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Neisserial Porins Inhibit Human Neutrophil Actin Polymerization, Degranulation, Opsonin Receptor Expression, and Phagocytosis but Prime the Neutrophils To Increase Their Oxidative Burst

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Porins are trimeric proteins that constitute water-filled pores that allow transmembrane diffusion of small solutes through the outer membrane layer of gram-negative bacteria. The porins are capable of inserting into the membranes of eucaryotic cells, and in the present study we have examined the in vitro effects on neutrophil functions of the following purified porins: meningococcal outer membrane protein classes 1 and 3 and gonococcal outer membrane protein 1B (P1B). The neisserial porins inhibited human neutrophil chemoattractant-induced actin polymerization and degranulation of both primary and secondary granules. The neutrophil expression of immunoglobulin G (IgG) Fc receptors II (FcγRII; CDw32) and III (FcγRIII; CD16), as well as the activation-dependent downregulation of FcγRIII, were reduced by the meningococcal and gonococcal porins. The neisserial porins impaired the upregulation of complement receptors 1 (CD35) and 3 (CD11b) and inhibited the phagocytic capacity of neutrophils, as evaluated by the uptake of meningococci (strain 44/76) in the presence of patient serum containing known amounts of IgG against meningococcal porins. The porins also primed neutrophils to increase their intracellular hydrogen peroxide production in response to FMLP, whereas no such priming was observed if the neutrophil protein kinase C was stimulated directly with phorbol myristate acetate. The neisserial porins influenced neutrophil functions in a time- and concentration-dependent manner. The meningococcal class 1 outer membrane protein and the gonococcal P1B tended to alter neutrophil functions more than the meningococcal class 3 protein. Thus, the neisserial porins inhibited human neutrophil actin polymerization, degranulation, opsonin receptor expression, and phagocytosis but primed the neutrophils to increase their oxidative burst. It remains to be determined whether these in vitro observations reflect mechanisms that may be of importance for the interaction between neutrophils and *Neisseria* species in vivo.

The importance of serum bactericidal antibodies for an effective human host defense against neisserial infections is well documented (17, 18, 42). In addition, several lines of evidence suggest that opsonizing complement-binding antibodies and phagocytic leukocytes may play a role in protection against both meningococcal and gonococcal disease (15, 20, 25, 43–45, 47). Hence, human neutrophils have been shown to effectively attach, ingest, and kill *Neisseria meningitidis* (15, 20, 43, 45) as well as *Neisseria gonorrhoeae* (44, 47). However, significant heterogeneity of neutrophil phagocytosis and oxidative burst has been demonstrated following interaction with serum opsonins and various meningococcal serogroups and serotypes (20). In addition, some strains of gonococci seem to resist neutrophil phagocytosis and killing (47), indicating that both *N. meningitidis* and *N. gonorrhoeae* may be capable of applying antiphagocytic strategies to reduce the protective efficacy of neutrophils.

Porins are trimeric proteins that constitute pores that allow diffusion of small solutes through the outer membrane layer of gram-negative bacteria (39). The neisserial porins, the most abundant proteins of the outer membrane of these bacteria, show extensive structural homology (1, 12, 19, 28, 55). The meningococcal class 1 outer membrane proteins lack gonococcal equivalents, whereas the meningococcal class 2 proteins are homologous to the gonococcal outer membrane protein 1B (P1B), and the class 3 proteins are homologous to the gonococcal outer membrane protein 1A (1, 12, 19, 28, 55).

The meningococcal and gonococcal porins are important immunodeterminants in humans after infection and immunization (36, 37, 40, 52–54). On the other hand, neisserial porins are capable of translocating from live meningococci and gonococci and being directionally inserted into foreign membranes, both lipid bilayers and human cells (9, 34). Consistently, when neutrophils are exposed to purified gonococcal P1B, the cell changes its membrane potential, and degranulation of cytoplasmic granules carrying microbicidal substances is inhibited (23). It is not known whether this occurs following exposure of neutrophils to meningococcal porins or whether this phenomenon may be of relevance for the in vivo situation.

In the present study, the effects of neisserial porins on human neutrophil actin polymerization, degranulation, opsonin receptor expression, phagocytosis, and oxidative burst were studied in vitro using purified meningococcal outer membrane protein class 1 and class 3 as well as gonococcal P1B.

MATERIALS AND METHODS

Porins. Meningococcal class 1 and class 3 outer membrane proteins were isolated by detergent solubilization and chromatographic purification from *N. meningitidis* strains lacking class 3 and class 4 proteins (strain 44/76D3D4 [22]) or lacking class 1 and class 4 proteins (strain 44/76D1D4 [21]). The gonococcal P1B was purified as described earlier from the serum-sensitive and Rmp-negative gonococcal strain Pgh 3-2 (strain PghD*rmp*) (53, 54). There was no contamination with other outer membrane proteins as evaluated by gel electrophoresis and

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Western blot (immunoblot) (31). Endotoxin (lipopolysaccharide [LPS]) contamination was less than 0.01%, as judged by gel electrophoresis and silver staining (50). Stock solutions of 1 μM neisserial outer membrane protein in 0.001% Empigen-BB (alkyl-dimethylamine-betaine; Albright and Wilson Ltd.) were prepared and used in all experiments.

Leukocytes. Venous blood from healthy nonsmoking individuals between 30 and 40 years old was drawn into Vacutainers containing ACD solution A (Becton Dickinson) and immediately diluted 1:10 in a solution containing 0.8% NH_4Cl, 0.08% $NaHCO_3$, and 0.08% EDTA (pH 6.8, 20°C) to lyse erythrocytes (16). After 10 min, the leukocytes were pelleted by centrifugation at 180 × *g* for 5 min, washed once in phosphate-buffered saline (PBS) before being washed and resuspended in PBS with 0.5% glucose. The PBS used in all experiments contained 0.1 g of $CaCl_2$ and 0.1 g of $MgCl_2$ per liter (Flow Laboratories). Leukocyte viability was ≥99% in all experiments, as evaluated by trypan blue dye (0.1% [wt/vol] in PBS) exclusion.

Actin polymerization. The effect of neisserial porins on chemoattractant-induced neutrophil actin polymerization was studied by flow cytometry (8, 26), using a method recently modified to include the new fluorescent marker Bodipy-phallacidin (Molecular Probes) to stain filamentous actin (F-actin) (4). Briefly, 5 × 10^4 leukocytes were preincubated with PBS, Empigen-BB, or porins for 10 to 30 min in V-bottomed polystyrene microwell plates (Nunc) at 37°C. The leukocytes were then exposed to 1 μM formyl-Met-Leu-Phe (FMLP) (dissolved in dimethyl sulfoxide; final concentration, 0.01%) or 0.1 μM recombinant human C5a (dissolved in PBS) (both from Sigma Chemicals) for 30 s to induce actin polymerization, before the cells were fixed and permeabilized for 15 min with 35 μl of an ice-cold solution containing 1,225 μl of 37% formaldehyde (Merck), 475 μl of lysophosphatidylcholine (2 mg/ml in PBS) (Sigma Chemicals), and 1,450 μl of PBS. F-actin was stained by the addition of saturating amounts of Bodipy-phallacidin in PBS. The leukocytes were incubated in the dark on ice for another 30 min, then washed twice, resuspended in PBS, and stored in the dark on ice until analyzed by flow cytometry.

The neutrophils were distinguished from the other leukocytes by flow cytometric measurements of cellular light scatter, and their Bodipy-phallacidin fluorescence was gated to separate histograms (4, 8, 26). The neutrophil content of polymerized actin was expressed as mean cellular fluorescence.

Degranulation. The neutrophil upregulation of surface-expressed granule-associated antigens was determined by immunofluorescence and flow cytometry (4, 33). To convert degranulation into the phagosome to exocytosis, the neutrophils were treated with cytochalasin B (see below). The complement receptor 3 (adhesion-linked integrin molecule CD11b) is associated with the secondary granules of neutrophils and is translocated to the plasma membrane during degranulation (11, 33). In addition, there are indications that CD11b is in part contained in the gelatinase-bearing secretory granules and the latent alkaline phosphatase compartment. The CD63 molecules are associated with the primary granules and are upregulated during degranulation of these granules (30).

To study the effect of neisserial porins on neutrophil degranulation, 5 × 10^4 leukocytes were incubated in PBS with or without cytochalasin B (5 μg/ml) for 5 min in V-bottomed polystyrene microwell plates (Nunc) at 37°C (4, 33). PBS, Empigen-BB, or porins were then added in various concentrations, and the leukocytes were incubated for another 10 to 30 min. To induce degranulation, the leukocytes were exposed to 1 μM FMLP (Sigma Chemicals) for 10 min, then washed once in ice-cold PBS, and labeled on ice for 25 min with the monoclonal antibody (MAb) Mo1 (anti-CR3/CD11b) (Coulter) or CLB-gran/12 (anti-CD63) (Caltag Laboratories). The leukocytes were washed in ice-cold PBS and stained on ice for 25 min with immunoglobulin M (IgM)- or IgG-specific fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgG (Jackson Immunoresearch). The cells were then washed once, fixed in 1% (wt/vol) paraformaldehyde in PBS, and stored on ice until analyzed by flow cytometry.

The neutrophils were distinguished from the other leukocytes by flow cytometric measurements of cellular light scatter, and their FITC fluorescence was gated to separate histograms (4, 33). The neutrophil expression of granule-associated proteins was expressed as mean cellular fluorescence.

Surface expression of opsonin receptors. To study the effect of neisserial porins on the neutrophil surface expression of IgG Fc receptor II (FcγRII) (CDw32), FcγRIII (CD16), and complement receptor 1 (CD35), leukocytes were incubated with cytochalasin B, exposed to PBS, Empigen-BB, or porins, and stimulated with FMLP as described above. After a washing, the cells were labeled with MAb IV.3 (anti-CDw32) (MedaRex), Vep-13 (anti-CD16) (Boehringer, Mannheim, Germany), or anti-CR1 (anti-CD35) (Becton Dickinson), washed again, and stained with IgM- or IgG-specific FITC-conjugated goat anti-mouse IgG (Jackson Immunoresearch). The cells were then washed once, fixed in 1% paraformaldehyde, and stored on ice until analyzed by flow cytometry as described above. The neutrophil expression of opsonin receptors was expressed as mean cellular fluorescence.

Phagocytosis. Neutrophil phagocytosis of meningococci was studied by a flow cytometric technique described earlier with minor modifications (5–7, 20). *N. meningitidis* 44/76 was fixed in ethanol, stained with FITC (0.25 mg/ml) (Molecular Probes), resuspended in Hanks' balanced salt solution (concentration, 5 × 10^8 per ml), and stored in aliquots at −70°C as described earlier (20). To allow opsonization, bacteria and serum (final concentration, 5%) were rotated end over end for 7.5 min at 37°C. Leukocytes that had been preincubated for 10 min with PBS, Empigen-BB, or porins were then added (bacteria-to-nonlymphocyte ratio, 20:1), and the mixture was rotated for another 7.5 min at 37°C. The phagocytosis was terminated by the addition of 1 ml of ice-cold PBS containing 0.02% EDTA (7). The tubes were then kept on ice until the contents were analyzed by flow cytometry.

The free, extracellular *N. meningitidis*, nonphagocytes, and phagocytes were distinguished and quantified by combined measurements of FITC fluorescence and light scatter (5–7). The number of associated bacteria per phagocyte was determined from the difference between the initial and final counts of free extracellular bacteria divided by the number of phagocytosing leukocytes (5–7).

The numbers of attached and internalized meningococci per phagocyte were determined by a fluorescence quenching technique (for details, see references 6 and 7). Briefly, trypan blue dye in PBS was added to the reaction mixtures (final concentration, 0.5 mg/ml), quenching the FITC fluorescence of free extracellular and attached bacteria. After flow cytometric analysis, the mean numbers of attached (N_a) and internalized (N_i) meningococci per phagocyte were then determined from the following equations: $N_a = (F - F_q)/F_e$ and $N_i = N - N_a$, where F is the mean FITC fluorescence of the phagocyte population before and F_q is the mean FITC fluorescence of the phagocyte population after quenching, F_e is the mean FITC fluorescence of free meningococci, and N is the total number of meningococci associated per phagocyte (5, 6).

Oxidative burst. The effect of neisserial porins on neutrophil hydrogen peroxide production was studied by a flow cytometric method reported earlier with slight modifications (8, 32, 33). Briefly, 5 × 10^4 leukocytes were incubated in PBS with or without cytochalasin B (5 μg/ml) for 5 min in V-bottomed polystyrene microwell plates (Nunc) at 37°C (8). PBS, Empigen-BB, or porins were then added, and the leukocytes were incubated for another 10 to 30 min. To induce oxidative burst, the leukocytes were exposed to 0.04 to 4 μM FMLP or 5 to 50 ng of phorbol myristate acetate (PMA) per ml (both from Sigma Chemicals) in the presence of the fluorescent indicator dihydrorhodamine-123 (10 μg/ml; Molecular Probes) (8). After 20 min of incubation, the reaction was stopped by adding 100 μl of ice-cold PBS containing 0.02% paraformaldehyde. The samples were kept on ice until analyzed by flow cytometry.

The neutrophils were distinguished from the other leukocytes by flow cytometric measurements of cellular light scatter, and their rhodamine-123 fluorescence was gated to separate histograms (8, 32, 33). The neutrophil oxidative burst was expressed as mean cellular fluorescence.

Controls. To determine how long preincubation had to be to obtain maximal effect of the neisserial porins on neutrophil function, the leukocytes were incubated with 500 nM meningococcal outer membrane protein class 1 and 3 or gonococcal P1B for 0 to 60 min. Neutrophil actin polymerization, degranulation, and oxidative burst were then measured following stimulation with 1 μM FMLP (see above). In all experiments (n = 8), maximal inhibition of neutrophil actin polymerization and degranulation, as well as stimulation of oxidative burst, was observed within 7.5 min (not shown). Preincubation periods of 10 to 30 min were therefore chosen in the following experiments.

To test whether the concentrations of neisserial porins used in the experiments were noncytotoxic, we compared the viability of leukocytes incubated with 0 to 1,000 nM menincococcal outer membrane protein class 1 and 3 or gonococcal P1B for 0 to 60 min at 37°C. Leukocyte viability remained ≥91% in all experiments (n = 4) as evaluated by trypan blue dye exclusion.

To test whether the effects of the neisserial porins were reversible, the neutrophils were washed after incubation with porin and prior to stimulation, and the results were compared with those obtained in parallel experiments without washing. In the six experiments performed, washing did not influence the effects of the neisserial porins on neutrophil function (not shown).

To test whether a possible direct interaction between the neutrophil stimuli and the porins could influence the responses observed, FMLP (1 μM), C5a (0.1 μM), or PMA (50 ng/ml) was incubated with each of the neisserial porins (final concentration, 500 nM) for 0 or 60 min and then added simultaneously to the neutrophils. Coincubation of stimulus and porin for 60 min did not influence the effects of the porins on the neutrophil functions studied (not shown).

To rule out the possibility that monocytes and lymphocytes present in the reaction mixtures could interfere with the results, purified human neutrophils were used in eight experiments. The results obtained in these control experiments were similar to those obtained with mixed leukocyte suspensions (not shown).

To test whether the effects of neisserial porins were influenced by blockade of neutrophil surface CD14 (the receptor for the complex of LPS and the LPS-binding protein [35]), the cells were incubated at room temperature with saturating amounts of the MAb Mo2 (anti-CD14 [mouse IgM; Coulter]). After 20 min, the leukocytes were washed once and incubated with PBS, porins, or Empigen as described above. MAb Mo2 itself did not influence neutrophil functions (not shown).

Flow cytometry. The flow cytometric analyses were performed with a Coulter Profile II (Coulter Electronics). A 488-nm excitation source and standard fluorescein filters were used for the measurements of both Bodipy-phallacidin, FITC, and rhodamine-123 fluorescence. Before each experiment, the flow cytometer was calibrated using the same batch of fluorescent microspheres.

Statistics. Significance of difference was determined by a Student t test.

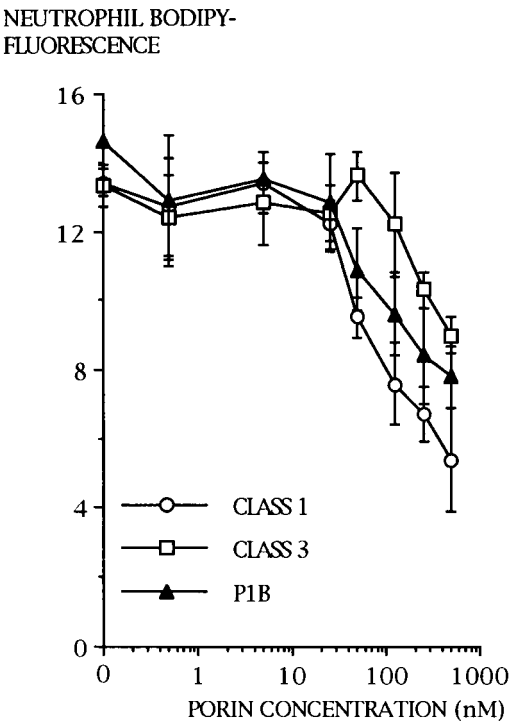

FIG. 1. Effect of meningococcal class 1 and 3 outer membrane proteins and gonococcal outer membrane P1B on neutrophil actin polymerization. The neutrophils were incubated with neisserial porins for 30 min before being stimulated with 1 μM FMLP. The amount of F-actin per neutrophil following stimulation with FMLP is indicated by the mean neutrophil Bodipy-phallacidin fluorescence and given as mean channel fluorescence ± standard deviation (SD) ($n = 4$). The fluorescence of unstimulated control neutrophils was 3.2 ± 0.7, $n = 4$.

RESULTS

Actin polymerization. Both the meningococcal and gonococcal porins inhibited the neutrophil actin polymerization in response to FMLP in a concentration-dependent manner (Fig. 1). Following incubation of neutrophils for 30 min at a concentration of meningococcal class 1 protein of 500 nM, the mean cellular F-actin-related fluorescence in response to FMLP was about 60% lower than that of neutrophils preincubated in the absence of porin. In comparison, the FMLP-induced actin polymerization in neutrophils exposed to the same concentrations of meningococcal class 3 outer membrane protein and gonococcal P1B was about 33 and 42% lower than that in the controls, respectively (Fig. 1). The lowest porin concentration inducing significant inhibition of the neutrophil FMLP-induced formation of F-actin was 50 nM for meningococcal class 1 protein, whereas a significantly reduced neutrophil actin polymerization was observed at concentrations of meningococcal class 3 protein and gonococcal P1B of 250 and 125 nM, respectively (Fig. 1). The neisserial porins also reduced C5a-induced neutrophil actin polymerization, showing that the inhibitory effect was not unique to FMLP (Table 1).

Preincubation of leukocytes with saturating amounts of an MAb against CD14 did not influence the porin-induced inhibition of FMLP-induced neutrophil actin polymerization (not shown). In addition, Empigen-BB at a concentration of 0.001% did not influence actin polymerization, and neither the porins nor the detergent altered the basal neutrophil level of F-actin (not shown).

Degranulation. The meningococcal and gonococcal porins inhibited degranulation of cytochalasin B-treated neutrophils in response to FMLP in a concentration-dependent manner (Fig. 2 and 3; Tables 2 and 3). The porins did not influence the basal neutrophil surface expression of CD63, indicating that the neisserial outer membrane proteins did not themselves induce degranulation of primary granules (Fig. 2). In contrast, the upregulation of CD63 in response to FMLP was significantly lower in neutrophils incubated with meningococcal and gonococcal porins than in neutrophils incubated with PBS or Empigen-BB (Fig. 2). At a porin concentration of 1,000 nM, the FMLP-induced upregulation of CD63 was almost completely inhibited (Table 2). The lowest porin concentration inducing significant inhibition of the neutrophil FMLP-induced upregulation of CD63 was 100 nM for meningococcal class 1 protein, whereas it was 500 and 100 nM for meningococcal class 3 protein and gonococcal P1B, respectively (Table 2).

None of the porins themselves influenced the neutrophil surface expression of complement receptor 3 (CD11b), indicating that the neisserial outer membrane proteins did not induce degranulation of secondary granules (Fig. 3). In contrast, the upregulation of CD11b in response to FMLP was

TABLE 1. Effect of meningococcal class 1 and 3 porins and gonococcal P1B on neutrophil actin polymerization[a]

Stimulus	Mean cellular Bodipy fluorescence ± SD ($n = 4$)				
	PBS	Empigen-BB	Class 1	Class 3	P1B
None	3.2 ± 0.7	3.4 ± 0.7	3.0 ± 0.4	3.3 ± 0.7	3.2 ± 0.5
FMLP	13.4 ± 0.4	13.0 ± 1.2	5.4 ± 1.5[b]	9.0 ± 0.5[b]	7.8 ± 0.9[b]
C5a	14.7 ± 0.9	14.0 ± 0.8	6.2 ± 1.1[b]	8.1 ± 1.0[b]	7.0 ± 0.7[b]

[a] The neutrophils were incubated with porins (500 nM) for 30 min. Actin polymerization was then measured before and after stimulation with 1 μM FMLP or 0.1 μM C5a for 30 s.

[b] $P \leq 0.05$, indicating that the fluorescence of stimulated neutrophils was significantly lower than that observed in the absence of Empigen-BB or porins.

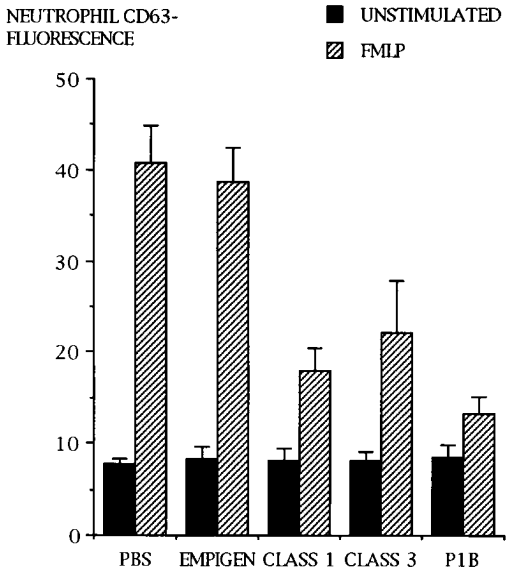

FIG. 2. Effect of meningococcal class 1 and 3 outer membrane proteins and gonococcal outer membrane P1B on neutrophil degranulation of primary granules as evaluated by upregulation of CD63. The cytochalasin B-exposed neutrophils were incubated with neisserial porins for 30 min before stimulation with 1 μM FMLP. The amount of CD63 per neutrophil is indicated by the mean neutrophil CD63 FITC fluorescence and given as mean channel fluorescence ± SD ($n = 4$).

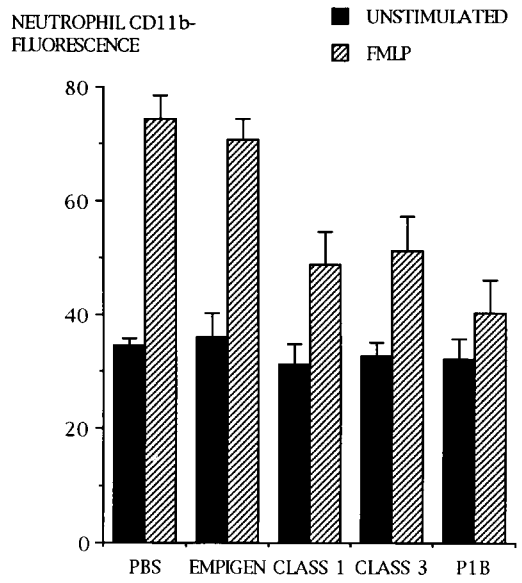

FIG. 3. Effect of meningococcal class 1 and 3 outer membrane proteins and gonococcal outer membrane P1B on neutrophil degranulation of secondary granules as evaluated by upregulation of CR3 (CD11b). The cytochalasin B-exposed neutrophils were incubated with neisserial porins for 30 min before stimulation with 1 μM FMLP. The amount of CR3 per neutrophil is indicated by the mean neutrophil CD11b FITC fluorescence and given as mean channel fluorescence ± SD (n = 4).

significantly lower in neutrophils incubated with meningococcal or gonococcal porins than in the controls (Fig. 3). At a porin concentration of 1,000 nM, the FMLP-induced upregulation of CD11b was almost completely inhibited (Table 3). The lowest porin concentration inducing significant inhibition of the neutrophil FMLP-induced upregulation of CD11b was 100 nM for all neisserial porins tested (Table 3).

Preincubation of leukocytes with saturating amounts of an MAb against CD14 did not influence the porin-induced inhibition of neutrophil degranulation (not shown). In addition, Empigen-BB at a concentration of 0.001% influenced neither basal neutrophil expression of CD63 or CD11b nor the FMLP-induced degranulation of primary and secondary granules from cytochalasin B-treated neutrophils (Fig. 2 and 3).

Opsonin receptor expression. Incubation of neutrophils with neisserial porins induced significantly reduced surface expression of FcγRII (Table 4). Following incubation of cytochalasin B-treated neutrophils for 10 min at a concentration of gonococcal P1B of 500 nM, the mean cellular FcγRII-related fluorescence was about 41% lower than that of neutrophils preincubated with PBS. In comparison, the FcγRII expression of neutrophils exposed to meningococcal outer membrane proteins of classes 1 and 3 was about 25 and 28% lower than that of the controls, respectively (Table 4). Stimulation of both porin-exposed and control neutrophils with FMLP did not alter the surface expression of FcγRII any further (Table 4). Incubation of neutrophils with the neisserial outer membrane proteins in the absence of chemoattractant also reduced the expression of FcγRIII by 26 to 44% (Table 4). In addition, both the meningococcal and gonococcal porins inhibited the FMLP-induced downregulation of FcγRIII in cytochalasin B-treated neutrophils (Table 4). The neisserial porins did not affect the basal neutrophil surface expression of complement receptor 1, i.e., the receptor for C3b (Table 4). In contrast, both the meningococcal and gonococcal porins slightly inhibited the neutrophil upregulation of complement receptor 1 in response to FMLP compared with controls (Table 4).

TABLE 2. Effect of meningococcal class 1 and 3 porins and gonococcal P1B on neutrophil degranulation of primary granules as measured by upregulation of surface CD63 level[a]

Porin	Mean cellular CD63 FITC fluorescence ± SD (n = 4) at porin concn:				
	0 nM	50 nM	100 nM	500 nM	1,000 nM
Class 1	40.4 ± 2.3	39.1 ± 3.1	30.8 ± 2.9[b]	16.8 ± 2.9[b]	9.2 ± 1.8[b]
Class 3	40.5 ± 2.9	40.6 ± 2.3	39.6 ± 1.4	19.9 ± 3.6[b]	11.7 ± 2.0[b]
P1B	40.1 ± 1.7	41.3 ± 2.7	27.9 ± 1.4[b]	13.4 ± 1.6[b]	8.6 ± 1.6[b]

[a] Cytochalasin B-treated neutrophils were preincubated with neisserial porins for 30 min and then stimulated with 1 μM FMLP.
[b] $P \leq 0.05$, indicating that the neutrophil CD63 fluorescence was significantly lower than that observed in the absence of Empigen-BB or porins.

Preincubation of leukocytes with saturating amounts of an MAb against CD14 did not influence the porin-induced alterations of neutrophil FcγRs or complement receptor 1 (not shown). In addition, Empigen-BB at a concentration of 0.001% influenced neither the basal nor the stimulated neutrophil expression of FcγRs or complement receptor 1 of cytochalasin B-treated neutrophils (Table 4).

The effects of the meningococcal outer membrane proteins of classes 1 and 3 as well as gonococcal P1B on the neutrophil expression of complement receptor 3 (CD11b), i.e., the receptor for C3bi, are described above (Fig. 3 and Table 4).

Phagocytosis. In these experiments, serogroup B meningococci (strain 44/76) and sera from two different patients who had undergone meningococcal disease were used. Serum from the first patient (serum A) has earlier been shown to contain large amounts of IgG specific for meningococcal outer membrane protein class 3 (7.8 μg/ml) and less anti-class 1 IgG (0.6 μg/ml), whereas serum from the other patient (serum B) contained more anti-class 1 IgG (0.7 μg/ml) than anti-class 3 IgG (0.3 μg/ml) (21, 22).

The meningococcal and gonococcal porins both inhibited phagocytosis of *N. meningitidis* (Fig. 4). Following incubation of neutrophils for 10 min at a porin concentration of 500 nM, the mean number of meningococci per phagocyte was reduced by about 40 to 50%, irrespective of the serum used (Fig. 4). In four experiments with serum A, a fluorescence quenching technique was used to demonstrate that both the attachment and ingestion of meningococci were inhibited by the porins (Table 5). Preincubation of leukocytes with saturating amounts of an MAb against CD14 did not influence the porin-induced inhibition of neutrophil phagocytosis (not shown). In addition,

TABLE 3. Effect of meningococcal class 1 and 3 porins and gonococcal P1B on neutrophil degranulation of secondary granules as measured by upregulation of surface CD11b[a]

Porin	Mean cellular CD11b FITC fluorescence ± SD (n = 4) at porin concn:				
	0 nM	50 nM	100 nM	500 nM	1,000 nM
Class 1	74.6 ± 5.4	71.1 ± 2.4	65.2 ± 5.2[b]	48.6 ± 3.9[b]	37.1 ± 3.8[b]
Class 3	72.1 ± 3.8	72.0 ± 3.6	67.9 ± 3.2[b]	52.8 ± 4.8[b]	41.0 ± 4.8[b]
P1B	70.4 ± 2.3	71.4 ± 3.5	60.3 ± 3.5[b]	43.9 ± 5.7[b]	36.3 ± 3.7[b]

[a] Cytochalasin B-treated neutrophils were preincubated with neisserial porins for 30 min and then stimulated with 1 μM FMLP.
[b] $P \leq 0.05$, indicating that the neutrophil CD11b fluorescence was significantly lower than that observed in the absence of Empigen-BB or porins.

TABLE 4. Effect of meningococcal class 1 and 3 porins and gonococcal P1B on neutrophil opsonin receptor expression[a]

Receptor	Mean cellular FITC fluorescence ± SD[b]									
	Unstimulated					FMLP stimulated				
	PBS	Empigen-BB	Class 1	Class 3	P1B	PBS	Empigen-BB	Class 1	Class 3	P1B
FcγRII (CDw32)	31.5 ± 3.1	31.1 ± 3.4	23.5 ± 4.7*	22.7 ± 1.2*	18.5 ± 0.4*	31.7 ± 2.2	30.2 ± 1.6	19.5 ± 0.9†	23.5 ± 1.4†	17.8 ± 1.2†
FcγRIII (CD16)	129.7 ± 8.9	131.0 ± 6.1	87.2 ± 7.6*	111.1 ± 5.4*	72.0 ± 3.7*	87.7 ± 6.4	90.5 ± 4.1	76.7 ± 4.9†	93.8 ± 5.3	57.1 ± 2.6†
CR1 (CD35)	22.5 ± 1.3	21.0 ± 1.8	21.1 ± 2.5	20.7 ± 2.1	22.0 ± 1.2	33.9 ± 0.9	33.6 ± 1.4	28.1 ± 1.2†	30.8 ± 0.4†	27.5 ± 0.7†
CR3 (CD11b)	34.4 ± 4.0	36.1 ± 4.3	31.3 ± 3.8	32.6 ± 2.7	32.0 ± 3.9	74.3 ± 4.1	70.6 ± 3.6	48.7 ± 5.9†	51.1 ± 6.9†	40.2 ± 5.0†

[a] Neutrophil opsonin receptor expression was measured before and after stimulation with FMLP. The results are given as mean cellular FITC fluorescence ($n = 3$ for all receptors except CR3 [$n = 8$]).

[b] *, $P \leq 0.05$, indicating that the unstimulated neutrophil surface expression of opsonin receptor was significantly lower than that observed in the absence of Empigen-BB or porins; †, $P \leq 0.05$, indicating that the FMLP-stimulated neutrophil surface expression of opsonin receptor was significantly lower than that observed in the absence of Empigen-BB or porins.

Empigen-BB at a concentration of 0.001% did not influence neutrophil phagocytosis of meningococci (Fig. 4).

Oxidative burst. The meningococcal and gonococcal porins augmented the neutrophil oxidative burst in response to FMLP in a concentration-dependent manner (Fig. 5). Following incubation of neutrophils for 30 min at a P1B concentration of 500 nM, the mean hydrogen peroxide production in response to FMLP was 5.3 times larger than in unprimed cells. The FMLP-induced hydrogen peroxide production in neutrophils exposed to meningococcal class 1 protein was similar to that demonstrated for gonococcal P1B, whereas neutrophils exposed to class 3 meningococcal protein showed a less pronounced priming effect (Fig. 5). The lowest porin concentration inducing significant priming of the neutrophil FMLP-induced oxidative burst was 50 nM for meningococcal class 1 protein and gonococcal P1B and 125 nM for meningococcal class 3 protein (Fig. 5). The neisserial porins also primed cytochalasin B-treated neutrophils to increase their FMLP-induced hydrogen peroxide production (Table 6). The priming of neutrophils by neisserial porins was, however, restricted to FMLP, as the oxidative burst induced by PMA (5 to 50 ng/ml) was not affected by these proteins (Table 6). Preincubation of leukocytes with saturating amounts of an MAb against CD14 did not influence the porin-induced stimulation of neutrophil oxidative burst (not shown). In addition, Empigen-BB at a concentration of 0.001% did not influence the FMLP-induced oxidative burst, and neither the porins nor the detergent itself increased neutrophil oxidative metabolism above background levels (Table 6).

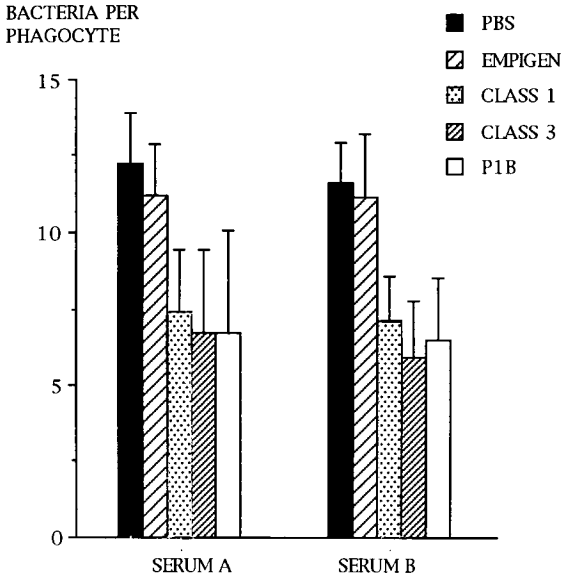

FIG. 4. Effect of meningococcal class 1 and 3 outer membrane proteins and gonococcal outer membrane P1B on neutrophil phagocytosis of FITC-labeled *N. meningitidis*. The neutrophils were incubated with neisserial porins (500 nM) for 10 min before incubation with meningococci. The results are given as the mean number of bacteria per phagocyte ± SD ($n = 4$).

DISCUSSION

The present study demonstrated that neisserial porins severely impair human neutrophil function in vitro. Both class 1 and class 3 meningococcal outer membrane proteins and gonococcal P1B inhibited neutrophil actin polymerization, degranulation, opsonin receptor expression, and phagocytosis but primed the neutrophils to increase their oxidative burst, as evaluated by intracellular hydrogen peroxide production.

The porins are voltage-dependent ion channels, where the meningococcal class 1 protein is cation selective and the meningococcal class 2 and 3 proteins and the gonococcal P1B are anion selective (34, 49). After incorporation of gonococcal P1B into the neutrophil surface membrane, Haines et al. have demonstrated that the cellular membrane potential and the intracellular signalling events change significantly after stimulation with FMLP (23, 24). Binding of FMLP normally induces association between its receptor and G-proteins in the neutrophil plasma membrane, initiating intracellular signalling events and functional responses, e.g., actin polymerization, degranulation, and oxidative burst (reviewed in reference 48). The chemoattractant–G-protein complex activates the phospholipase C, which catalyzes breakdown of phosphatidylinositol 4,5-bisphosphate to inositol 1,4,5-trisphosphate and diacylglycerol (DAG).

TABLE 5. Effect of meningococcal class 1 and 3 porins and gonococcal P1B on the attachment and ingestion phases of neutrophil phagocytosis[a]

Phase	Mean no. of meningococci/phagocyte ± SD ($n = 4$)				
	PBS	Empigen-BB	Class 1	Class 3	P1B
Attachment	2.5 ± 0.5	2.6 ± 0.7	0.9 ± 0.2[b]	0.9 ± 0.3[b]	0.8 ± 0.1[b]
Ingestion	11.5 ± 0.8	10.5 ± 1.8	5.3 ± 0.7[b]	5.1 ± 0.8[b]	5.6 ± 0.9[b]

[a] The neutrophils were preincubated with porins (500 nM) for 30 min, and serum A was used for opsonization. Following incubation with meningococci, attachment and ingestion were determined by a flow cytometric fluorescence quenching technique.

[b] $P \leq 0.05$, indicating that the number of meningococci per phagocyte was significantly lower than that observed in the absence of Empigen-BB or porins.

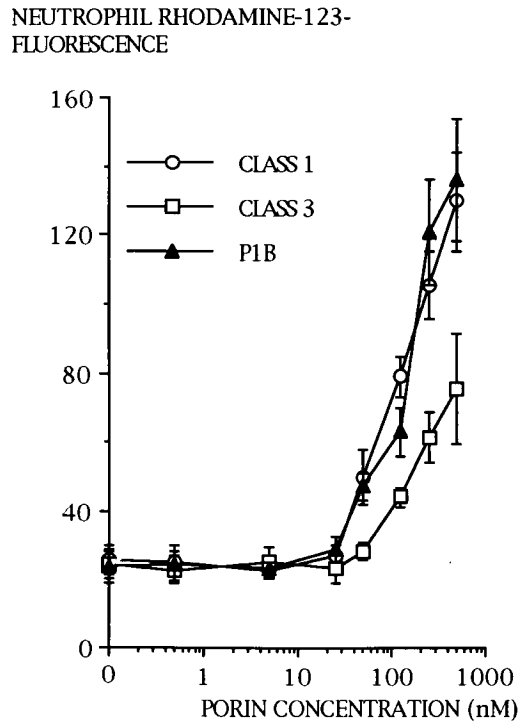

FIG. 5. Effect of meningococcal class 1 and 3 outer membrane proteins and gonococcal outer membrane P1B on neutrophil oxidative burst. Neutrophils were incubated with neisserial porins for 30 min before stimulation with 1 μM FMLP. The amount of hydrogen peroxide per neutrophil following stimulation with FMLP is indicated by the mean neutrophil rhodamine-123 fluorescence and given as mean channel fluorescence ± SD (n = 4). The fluorescence of unstimulated control neutrophils was 4.4 ± 1.1 (n = 4).

Inositol 1,4,5-trisphosphate and DAG are considered responsible for the release of calcium from intracellular stores and activation of protein kinase C, respectively (48). The accumulation of DAG in FMLP-stimulated neutrophils is biphasic, with a rapid and short as well as a slow and sustained DAG peak (24, 48). The rapid signalling response seems to be linked mainly to phosphatidylinositol 4,5-bisphosphate hydrolysis and to neutrophil-directed cell migration (48). In contrast, the slow DAG formation has been claimed to be linked more to hydrolysis of phophatidylcholine by the calcium-activated phospholipase D and to neutrophil microbicidal activities (degranulation and oxidative burst) (see reference 48). However, Haines et al., using the gonococcal outer membrane protein P1B to study the neutrophil intracellular signalling events, have presented data suggesting that the slow DAG response is not required to provoke activation of protein kinase C (24). Consistently, others have reported that it is generation of phosphatidic acid rather than the slow generation of DAG that may be the important signal for the assembly of the NADPH oxidase and the initiation of the oxidative burst (46). As the gonococcal P1B has been shown to influence neutrophil signalling events (23, 24) and this study documents that other neisserial porins also significantly alter several downstream function-linked processes, this group of bacterial proteins may represent a useful tool for further studies of neutrophil intracelluar signalling pathways.

Chemoattractants trigger rapid neutrophil actin polymerization, and the formation of F-actin seems to be linked to mediators of the phosphatidylinositol pathway. The relative roles of calcium ions and phospholipase C in the formation of polymerized actin are, however, still unclear (2, 3, 57). In the present study, both the meningococcal and gonococcal porins inhibited FMLP- and C5a-induced neutrophil actin polymerization. It has earlier been shown that the gonococcal outer membrane protein P1B does not influence the calcium fluxes induced by FMLP (23). Accordingly, the inhibition of F-actin formation may be linked to alterations of the phosphatidylinositol pathway, possibly by inhibiting the rapid formation of DAG.

The gonococcal P1B has been shown to inhibit the FMLP-induced neutrophil degranulation of primary granules, closely associated with the slow intracellular accumulation of DAG (23, 24). Our study demonstrated that the meningococcal porins inhibited neutrophil degranulation to the same extent as the gonococcal P1B and that neutrophil degranulation of both primary and secondary granules was inhibited by the neisserial porins. This suggests a common mechanism for the impairment of neutrophil degranulation by neisserial outer membrane proteins, and it is possible that this is linked to reduced slow generation of DAG.

The meningococcal and gonococcal porins inhibited the neutrophil attachment and ingestion of meningococci. The neutrophil expression of FcγRs as well as the activation-related upregulation of complement receptors 1 and 3 were reduced by the neisserial porins. It is known from earlier studies that the phagocytosis of meningococci is dependent on both immunoglobulins and complement (20, 25, 45). The reduced neutrophil expression of opsonin receptors may therefore have contributed to the impaired uptake of bacteria in the presence of porins. In addition, the reduced capacity of actin polymerization may also have impaired neutrophil pseudopod formation during ingestion, further inhibiting the uptake of meningococci.

It should be noted that all the neisserial porins examined inhibited the neutrophil phagocytosis of meningococci and that the inhibition was of similar magnitude irrespective of the content of antiporin antibodies in the serum used. This indi-

TABLE 6. Effect of meningococcal class 1 and 3 porins and gonococcal P1B on neutrophil oxidative burst

Porin	Mean cellular rhodamine-123 fluorescence ± SD (n = 4) after indicated treatment:				
	PBS preincubation			Cytochalasin B preincubation	
	PBS	FMLP	PMA	PBS	FMLP
None (PBS control)	4.4 ± 1.1	25.7 ± 4.3	203.9 ± 11.9	4.2 ± 0.6	43.5 ± 3.2
None (Empigen-BB control)	4.3 ± 0.7	25.3 ± 4.9	209.2 ± 12.8	4.5 ± 0.6	46.1 ± 4.1
Class 1	5.0 ± 0.9	129.7 ± 14.5[a]	204.5 ± 19.1	5.1 ± 1.4	182.8 ± 13.9[a]
Class 3	4.3 ± 0.7	75.6 ± 15.9[a]	207.9 ± 7.3	3.9 ± 0.4	146.6 ± 22.3[a]
P1B	4.4 ± 0.6	135.9 ± 17.7[a]	211.5 ± 13.1	4.4 ± 0.6	195.9 ± 15.1[a]

[a] $P \leq 0.05$, indicating that the neutrophil oxidative burst was significantly higher than that observed in the absence of Empigen-BB or porins.

cates that the reduced phagocytosis was due to a direct effect on the neutrophils rather than a lack of opsonizing antibodies due to complex formation between free porins and serum antiporin IgG.

The enzyme responsible for the neutrophil oxidative burst, the NADPH oxidase, catalyzes the conversion of oxygen to superoxide anions (29). This product is toxic but is further converted to bactericidal hydrogen peroxide by intracellular superoxide dismutase (29). Hydrogen peroxide also participates in the myeloperoxidase-mediated conversion of halides to a wide range of toxic compounds, including hypochloric acid and chloramines (29). In the present study, we have shown that neisserial porins significantly increased the neutrophil intracellular formation of hydrogen peroxide in response to FMLP in both the presence and absence of cytochalasin B. In addition, neither the meningococcal nor the gonococcal porins influenced the PMA-induced neutrophilic oxidative burst, indicating that the neisserial porins did not interfere with protein kinase C activity. PMA directly activates protein kinase C, bypassing the receptor-ligand interactions and the phosphatidylinositol pathway. Thus, the priming of human neutrophils to increase their FMLP-induced hydrogen peroxide production by the meningococcal and gonococcal outer membrane proteins may therefore be due to altered production of DAG or phosphatidic acid (24, 46, 48) (see also above).

Haines et al. have reported that the gonococcal porin P1B did not alter the neutrophil extracellular release of superoxide in response to FMLP (23, 24), whereas this study demonstrated significant increased FMLP-induced intracellular production of hydrogen peroxide in neutrophils exposed to both gonococcal and meningococcal porins. Even though the source of gonococcal P1B was different in the two studies (our P1B was from the gonococcal strain Pgh 3-2 [serotype P1-1] [53], whereas the P1B used by Haines et al. was from strain R10 [serotype P1-9] [23]), these findings suggest that neisserial porins can selectively stimulate the neutrophil intracellular generation of reactive oxygen intermediates. It is known from earlier reports that professional phagocytes can have their oxidative burst modified in such a way that the extracellular release and the intracellular production of superoxide and hydrogen peroxide are altered independently (38, 51). Interestingly, Naids and Rest have reported that gonococci induce the generation of reactive oxygen intermediates in the phagocytic vacuoles of human neutrophils without any detectable extracellular release of either superoxide or hydrogen peroxide (38). Whether this phenomenon is related to the effects of porins on neutrophils remains to be determined.

The porin-induced impairment of degranulation of both primary and secondary granules may impair the neutrophil intracellular killing of *Neisseria* species. Densen and Mandell have shown that phagolysosome formation is required for killing of gonococci (14). As the granules contain a variety of bactericidal cationic proteins and enzymes, and neutrophils from patients with chronic granulomatous disease effectively kill gonococci (41), it has been claimed that the neutrophil oxygen-independent microbicidal systems are especially active against gonococci (47). Consistently, others have reported that neutrophils kill ingested gonococci as well under anaerobic as under aerobic conditions if an incubation period of 2 h is used (13). Thus, the reduced neutrophil degranulation following interaction with gonococcal P1B may impair the oxygen-independent intracellular killing of these bacteria. Moreover, impaired degranulation can lower the amount of myeloperoxidase available in the phagolysosome, inhibiting the formation of halide-containing bactericidal compounds (29). It is therefore possible that our findings may be of importance for the efficacy of neutrophil gonococcal intracellular killing in vivo. As the relative importance of neutrophil oxygen-dependent and oxygen-independent bactericidal systems has not been examined for meningococci, it is not known whether our findings may have relevance for the intracellular killing of these bacteria in vivo.

Even though we were unable to detect LPS in our porin suspensions, it might be argued that very small amounts ($<0.01\%$) could have been responsible for the alterations of neutrophil functions observed. However, even though LPS, like the porins, primes neutrophils to increase their oxidative burst (10, 56), LPS also primes neutrophils to increase their actin polymerization and phagocytosis (10, 27). In addition, LPS directly induces neutrophil degranulation and upregulation of surface complement receptor 3 (CD11b) (56). Thus, the alterations of neutrophil functions induced by neisserial porins were not similar to those described earlier for LPS. In addition, saturating amounts of an MAb against CD14 (the receptor for the complex of LPS and LPS-binding protein) did not influence the porin-induced effects on neutrophils, further suggesting that LPS could not have been responsible for the functional alterations observed (35).

In conclusion, class 1 and 3 meningococcal outer membrane proteins as well as gonococcal outer membrane protein P1B inhibited human neutrophil actin polymerization, degranulation, and phagocytosis but primed the neutrophils to increase their oxidative burst. It remains to be demonstrated whether these in vitro observations indicate a neisserial antineutrophil strategy which may influence the virulence of the bacteria and impair the protective capacity of neutrophils against *N. meningitidis* or *N. gonorrhoeae* in vivo.

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