Differential Response of Human Monocytes to *Neisseria gonorrhoeae* Variants Expressing Pili and Opacity Proteins

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Experiments in vitro suggest that *Neisseria gonorrhoeae* surface variation plays a key role in gonococcal pathogenesis by providing the appropriate bacterial phenotypes to go through different stages of the infection. Here we report on the effects of phase and antigen variation of two major gonococcal adhesins, pili and opacity (Opa) outer membrane proteins, on the interaction of the gonococci with human monocytes. Using a set of recombinants of gonococcus strain MS11 that each express 1 of 11 genetically defined Opa proteins or a defined type of pilus, we found that both Opa proteins and pili promote bacterial phagocytosis by monocytes in the absence of serum and that this feature largely depends on the type of protein that is expressed. One of the Opa proteins (Opa₅₀) strongly promoted uptake by monocytes but had little effect on the interaction with polymorphonuclear leukocytes under the conditions employed. Similarly, the phagocytosis-promoting effect of the pili was much more pronounced in monocytes than in neutrophils (4-fold versus 22-fold stimulation of uptake, respectively). Only a subpopulation of both types of phagocytes actively ingested bacteria, as has been observed during natural infections. Measurements of luminol-enhanced chemiluminescence demonstrated that phagocytosis of opaque but not piliated gonococci was accompanied by an increase in oxygen-reactive metabolites. These findings demonstrate that the monocyte response towards gonococci is highly dependent on the bacterial phenotype and differs from the neutrophil response. This diversity in bacterial behavior towards various types of human phagocytic cells underlines the biological impact of gonococcal surface variation and may explain previous contradictory results on this subject.

Two gonococcal surface structures, pili and opacity (Opa) outer membrane proteins, have been implicated in conferring the primary contact between the bacteria and the various types of host cells encountered during gonococcal infection (27, 31). Pili carrying the minor pilus-associated PilC protein (37, 38) are thought to facilitate the initial anchoring of gonococci to mucosal epithelial cells. Once this is established, Opa proteins come into play, strengthening the initial attachment and likely contributing to the penetration to the mucosal barrier. At a later stage of infection, Opa proteins promote the interaction of bacteria with professional phagocytes. Opa proteins can act as bacterial opsonins, directing the uptake of the gonococci by polymorphonuclear cells (PMNs) in the absence of serum factors; they also promote the induction of a neutrophil oxidative response (1, 13, 18, 22, 40, 42, 48). A key feature in gonococcal pathogenesis appears to be that both pili and Opa proteins undergo phase and antigenic variation, resulting in variant molecules with different biological properties (27, 31). For pili, variation in the amino acid sequence of the major subunit has been demonstrated to influence the adhesive properties of bacteria towards various types of epithelial cells (23, 37). Opa proteins comprise a family of up to 11 outer membrane proteins that each can be expressed independently, resulting in both nonopaque variants and those having one to several Opa proteins inserted into the outer membrane (3, 7, 21, 41). A

number of studies suggest that distinct Opa proteins preferentially promote bacterial interaction with either epithelial cells (24, 46, 50, 52) or PMNs (1, 13, 21, 22), thus contributing to the cell tropism displayed by gonococci (21). Whether pili also support bacterial contact with phagocytes or interact uniquely with epithelial cells is still subject to debate (40). The majority of studies suggest that piliated gonococci may variably attach to PMNs but resist phagocytosis (9, 10, 14, 40, 48). Others indicate that piliated gonococci, particularly those passaged in vivo, do not uniformly resist ingestion by human leukocytes and that a small percentage may even survive intracellularly (34, 43, 47). Possibly, these variable results may point to the existence of pilus variants with different biological properties towards phagocytic cells.

In this study we focused on the role of pili and Opa proteins as cell tropism-directing adhesins by studying human monocytes as target cells for gonococcal infection. This was done with the rationales that short-lived neutrophils may be a primary defense against the invading organisms and that monocytes and monocyte-derived macrophages may be a niche during the persistence of the infection. Gonococci are ingested by mononuclear phagocytes (4, 33, 47, 53), but the bacterial features that render them susceptible to nonopsonic phagocytosis are not well characterized. Nonpiliated gonococci expressing opacity protein have been reported to become readily phagocytosed (11, 28) as well as to resist contact with monocytes (12), suggesting that variable gonococcal surface components direct the gonococcus-monocyte interaction. Whether the observed Opa-mediated phagocytosis requires the same or different Opa proteins as those interacting with PMNs is unknown. Similarly, the roles of pili and pilus variation are ill defined. Both phagocytosis-promoting (33) and indifferent or inhibitory effects of pili (4, 17, 53) have been reported, perhaps

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reflecting differences in pilus structure. The purpose of the present study was to evaluate the role of gonococcal pili and opacity proteins in the interaction of gonococci with monocytes by using sets of genetically defined pilus and opacity protein variants of gonococcus strain MS11. Bacterium-directed phagocytosis and elicited luminol-enhanced chemiluminescence were compared with results obtained with PMNs. Our data indicate that both pilus and Opa variations have a major impact on the nature of the monocyte response towards gonococci.

MATERIALS AND METHODS

Bacteria. Gonococci were grown on GC agar base plus vitamin supplement (Becton Dickinson, Meylan, France) at 37°C in a humidified 5% CO₂ atmosphere and passaged daily. The characteristics of *Neisseria gonorrhoeae* MS11 and its derivatives expressing genetically defined Opa proteins (Opa₅₀ to Opa₆₀ from strain MS11) from the ptdM25.2 plasmid are detailed by Kupsch et al. (21). The recombinant strains produced less Opa protein but showed Opa-related cell tropism typical of that reported for natural Opa variants (5, 21). Lack of Opa-specific antibodies did not allow a direct functional comparison of all available recombinant and natural Opa variants. In contrast to natural Opa variants, the recombinant Opa proteins were not subject to phase variation as a result of modification of the repetitive pentameric nucleotide repeat involved in Opa phase variation (21). The characteristics and pilE sequences of the pilated MS11 variants have been previously described (34). The MS11 *recA* derivatives MS11-F3* and MS11-A1* were constructed as described for MS11-E1* (38). MS11-B2 is a nonpilated, spontaneous mutant with *pilE1* and *pilE2* deleted (39). The nonpilated VP1 variants carrying either no Opa protein or the epithelial cell invasion-associated Opa_{27.5} have been previously described (24).

Isolation of human neutrophils and monocytes. PMNs were isolated from fresh human heparinized (25 U/ml) blood by Percoll gradient centrifugation as described previously (21). Human monocytes were isolated from freshly isolated buffy coats derived from 500 ml of human blood provided by the Transfusionsmedizin, Universitätsklinikum, Tübingen, Germany. One buffy coat was diluted with 50 ml of phosphate-buffered saline (PBS) (10 mM sodium phosphate, 140 mM NaCl, 2.5 mM KCl [pH 7.4]) containing 1 mM CaCl₂ and 1 mM MgCl₂ (PBS+), and 25 ml of this suspension was layered onto 20 ml of Ficoll (density, 1.077 g/cm³; Biochrom, Berlin, Germany) in a 50-ml polypropylene centrifuge tube. After centrifugation (30 min, 400× g, 20°C), the mononuclear cells (2 × 10⁸ to 4 × 10⁸ from one buffy coat; approximately 10 to 20% monocytes) were collected, washed twice with cold PBS+, again collected by centrifugation (10 min, 400× g, 4°C), and finally diluted in RPMI 1640 tissue culture medium at 5 × 10⁶ cells per ml. For phagocytosis experiments, 1 ml of this suspension was added to circular glass coverslips (diameter, 12 mm) in 24-well tissue culture plates (Nunc) and incubated for 45 min at 37°C to enrich for monocytes. Nonadherent cells were removed by three washes with warm (37°C) medium, and the remaining adherent cells (approximately 10⁶ cells per coverslip) were used immediately for phagocytosis experiments.

Phagocytosis assays. Phagocytosis experiments were essentially carried out as described previously (21). In brief, 5 × 10⁷ agar plate-grown bacteria were added to either adherent mononuclear cells in 1 ml of RPMI 1640 (see above) or to 5 × 10⁶ PMNs seeded onto glass coverslips in a 24-well plate in PBS+ (total volume, 0.5 ml). Phagocytosis was allowed to proceed for 1 h at 37°C. Then, the cells were washed three times with PBS+ and either stained with Wright stain (0.5 ml of 0.3% solution [Sigma] in methanol for 3 min, followed by the addition of 1 ml of distilled water and further incubation for 6 min, stopped by two rinses in distilled water) or subjected to the immunogold-silver staining procedure, which facilitates discrimination of extra- and intracellular bacteria (44). Phagocytosis was detected by microscopy and confirmed by electron microscopy. All experiments were carried out in duplicate, each time with cells from two different donors. Phagocytosis by neutrophils is given as absolute numbers of adherent and intracellular gonococci per cell. For estimations of phagocytic activity of monocytes, at least 100 cells were grouped into those having 0, 1 to 5 (average, 3), 6 to 19 (average, 13), or >20 (average, 25) ingested gonococci (see Fig. 5). The percentage of each particular group was multiplied by the average number of gonococci for that group (e.g., 20% of the cells carrying on average 13 gonococci gave a total of number of 2.6 bacteria for that group), and the total of the four groups was considered to be a measure of the interaction between the bacteria and the phagocytic cells. Data presented are the mean numbers of phagocytosed gonococci per cell from at least three experiments unless indicated otherwise.

Luminol-enhanced chemiluminescence assay. Bacterium-induced generation of oxygen-reactive metabolites by leukocytes was evaluated by measurement of myeloperoxidase-hydrogen peroxide-halide production as reflected by luminol-dependent chemiluminescence. For this purpose, 5 × 10⁵ polymorphonuclear or mononuclear blood cells (about 20% monocytes) were mixed with 3 µl of 10 mM luminol (5-amino-2,3-dihydro-1,4-phthalazinedione; Sigma) solution in a final volume of 200 µl of PBS and incubated for 10 min at 37°C. Then, 100 µl of bacterial suspension (5 × 10⁶ bacteria) (final concentration of luminol, 100 µM) was added and luminol-augmented chemiluminescence was measured in a luminometer (Berthold Lumat LB 9501) at 10-min intervals. Chemiluminescence was

expressed as relative light units (RLU) per 10 s. For each experiment, control incubations without bacteria and with the addition of latex particles were included in the assay.

Electrophoresis and immunoblotting. Boiled (5 min) whole-cell lysates of gonococci were electrophoresed (2 × 10⁷ bacteria per lane) and immunoblotted essentially as described previously (52). Blots were incubated with the pilus-specific rabbit polyclonal antiserum AK35 (final dilution, 1/500; prepared by H. Fiedler) and the Opa-specific monoclonal antibody 4B12/CII (final dilution, 1/2,000; kindly provided by M. Achtman). Reactive bands were visualized with alkaline phosphatase-conjugated protein A and 5-bromo-4-chloro-3-indolylphosphate and Nitro Blue Tetrazolium as a substrate.

Electron microscopy. For electron microscopy, cells that had been allowed to internalize gonococci for 1 h were fixed (1 h, 20°C) in 2% paraformaldehyde–0.1% glutaraldehyde in PBS+ for 16 h. The cells were gently removed from the coverslips with a rubber policeman and collected by centrifugation in an Eppendorf Microfuge (3 min, 13,000 × g, 20°C). The pellet was postfixed in 1% OsO₄ in 50 mM cacodylate buffer, subjected to a graded series of ethanol, and embedded in Durcupan-ACM. Sections were poststained with 1% uranylacetate and lead citrate as described previously (42). Specimen were viewed in a Zeiss EM109 electron microscope.

RESULTS

Opacity protein-dependent interaction of gonococci with human monocytes. Variable expression of Opa outer membrane proteins has previously been recognized as a major determinant of gonococcal entry into epithelial cells and of nonopsonic phagocytosis of gonococci by polymorphonuclear phagocytes (1, 13, 21, 22, 24, 45, 50, 52). To further explore the apparent function of Opa proteins as cell tropism-conferring adhesins, we inoculated freshly isolated human mononuclear leukocytes with a complete set of recombinants of strain MS11 that each express from the conjugative gonococcal plasmid ptdM25.2 a single genetically defined member of the 11-member Opa protein family (MS11-Opa₅₀ to -Opa₆₀) (21). All strains were nonpilated, as demonstrated by transillumination microscopy on bacterial colonies and the absence of pilin in Western blots probed with a pilus-specific antiserum (data not shown). In a separate experiment, we measured the interaction of the same recombinants with PMNs to relate our findings with those of a previous published work (21). The phagocytic cells were inoculated at bacterium-cell ratios of 50 to 1 (monocytes) or 10 to 1 (neutrophils), incubated for a period of 1 h in the absence of serum, and analyzed by microscopy. The presence of adherent and intracellular gonococci was confirmed by the selective immunogold-silver staining method and by transmission electron microscopy. As illustrated in Fig. 1A, the recombinant strains exhibited a marked Opa type-dependent interaction with human monocytes. The strains expressing Opa₅₀, Opa₅₂, Opa₅₄, and Opa₅₇ to Opa₆₀ were readily phagocytosed, while bacteria carrying Opa₅₁, Opa₅₃, Opa₅₅, Opa₅₆, or the vector alone only poorly interacted with the monocytes. Adherent bacteria were rare. Basically the same pattern of Opa type-dependent phagocytosis was observed with PMNs, with one exception. MS11-Opa₅₀, which was efficiently ingested by monocytes, reacted as an Opa-negative strain with PMNs (Fig. 1B), consistent with previous results (21). Opa₅₀ differs from the other Opa proteins in that it promotes bacterial adherence and perhaps entry into epithelial cells through binding of cell surface heparan sulfate proteoglycan receptors (45). This interaction is inhibited by exogenous heparin (10 µg/ml) (45). Addition of heparin (up to 100 µg/ml) to the monocyte phagocytosis assay, however, did not reduce the interaction between MS11-Opa₅₀ and this cell type (data not shown), suggesting that the uptake-promoting effect of Opa₅₀ in both types of host cells is mediated by distinct cell surface receptors, both of which appear to be functionally absent in PMNs.

Pilus-mediated phagocytosis of *N. gonorrhoeae* by isolated human peripheral blood monocytes. The contribution of pilus expression and pilus antigenic variation to the interaction of

FIG. 1. Nonopsonic phagocytosis of gonococcal Opa variants by human monocytes (A) and PMNs (B). Cells were inoculated with nonpiliated MS11 recombinant strains expressing either no Opa (vector only) [(-)] or a single genetically defined Opa protein (Opa₅₀ to Opa₆₀). Numbers of total cell-associated bacteria (total bars) and intracellular bacteria (hatched bars) were determined by microscopy after 1 h of infection. Values are the means for six to seven (monocytes) and three to four (PMN) experiments. The experimental variation (standard deviation [error bars]) shown applies for the total number of cell-associated bacteria. Ngo, *N. gonorrhoeae*.

gonococci with professional phagocytes was addressed with a set of MS11 variants that expressed either no pili (MS11-B2) or distinct types of pili (MS11-F3*, MS11-A1*, MS11-E1*, MS11-F3, and MS11-C2) (Fig. 2) (37). These pili were built up from genetically defined pilin. In some variants (designations with asterisks), the *recA* gene was deleted to assure that no recombination among pilin genes could occur. Strain MS11-F3 with an intact *recA* gene was included as a control to exclude possible effects of the *recA* mutation. The various strains did not express Opa protein as demonstrated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and Western blotting

FIG. 2. Western blot showing expression of gonococcal pilin and Opa protein by the pilus variants used in this study. Whole-cell lysates of the MS11 variants were electrophoresed (sodium dodecyl sulfate–10% polyacrylamide gel), transferred onto nitrocellulose, and incubated with pilin-specific polyclonal antibody AK35 and the Opa-specific monoclonal antibody 4B12. Reactive bands were visualized with alkaline phosphatase-conjugated protein A and the appropriate chromogen. Strain MS11-Opa₅₀ served as a control.

FIG. 3. Nonopsonic phagocytosis of gonococcal pilus variants by human monocytes (A) and PMNs (B). Variants MS11-F3*, MS11-A1*, and MS11-E1* expressed pili in a *recA* mutant background. Variants MS11-F3 and MS11-C2 are spontaneous pilus variants carrying the intact *recA* gene. MS11-B2 is a nonpiliated strain and served as a negative control. All strains were negative for Opa expression. Numbers of total cell-associated (total bars) and ingested (hatched bars) gonococci were determined after 1 h of infection. Values are the means of three experiments. The experimental deviation shown (standard deviation [error bars]) applies for the total number of cell-associated bacteria. Ngo, *N. gonorrhoeae*.

using the Opa-specific monoclonal antibody 4B12 (Fig. 2). Evaluation of the interaction of the variants with freshly isolated monocytes and PMNs followed the procedure described for the MS11-Opa recombinants. The results are summarized in Fig. 3. Pili conferred a strong interaction of the various gonococcal variants with human monocytes, resulting in a greater-than-20-fold increase in the number of intracellular bacteria compared with the pilus-negative control strain (Fig. 3A). On average, four to nine intracellular gonococci per cell, depending on the type of pilus that was expressed, were observed. Adherent bacteria were rare, indicating that phagocytosis was rapid and efficient. In contrast to monocytes, ingestion of piliated gonococci by PMNs was low (one to two bacteria per cell), although with some variants a fourfold increase in the number of intracellular bacteria over the nonpiliated variant (MS11-B2) was observed (Fig. 3B). These data suggest a basic difference between monocytes and PMNs in their behavior towards piliated gonococci. Electron microscopy revealed no obvious ultrastructural differences in bacterial or monocyte morphology whether gonococci were ingested through the pilus- or Opa-mediated phagocytosis pathway. Both types of bacteria appeared to be contained in membrane-bound vesicles and showed heterogeneity in their morphology, ranging from apparently intact gonococci with well-defined three-layered membranes and a translucent cytoplasm to seemingly disintegrating organisms with ruffled outer membranes and condensed cytoplasm (Fig. 4).

Heterogeneity among monocytes in their ability to interact with gonococci. Microscopy on the infected PMNs and mononuclear phagocytic cells revealed that only a subset of both cell populations contained bacteria and that the number of ingested gonococci per cell was highly variable. Since this heterogeneity in phagocytic behavior also seems to exist during natural infections (19, 47), we systematically classified pilus- and Opa variant-exposed monocytes according to their numbers of intracellular bacteria. This revealed that interacting Opa-positive gonococci were ingested by a slightly larger subset of cells than were piliated bacteria (55 to 80% versus 35 to 60% of infected cells, respectively) (Fig. 5). At lower and higher multiplicities of infection, this difference was maintained, although the percentage of infected cells increased at higher bacterium-host cell ratios (data not shown). A similar

FIG. 4. Transmission electron micrographs illustrating the intracellular location of piliated (MS11-F3*) (A and B) and Opa-expressing (MS11-Opa₅₀) (C and D) gonococci in human monocytes (1 h of infection). Note that both morphologically intact and disintegrating gonococci are present and that they appear to be contained in membrane-bound vacuoles (B and D). Magnifications for panels A and C, $\times 7,000$; magnifications for panels B and D, $\times 20,000$.

effect was observed when infection was allowed to continue for longer periods (3 to 6 h), suggesting that the number of active monocytes within the population increased with time. Further quantitation of the number of gonococci ingested by single cells showed the existence of subpopulations of highly active and less active monocytes (Fig. 5). Only a minority of monocytes (5 to 20%, depending on the gonococcal strain) contained more than 20 bacteria per cell after 1 h of infection at a multiplicity of infection of 50. This heterogeneity in phagocytic behavior was observed both for piliated and opaque-protein-bearing gonococci of strain MS11 and for VP1 expressing Opa_{27.5} (Fig. 5). We were unable to determine whether both bacterial phenotypes were ingested by the same subpopulation of monocytes.

Association between bacterial phenotype and induction of respiratory burst. In an attempt to further unravel the possible biological impact of the presence of two bacterial surface adhesins (pilus and Opa) that promote nonopsonic phagocytosis by human monocytes, we measured the abilities of Opa-posi-

tive and of piliated gonococci to induce a monocyte oxidative response. As a marker of produced oxygen-reactive metabolites, we determined the luminol-enhanced chemiluminescence generated by the hydrogen peroxide (H₂O₂)-myeloperoxidase-halide system (25). Suspensions of freshly isolated monocytes were incubated with MS11 recombinants expressing a defined Opa and piliated gonococci (MS11-F3) in the presence of 100 μ M luminol, and elicited chemiluminescence was monitored at 10-min intervals for up to 50 min. As shown in Fig. 6, MS11 strains expressing Opa₅₀, Opa₅₂, or Opa₆₀ caused a marked oxidative response which lasted for up to 50 min, in contrast to MS11-Opa₅₁ and the Opa-negative strain (Fig. 6). Analysis of all the Opa variants showed a reactivity pattern that reflected that observed for the phagocytosis-promoting activity (Fig. 7A), suggesting an interrelationship between the two events. A similar Opa protein-dependent increase in oxidative response was observed for PMNs, again with the exception of MS11-Opa₅₀ (Fig. 7C). This Opa variant stimulated chemiluminescence in monocytes only, consistent with its variable phagocy-

FIG. 5. Heterogeneity of human monocytes in their ability to ingest various gonococcal pilus and Opa variants. Cells were infected with nonpilated MS11 recombinant strains expressing either no Opa [(-)] or a single genetically defined Opa (Opa₅₀, Opa₅₁, Opa₅₂, or Opa₆₀); non-pilated VP1 variants expressing either no Opa or Opa_{27.5}; MS11 pilus variants (E1 and F3) and the nonpilated control MS11-B2; and the nonpathogenic *Neisseria mucosa* (N. muc). The percentage of interacting monocytes and the number of intracellular bacteria per cell were determined after 1 h of infection. Monocytes were classified as having 0, 1 to 5, 6 to 19, or more than 20 intracellular gonococci per cell. Data are the averages of three to four experiments.

tosis-promoting activity (Fig. 1). It should be noted that the variable oxidative response among the Opa variants was much less prominent at higher bacterium-host cell ratios (>100 to 1), possibly due to the presence of more released lipopolysaccharide at higher bacterial densities.

Remarkably, pilated gonococci did not stimulate luminol-enhanced chemiluminescence (Fig. 7). Measurements over a 2-h incubation period showed the same stable low level of chemiluminescence for both MS11-F3* and the nonpilated control strain MS11-B2. This profound difference with Opa-expressing gonococci was maintained for all pilus variants that were tested (Fig. 7B and D). The lack of response was not the result of a poor interaction of the pilated gonococci with the monocytes, as microscopy on cytospin preparations of monocytes indicated that pilus-mediated phagocytosis had occurred. Gonococci that expressed both pili and Opa₅₀ induced a typical MS11-Opa₅₀ response (data not shown), indicating that the

FIG. 6. Opa protein-dependent stimulation of luminol-enhanced chemiluminescence (CL) after infection of human monocytes with MS11 recombinant strains expressing either no Opa [(-)] or a single genetically defined Opa at a bacterium-host cell ratio of 10 to 1. The elicited chemiluminescence was monitored and expressed as relative chemiluminescence units (RLU) as measured in 10 s. Data are from a typical experiment.

FIG. 7. Comparison of monocyte (A and B) and PMN (C and D) luminol-enhanced chemiluminescence (CL) after 45 min of infection with MS11 recombinants expressing various types of Opa (A and C) and pili (B and D). Chemiluminescence is expressed as relative chemiluminescence units (RLU) per 10 s. Note that Opa proteins that promote phagocytosis (cf. Fig. 1) also stimulate chemiluminescence. Pilated variants differing in pili (PilE) protein do not significantly increase monocyte hydrogen peroxide production compared to the nonpilated control. Data are the means of three independent experiments (error bars, standard deviations).

effect of this Opa was dominant over that of pili and that pili did not suppress the respiratory burst.

DISCUSSION

The interaction of *N. gonorrhoeae* with the human host is influenced by a number of pathogenicity factors including pili, opacity-associated outer membrane (Opa) proteins, and lipopolysaccharide. All these components are variably expressed and can undergo structural variation during natural infections, thus generating a heterogeneous population of bacteria that may differ in antigenic and functional properties (for a review, see reference 27). Here we demonstrate that both pili and Opa proteins direct interactions between *Neisseria gonorrhoeae* and human monocytes and give rise to a diverse biological response. The fact that gonococci have evolved two variably expressed surface constituents to finely tune the monocyte response may indicate that this cell type is a potential target for gonococcal infection. During natural infections, gonococci appear to be primarily associated with PMNs (19, 32, 47, 51), but we emphasize that these observations reflect the acute stage of the infection. It can be imagined that during prolonged gonococcal infection, cells of the monocyte/macrophage lineages are involved in the persistence and spread of the microorganisms.

The findings that Opa-mediated interactions of gonococci with human monocytes were limited to distinct Opa phenotypes indicate functional diversity among members of the Opa protein family towards this cell type. The repertoire of Opa proteins that promoted phagocytosis and the stimulation of respiratory burst in monocytes was similar to that observed for PMNs (Fig. 7) (21), with the exception of Opa₅₀. This Opa protein, which has previously been demonstrated to promote gonococcal uptake by Chang epithelial cells, conferred inter-

actions with monocytes but was poorly recognized by PMNs. The molecular basis for the variable reactivity of MS11-Opa₅₀ with monocytes and PMNs is unknown. Opa₅₀ promotes gonococcal uptake into cultured epithelial cells through binding of a cell surface heparan sulfate-containing proteoglycan receptor (45), but inhibition experiments using soluble heparin as a receptor analog indicate that the MS11-Opa₅₀ interaction with monocytes does not involve proteoglycans and thus involves a different class of receptors.

The observed Opa tropism towards various cell types is consistent with the existence of three functional groups of Opa proteins in gonococcus strain MS11: one group promoting bacterial uptake by epithelial cells and monocytes (Opa₅₀), a second group conferring interactions with monocytes and PMNs (Opa₅₂, Opa₅₄, and Opa₅₇ to Opa₆₀), and a third group (Opa₅₁, Opa₅₃, Opa₅₅, and Opa₅₆) that poorly promotes phagocytosis by either of these cell types, although some adherence to PMNs was observed (21). This functional classification, which appears to be maintained with recombinant *Escherichia coli* strains expressing the various Opa proteins (1), parallels structural similarities among the members of the different groups as deduced from protein sequence analyses of the various proteins (3, 21). The predicted outer membrane topology of the Opa proteins includes four surface exposed loops, of which one is semivariable, two are hypervariable, and one is conserved. Analyses of the semivariable regions indicate that both Opa₅₀ and Opa₆₈ (the cloned Opa₅₀ homolog in strain VP1 that is probably identical to epithelial cell invasion- and phagocytosis-promoting VP1-Opa_{27.5}) (21) lack multiple negatively charged amino acid residues and contain a similar array of positive charges in this region. Opa proteins that do not interact with monocytes (Opa₅₁, Opa₅₃, Opa₅₅, and Opa₅₆) are also more closely related in their semivariable region, as are the phagocytosis-mediating Opa₅₂, Opa₅₇, Opa₅₈, and Opa₆₀. Opa₅₄ and Opa₅₉ can be considered to belong to a separate category. Whether the semivariable domains perhaps in conjunction with a hypervariable region (29) are directly involved in receptor recognition or only provide the appropriate protein or membrane configuration required for binding to one or more classes of host receptor molecules awaits further characterization of the involved Opa-binding domains. Recently, members of the CD66 receptor family have been implicated to function as receptors for certain gonococcal and meningococcal Opa proteins and have been postulated to act as PMN receptors for these bacterial pathogens (6, 14a, 49). Moreover, systemic analysis of the effect of Opa variation on CD66 receptor recognition (using the same recombinant strains as applied in the present study) revealed a reaction pattern that grossly followed the interaction of the various MS11-Opa recombinants with PMNs reported here, including the lack of reactivity with MS11-Opa₅₀ (5). Thus, differential recognition of CD66 receptors on PMNs (and perhaps monocytes) may account for the observed tropism of gonococcal Opa variants towards phagocytic cells.

Pili are thought to contribute to gonococcal pathogenesis by promoting the initial anchoring of the microorganisms to the mucosal surface. Our data indicate that these surface appendages may also function at a later stage of the infection during exposure to human monocytes. Pili definitively promote non-opsonic ingestion of gonococci by human monocytes in the absence of Opa proteins or serum factors, although the effect varies with the structure of the major pilin subunit, PilE (Fig. 3). A similar pilus structure-dependent biological response has been reported for the interaction of pili with epithelial cells (23, 37), feeding the concept that antigen variation may act as a mechanism for providing a functional diversity that is re-

quired to establish infection. Whether pilus variation also accounts for the reported failure of piliated meningococci to interact with monocytes (26) remains to be established. For conferring adherence to epithelial cells, pili demand expression of the minor pilus-associated protein PilC (37, 38), which is supposed to act as a pilus-tip adhesin (38). At this time, we do not know whether PilC is also required for the pilus-mediated recognition of gonococci by mononuclear phagocytes. Comparison of both types of adherence may provide valuable information about pilus-related cell tropism of gonococci and perhaps the nature and distribution of involved host cells receptor(s) for gonococcal pili. The fact that our pilus variants only slightly promoted interactions between gonococci and PMNs (maximum increase of uptake, 4-fold [versus 22-fold for monocytes]) suggests that this cell type is not a primary target of piliated gonococci, although it cannot be excluded that additional pilus (or PilC) variants can be generated that show a different behavior, or that phagocytic cells at the infection site are more susceptible for gonococcal pili. The observed heterogeneity among the population of PMNs and monocytes that actively interact with gonococci, a feature which is also observed during natural infections (19, 47), indicates that expression of functional receptors for pili and Opa proteins on phagocytic cells may be subject to regulation.

Both neutrophils and monocytes respond to a variety of external stimuli with activation of the system producing myeloperoxidase-hydrogen peroxide-halide and generation of superoxide anions (20). Measurements of luminol-enhanced chemiluminescence (reflecting the activity of the myeloperoxidase system) indicated that also certain Opa-expressing gonococci are able to trigger respiratory burst in PMNs (1, 13, 30, 36, 48). Our findings with the whole repertoire of genetically defined Opa variants confirm these results and suggest an association between Opa-mediated phagocytosis and the induction of an oxidative response. Basically similar results, albeit with less vigorous responses, were obtained for monocytes, again with the exception of MS11-Opa₅₀. The fact that this Opa variant is phagocytosed and increases the luminol-enhanced chemiluminescence in monocytes but not in PMNs may point to recognition by this variant of monocyte receptors that are functionally absent from PMNs and that may differ from those recognized by the other Opa variants that apparently do not discriminate between monocytes and PMNs. The observation that the level of chemiluminescence was generally lower in monocytes than in PMNs (cf. Fig. 7A and C) is consistent with the lower levels of myeloperoxidase activity in freshly isolated monocytes (8, 25).

In contrast to Opa-mediated phagocytosis, ingestion of piliated gonococci by monocytes was not accompanied by increased luminol chemiluminescence, pointing to a fundamental difference in the interaction of piliated and opaque gonococci with human monocytes. As additional Opa₅₀ protein expression by piliated gonococci restored respiratory burst activity, the effect of pili is probably very specific and not caused by a general suppression of the oxidative response. The identity of the monocyte receptors recognized by piliated gonococci and whether the bacteria are also unable to generate increased superoxide anion levels are as yet unknown. It has been demonstrated that ligand binding to the phagocyte CR3 complement receptor complex is not always accompanied by a respiratory burst, in contrast to binding to Fc receptors (2, 16, 54, 55). Thus, CR3 may be a candidate receptor for mediating pilus-mediated phagocytosis of gonococci, perhaps in cooperation with other cell surface molecules, as has been reported for *Bordetella pertussis* (15, 35). The existence of a differential response in monocytes towards different gonococcal pheno-

types raises the intriguing possibility that the bacterial phenotype determines the intracellular fate of the gonococci, depending on the stimulation of toxic oxygen derivatives. This topic will be the subject of further investigation.

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