Opa$^+$ or Opa$^-$ gonococci to neutrophils. Also, the ability of Opa proteins to mediate gonococcal adherence to neutrophils is unaltered by anaerobic growth.

Stimulation of neutrophil LDCL by anaerobic and aerobic gonococci. After treatment with an appropriate agent, neutrophils use NADPH oxidase to generate reduced oxygen intermediates (O_2^- and H_2O_2), which are associated with oxygen-dependent killing of certain bacteria by these cells (15). The oxidase is stimulated by soluble agents, such as PMA, or by serum-treated particles, such as zymosan or gonococci (22, 34). In the absence of serum, Opa$^+$ gonococci stimulate the neutrophil oxidative burst, while Opa$^-$ gonococci do not. (14). We used unopsonized anaerobic and aerobic gonococci to determine whether Opa-mediated stim-

TABLE 1. Effect of anaerobic growth on gonococcal adherence to human neutrophils

Expt	Avg no. of gonococci/neutrophil[a]					
	F62 Opa4			F62 Opa$^-$		
	AnO_2	O_2	$O_2 + NO_2$	AnO_2	O_2	$O_2 + NO_2$
1	8.5	7.5	7.5	ND	ND	ND
2	5.3	5.4	4.9	0.4	0.4	0.4
3	4.5	ND	4.2	0.3	0.3	ND
4	4.9	4.8	4.2	0.4	0.4	0.4
5	3.9[b]	4.0	ND	0.4	ND	0.4
6	4.3[b]	4.8	ND	0.4	ND	0.3
Avg	5.2 ± 1.6	5.3 ± 1.3	5.2 ± 1.5	0.4 ± 0.0	0.4 ± 0.0	0.4 ± 0.0
%C[c]	98.1 ± 30.2	100 ± 24.5	98.1 ± 30.2	7.5 ± 0.0	7.5 ± 0.0	7.5 ± 0.0

[a] Opa4 or Opa$^-$ gonococci of strain F62 were mixed with stimulated neutrophils, and adherence was measured as detailed in Materials and Methods. Gonococci were grown anaerobically with nitrite (AnO_2), aerobically without nitrite (O_2), or aerobically with nitrite ($O_2 + NO_2$). ND, not done.

[b] Assay was performed in an anaerobic chamber to control for possible reversion of anaerobic gonococci to an aerobic phenotype.

[c] %C, percentage relative to control. Adherence of aerobic Opa4 gonococci was taken as 100%.

TABLE 2. Effect of anaerobic growth on ability of gonococci to stimulate neutrophil oxidative metabolism

Expt	LDCL (% of control)[a]					
	F62 Opa4			F62 Opa$^-$		
	AnO_2	O_2	$O_2 + NO_2$	AnO_2	O_2	$O_2 + NO_2$
1	93.5	100.0[b]	103.5	ND	ND	ND
2	85.5	100.0	97.2	3.0	4.2	3.7
3	94.4	100.0	87.4	2.3	3.6	4.0
4	85.9	100.0	104.0	3.0	2.0	4.5
5	94.3[c]	100.0	ND	ND	ND	ND
6	93.5[c]	100.0	ND	ND	ND	ND
Avg	91.2 ± 4.3	100.0	98 ± 7.7	2.8 ± 0.4	3.3 ± 1.1	4.1 ± 0.4

[a] Opa4 or Opa$^-$ gonococci of strain F62 were mixed with unstimulated neutrophils, and neutrophil oxidative metabolism was measured as detailed in Materials and Methods. Gonococci were grown anaerobically with nitrite (AnO_2), aerobically without nitrite (O_2), or aerobically with nitrite ($O_2 + NO_2$). ND, not done.

[b] LDCL stimulated by aerobic Opa4 gonococci was taken as the control (100%). Peak LDCL was ~60.0 mV.

[c] To control for possible reversion of anaerobic gonococci to an aerobic phenotype, gonococcal protein synthesis was inhibited by incubation of 4×10^7 gonococci with 100 μg of gentamicin per ml (1 h, 37°C) before assays were performed.

ulation of the oxidative burst was altered by anaerobic growth.

F62 Opa4 and Opa$^-$ gonococci were grown aerobically, anaerobically with nitrite, or aerobically with nitrite and were mixed with unstimulated neutrophils, and respiratory burst activity was measured by LDCL. All F62 Opa4 gonococci stimulated neutrophils to a similar extent (within a range of ±8.8%) regardless of whether they had an anaerobic or aerobic phenotype, while all F62 Opa$^-$ gonococci were equally nonstimulatory (Table 2). The LDCL assay requires an average of 90 min for measurement of peak LDCL, during which time anaerobic gonococci might revert to an aerobic phenotype. However, oxidative metabolism requires molecular oxygen and thus cannot be measured in an anaerobic chamber. Therefore, in an attempt to prevent switching of anaerobic gonococci to an aerobic phenotype, gonococcal protein synthesis was inhibited and gonococci were killed by preincubation with gentamicin (100 μg/ml, 1 h, 37°C). Results similar to those observed with live gonococci were obtained under these circumstances. The presence of gentamicin in the LDCL assay did not alter the peak oxidative activity of neutrophils in response to either PMA or opsonized zymosan (data not shown). These data suggest that the PAN (and, conversely, the POX) proteins do not influence the stimulation of neutrophil oxidative metabolism by Opa$^+$ and Opa$^-$ gonococci. Also, the role of Opa proteins in oxidative burst activation appears to be unaltered by anaerobic growth.

Phagocytic killing of anaerobic and aerobic gonococci. In the absence of serum, phagocytosis and subsequent killing of gonococci by neutrophils are dependent upon gonococcal expression of Opa proteins (14, 33, 36, 45). We wanted to determine whether anaerobic growth modified the Opa-dependent phagocytic killing of gonococci. Unstimulated neutrophils were preincubated at 37°C in buffer alone or in buffer containing 5 μg of cytochalasin B per ml to prevent phagocytosis. Then, gonococci were added, and phagocytic killing assays were performed in both anaerobic and aerobic environments (see Materials and Methods). We used Opa4 and Opa$^-$ gonococci of strain F62, which were grown aerobically, anaerobically in the presence of nitrite, or aerobically in the presence of nitrite (to control for any nitrite-specific effects).

Anaerobic and aerobic Opa4 gonococci were killed by neutrophils to the same extent (within a range of ±4.2%) (Fig. 2). Aerobic Opa4 gonococci grown in the presence of nitrite were killed to the same extent as aerobic Opa4 gonococci grown in the absence of nitrite (within a range of ±2.4%) (data not shown), thus eliminating the possibility of nitrite effects. We also observed that, regardless of anaerobic or aerobic phenotype, Opa$^-$ gonococci were not phagocytically killed by neutrophils (Fig. 2). The results presented above suggest that the PAN (and, conversely, the POX) proteins do not affect the relative sensitivity or resistance of Opa$^+$ or Opa$^-$ gonococci to phagocytic killing by human neutrophils. Also, the ability of Opa proteins to facilitate phagocytic killing of gonococci is unaltered by anaerobic growth.

DISCUSSION

In the absence of serum, nonpiliated Opa$^+$ gonococci adhere to, stimulate, and are phagocytically killed by human neutrophils, while nonpiliated Opa$^-$ gonococci demonstrate none of these activities (14, 33, 36, 45). Pili, although implicated in gonococcal adherence to certain cell types, do

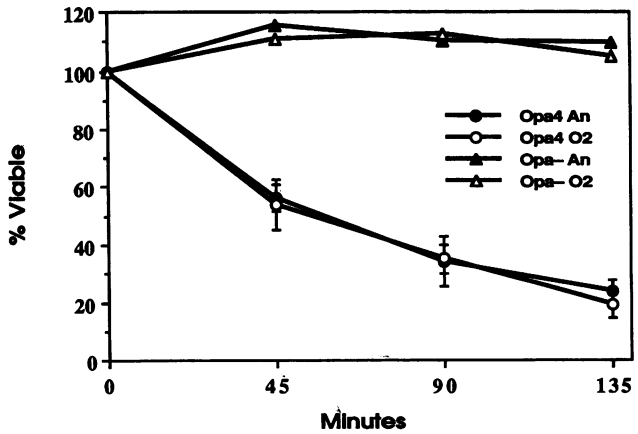

FIG. 2. Phagocytic killing of anaerobic and aerobic gonococci by human neutrophils in the absence of serum. Unstimulated neutrophils were mixed with strain F62 Opa4 gonococci grown anaerobically (●) or aerobically (○) or with strain F62 Opa$^-$ gonococci grown anaerobically (▲) or aerobically (△), and phagocytic killing was measured as detailed in Materials and Methods. The data for Opa4 gonococci represent three similar experiments, and the experiment using Opa$^-$ gonococci was conducted once as a control.

not appear to play a major role in gonococcal interactions with neutrophils (36, 42, 45). Thus far, the ability of Opa to mediate such interactions has been demonstrated exclusively in an aerobic environment. Given that anaerobic growth of gonococci occurs in vivo and may be important in the pathogenesis of gonorrhea, we wanted to determine whether anaerobic growth affected the Opa-mediated interactions of gonococci with neutrophils. We observed that neither PAN nor POX proteins appear to function in the adherence to or stimulation of neutrophils, nor do they modulate the ability of gonococci to be phagocytically killed by neutrophils. Thus, the role of Opa proteins in mediating these phenomena remains unaltered by anaerobic growth.

Recent investigation into the characteristics of anaerobically grown gonococci has led to several exciting discoveries. Gonococci can be isolated from infection sites in the presence of obligate anaerobes (4, 8) and can survive and grow without oxygen (20, 37, 38), suggesting that they are able to grow in a strictly anaerobic environment in vivo. Upon the realization that this ability is lost after subculture on laboratory media, the search began for the appropriate culture conditions for growing anaerobic gonococci (24, 37). After it was observed that gonococci grow anaerobically in the presence of nitrite and differentially express or repress several outer membrane proteins (9), it was proposed that anaerobiosis might be an important consideration in the pathogenesis of gonorrhea. Convalescent-phase serum contains antibody that reacts strongly with PAN 1 on Western blots, indicating that PAN 1 is expressed by gonococci during clinical infection, probably as a result of anaerobic growth or other environmental factors (10). Taken together, these results imply that the study of anaerobic gonococci could provide investigators with a more accurate picture of gonococcal pathogenesis in vivo and a more appropriate set of virulence factors with which to study vaccine development.

Using SDS-PAGE, we showed that Opa4, PI, and PIII were expressed by anaerobic gonococci of strain F62 and possessed relative mobilities identical to those of their counterparts expressed by aerobic gonococci. Antigenic variation of the Opa molecule is generated by differential expression of Opa genes encoding distinct hypervariable regions (30, 35) and typically is accompanied by a shift in the molecular weight of the Opa gene product (41). There are, at present, no specific environmental factors known to cause gonococci to alter their Opa phenotype, and genetic studies suggest that Opa variation is a random event (31). Our findings provide evidence that gonococci do not necessarily switch expression from one Opa gene to another when moved from an aerobic to an anaerobic environment, or vice versa, confirming the work of Clark et al. (9).

Differences between LOSs in outer membrane preparations from anaerobic and aerobic gonococci were also observed. It is unknown whether the smaller LOS region exhibited by anaerobic gonococci represents a reduction in the actual amount of LOS on the outer membrane or an alteration of LOS structure, resulting in the disappearance of one or more LOS bands. We are investigating these possibilities, using an SDS-PAGE system that resolves individual LOS bands. Regardless, the apparent change in the LOS phenotype of anaerobic gonococci did not affect their Opa-mediated interactions with neutrophils.

We compared anaerobic and aerobic gonococci and found no difference in the ability of Opa^+ gonococci, or in the inability of Opa^- gonococci, to adhere to neutrophils or to induce neutrophil oxidative metabolism. These results indicate that the PAN and POX proteins, and anaerobic growth in general, do not augment or reduce either of these Opa-mediated interactions of gonococci with human neutrophils.

Given the requirement for molecular oxygen in the respiratory burst, it is unlikely that neutrophils in anaerobic infection sites would be able to cause oxygen-dependent killing of gonococci. Despite this consideration, increased oxidative metabolism is an indication of neutrophil stimulation, including degranulation. Therefore, these studies are the first to suggest that anaerobic and aerobic gonococci stimulate neutrophil function to equal extents and that the role of Opa as a stimulator of neutrophil function is unchanged by anaerobic growth.

It seemed likely that the Opa-mediated phagocytosis of gonococci also would remain unaltered by anaerobic growth, considering the lack of differences between anaerobic and aerobic gonococci in their Opa-mediated adherence to and stimulation of neutrophils. However, it was possible that one of the PAN proteins was an antiphagocytic virulence factor, as is the pneumococcal capsule, staphylococcal protein A, streptococcal M protein and hyalouronic acid (23). This possibility has been ruled out following our observation that Opa^+ gonococci are equally sensitive, and Opa^- gonococci are equally resistant, to phagocytic killing by neutrophils, regardless of anaerobic or aerobic phenotype.

The phagocytic killing studies also suggest that anaerobic gonococci do not possess some inherent resistance to the oxygen-independent intracellular killing mechanisms of neutrophils, assuming that infection sites at which gonococci are phagocytically killed are oxygen limited or strictly anaerobic. Such resistance was thought to have been discovered by Casey et al. (5), who reported that bacteriostatic conditions imposed by anaerobiosis increased gonococcal resistance to crude neutrophil granule extracts and to an isolated cationic antimicrobial protein. However, when bacteriostasis was reversed by incubation of anaerobic gonococci with sodium nitrite, gonococci lost their resistance. This finding supports work by Buck and Rest (3) showing that granule proteins kill actively growing gonococci better than they kill stationary-phase gonococci. Also, in assays using anaerobic or aerobic whole neutrophils instead of granule extracts or proteins, gonococci were equally sensitive to phagocytic killing in the absence of nitrite (6). These data might indicate the presence of an electron acceptor similar or identical to nitrite during phagocytic killing. These results also support earlier work by Rest et al. (33) demonstrating that gonococci are killed equally well by normal neutrophils and neutrophils from individuals with chronic granulomatous disease, which are incapable of oxygen-dependent killing.

The fact that the Opa-mediated interactions of gonococci with neutrophils are similar in anaerobic and aerobic environments lends further support to the theory that Opa may be important in certain aspects of gonococcal pathogenesis during clinical infection. However, the role of Opa in mediating gonococcal interactions with human epithelial cells is only now becoming understood. Opa apparently mediates adherence to and invasion of Chang, HecIB, and ME-180 human epithelial cell monolayers (17a, 29, 37a, 47), possibly via a receptor or mechanism distinct from that which mediates adherence to neutrophils. Although growth of *Salmonella* spp. under oxygen-limited conditions results in up to 70-fold more adherence to and invasion of mammalian cells (27), work by Chen et al. (7) comparing the invasion of HecIB cells by anaerobic and aerobic gonococci revealed no differences.

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