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Up-Regulation of Human Neutrophil Receptors for *Neisseria gonorrhoeae* Expressing PII Outer Membrane Proteins

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In the absence of serum, nonpiliated gonococci expressing PII outer membrane proteins (PIIs) adhere to human neutrophils whereas non-PII-expressing (PII$^-$) gonococci do not. After an observation that neutrophils in monolayers bound more gonococci than neutrophils in suspension, we treated neutrophil suspensions with known stimulants of degranulation and measured subsequent gonococcal adherence to suspended neutrophils. The chemotactic peptide *N*-formyl-methionyl-leucyl-phenylalanine (fmlp), the potent secretagogue phorbol myristate acetate, and the calcium ionophore A23187 all caused increased adherence of PII$^+$ gonococci, but not PII$^-$ gonococci, to neutrophils in a dose-responsive manner. Increased adherence of gonococci to neutrophils was paralleled by increased degranulation of neutrophil myeloperoxidase, lysozyme, and lactoferrin. Inhibition of fmlp-induced neutrophil degranulation by pertussis toxin, the calmodulin inhibitors trifluoperazine and *N*-5-chloronaphthalene sulfonamide, or the intracellular calcium-binding agent trimethoxybenzoic acid also inhibited fmlp-induced gonococcal adherence to neutrophils. Neither undifferentiated nor myelocytically differentiated HL-60 cells, which possess primary but defective or nonexistent secondary granules, bound PII$^+$ or PII$^-$ gonococci. Gonococci did not adhere to human monocytes, monocyte-derived macrophages, lymphocytes, platelets, or erythrocytes, indicating that several receptors, such as the complement receptors CR1, CR3 (CD11b/CD18), and CR4 (CD11c/CD18) or the adherence complex LFA-1 (CD11a/CD18), were probably not involved in gonococcal adherence to human neutrophils.

Gonococci possess antigenically variable outer membrane proteins, termed PII proteins, that appear to mediate adherence to human polymorphonuclear leukocytes (neutrophils) (PMNs) (14, 19, 23, 26, 46, 51, 52) and to various human epithelial cell lines (17, 53). In fact, it has been shown that an anti-PII monoclonal antibody (MAb) abrogates adherence of nonpiliated gonococci to human neutrophils (11). PIIs may bind to carbohydrate moieties of glycoconjugates in a lectinlike manner, since glycosidase treatment of buccal cells and of neutrophils decreases gonococcal attachment (17, 37) and since some carbohydrates interfere with stimulation of human neutrophil oxidative metabolism by gonococci (37) and with PII-mediated adherence of gonococci to one another (7a).

Several pathogens interact with eucaryotic cells via lectinlike mechanisms, including uropathogenic *Escherichia coli* and *Streptococcus pneumoniae*, which bind the galactose-β1-4-galactose disaccharide and glucosamine-β1-3-galactose portion of glycolipids, respectively (44). Several bacterial species bind to lactosylceramide present in epithelial cell membranes (15, 16). The type 1 fimbriae of *E. coli* bind to gp150,95 (CD11c/CD18) of neutrophils, presumably via mannose in the leukocyte receptor (38). Recent studies demonstrated that gonococci bind to certain carbohydrate structures in glycolipids, specifically lactosyl-, gangliotriaosyl-, isoglobotriaosyl-, and gangliotetraosylceramides (43). Gonococci adhere to these purified, immobilized glycolipids regardless of piliation or PII content, suggesting that these glycolipids are not the receptors for pilus- or PII-mediated gonococcal adherence (43).

In this paper we report that stimulation and subsequent degranulation of neutrophils enhances adherence of PII$^+$ gonococci and that the neutrophil receptors for PII$^+$ gonococci appear to be stored within a subcellular granule population(s) other than the classically defined azurophil granules.

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MATERIALS AND METHODS

Reagents. The following chemicals were purchased from Sigma Chemical Co. (St. Louis, Mo.): Ficoll, Wright stain, trypan blue, dimethyl sulfoxide (DMSO), buffer reagents, phorbol myristate acetate (PMA), N-formyl-methionyl-leucyl-phenylalanine (fmlp), A23187, cytochalasin B, trimethoxybenzoic acid (TMB-8), trifluoperazine (TFP), *N*-5-chloronaphthalene sulfonamide (W-7), *o*-phenylenediamine, and *o*-dianisidine hydrochloride. Lysozyme substrate was obtained from Difco Laboratories (Detroit, Mich.). Lactoferrin and antilactoferrin were purified in our laboratory as described previously (7, 35). Peroxidase-conjugated antilactoferrin was purchased from Cooper Biomedical (Cochranville, Pa.). Pertussis toxin (PT) was a gift from Richard Friedman, University of Arizona Medical Center, Tucson. HL-60 cells were provided by Robin Chou and Robert McMichael, Hahnemann University. The MAbs used were kindly furnished by the following investigators: NCD-1, NCD-2, and NCD-3 from T. G. Cotter, Saint Patrick's College, Maynooth, Ireland; 60.3 from P. G. Beatty, University of Washington, Seattle; TS1/18 from T. A. Springer, Sidney Farber Cancer Institute, Boston, Mass. MAbs anti-Leu M5 and anti-CR1 from Becton-Dickinson and Co. and anti-C3b from Dako Scientific were kindly donated by D. Moser, Temple University, Philadelphia, Pa.

Gonococci. *Neisseria gonorrhoeae* FA1090 (a disseminated strain) and F62 (a strain which causes localized infections) were passaged daily on GC medium base (Difco) with supplements (36). Strain F62 was obtained from P. Fred Sparling, University of North Carolina, Chapel Hill. Strain FA1090 was obtained from Janne Cannon, University of

North Carolina, Chapel Hill. Nonpiliated PII^+ or PII^- colony phenotypes were assessed by the methods of Kellogg et al. (20, 21) and Swanson et al. (45, 48), as well as by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (25). Gonococci were grown to mid-log phase in GC broth with supplements (36), pelleted, suspended to 2×10^8 per ml in Dulbecco phosphate-buffered saline–0.1% gelatin (PBSG) without calcium or magnesium (pH 7.4), and maintained at room temperature.

Neutrophil purification. Human neutrophils were purified from heparinized venous blood by a single centrifugation through Ficoll-Hypaque (13). The neutrophil-rich interface was washed once in PBSG at $200 \times g$ for 10 min, and erythrocytes were hypotonically lysed once. Neutrophils were resuspended in calcium-magnesium-free PBSG at 10^7/ml and maintained on ice. Except where indicated, calcium (1 mM) and magnesium (1 mM) were added at the time of assay. This procedure yielded at least 97% neutrophils as seen with Wright stain. Cells remained approximately 97% viable throughout all of the treatments and assays described, as measured by trypan blue exclusion.

Maintenance of HL-60 cells. HL-60 cells were cultured in Falcon tissue culture flasks in RPMI 1640 supplemented with 10% fetal calf serum (both from GIBCO Laboratories, Grand Island, N.Y.) in 5% CO_2 in air. DMSO-induced cells were cultured in medium with 1.25% DMSO, and PMA-induced cells were grown in medium with 50 nM PMA. Differentiation was monitored with Wright stain and by the ability of the cells to reduce ferricytochrome *c* (Sigma) upon the addition of 1 ng of PMA per ml (1).

Degranulation. Between 1×10^6 and 10×10^6 neutrophils in PBSG with calcium and magnesium were first treated with 5 μg of cytochalasin B per ml for 5 min at 37°C, with tumbling at 10 rpm. Neutrophils were then stimulated with PMA (0.001 to 100 ng/ml), fmlp (10^{-9} to 10^{-6} M), or A23187 (10^{-10} to 10^{-7} M) for 10 min at 37°C at 10 rpm. Alternatively, neutrophils were first treated with cytochalasin B and TMB-8, TFP, or W-7 at the doses indicated below, followed by incubation with fmlp (10^{-7} M). For PT treatments, cells were incubated with 100 ng of PT per ml at 37°C for 2 h and then stimulated as described. Treated neutrophils were centrifuged at $200 \times g$ for 8 min at room temperature, and the supernatants were transferred to fresh tubes and frozen at −20°C until the measurements of degranulation could be performed.

Measurement of granule components. Myeloperoxidase (MPO) and lysozyme were measured spectrophotometrically in a Perkin-Elmer dual-beam spectrophotometer as described elsewhere (1). Briefly, MPO was measured by monitoring the reduction of *o*-dianisidine hydrochloride at 450 nm. The measurement of lysozyme was accomplished by measuring the decrease in optical density at 450 nm of a solution of *Micrococcus lysodeikticus* (lysozyme substrate). In assays using inhibitors of degranulation, neutrophils not treated with inhibitor were assigned control values. In the assay examining the effect on adherence and degranulation of various doses of PMA, Triton-lysed cells were assigned control values. Results are expressed as the percentages of control MPO or lysozyme release for a given number of cells. For each assay, Triton (0.1%)-lysed neutrophils and untreated neutrophils produced expected absorbance values. Lactoferrin release was evaluated by enzyme-linked immunosorbent assay (5) and is shown as the percentage of control lactoferrin measured. Lactoferrin standards were assayed in quadruplicate and sample supernatants were assayed in duplicate in a given assay. Typical lactoferrin assay absorbance values showed that 10^6 neutrophils secreted 20 to 300 ng of lactoferrin, depending on the treatment. Control values are presented in the figure legends.

Adherence. To 10^6 neutrophils or HL-60 cells, 200 μl of 2×10^8 gonococci per ml was added in a final volume of 1 ml of PBSG with or without calcium and magnesium. To measure adherence versus phagocytosis, neutrophils were always pretreated with 5 μg of cytochalasin B per ml. Previous assays which looked at adherence of gonococci to non-cytochalasin B-treated PMNs gave adherence values negligibly higher than those for cytochalasin B-treated cells (3.89 ± 0.65 versus 3.47 ± 0.89 gonococci per PMN, $n = 4$). No calcium or magnesium was added when TFP, W-7, or TMB-8 was used. Adherence mixtures were tumbled in 1.5-ml microcentrifuge tubes at 10 rpm for 30 min at 37°C. Samples (125 μl) were then centrifuged for 10 min at 200 rpm in a Shandon cytocentrifuge (Pittsburgh, Pa.) and Wright stained. One hundred to 200 contiguous neutrophils were examined under oil, and the average number of gonococci per neutrophil was calculated. Results are expressed as the average number of gonococci per neutrophil. Control values were assigned as described in figure legends and table footnotes.

MAbs. MAbs were incubated with neutrophils at the stated dilutions for 20 min at 37°C with rotation at 10 rpm. Gonococci and PBSG plus calcium-magnesium were then added to a final volume of 1 ml, and adherence assays were performed. An enzyme-linked immunosorbent assay showed that all the antibodies were able to adhere to PMA-stimulated PMNs. They were also checked in a ferricytochrome *c* reduction assay for their ability to inhibit an opsonized zymosan-induced neutrophil oxidative burst (1). As expected, MAbs 60.3, TS1/18, and anti-Leu M5 and antibodies to CR1 inhibited the burst greater than 85% at dilutions of 1:100 or less.

Statistics. *P* values were calculated according to the Mann-Whitney U test for adherence and degranulation results. Significance was evaluated by comparing experimental values to control (i.e., untreated) values for experiments done on at least three separate days.

RESULTS

Preliminary observations. In preliminary studies we noticed that neutrophils adherent to glass coverslips bound severalfold more PII^+ (but not PII^-) gonococci than did neutrophils in suspension (13.53 ± 1.05 versus 5.80 ± 0.79 gonococci per neutrophil, $n = 3$). These results suggested that activation of neutrophils i.e., the act of adhering to and spreading on a glass substrate (29), caused them to bind more PII^+ gonococci. We tested this hypothesis by performing the experiments described in this paper, in which we stimulated neutrophils in suspension with various stimulants and measured their ability to bind gonococci. Preliminary characterization studies showed that gonococci adhered only to neutrophils and not to erythrocytes, monocytes, eosinophils, or lymphocytes (for all cells, <0.2 gonococcus per cell). In addition, monocyte-derived macrophages (cultured on Lab-Tek plastic chamber slides for 1 week) did not bind PII^+ or PII^- gonococci. To assure that we were observing only binding, and not phagocytosis, of gonococci to neutrophils, we used 5 μg of cytochalasin B per ml in all experiments described in this paper. In fact, in the relatively short adherence assay (30 min, see Materials and Methods), we noticed no significant effect of cytochalasin B on binding of gonococci to resting or stimulated neutrophils. Strains

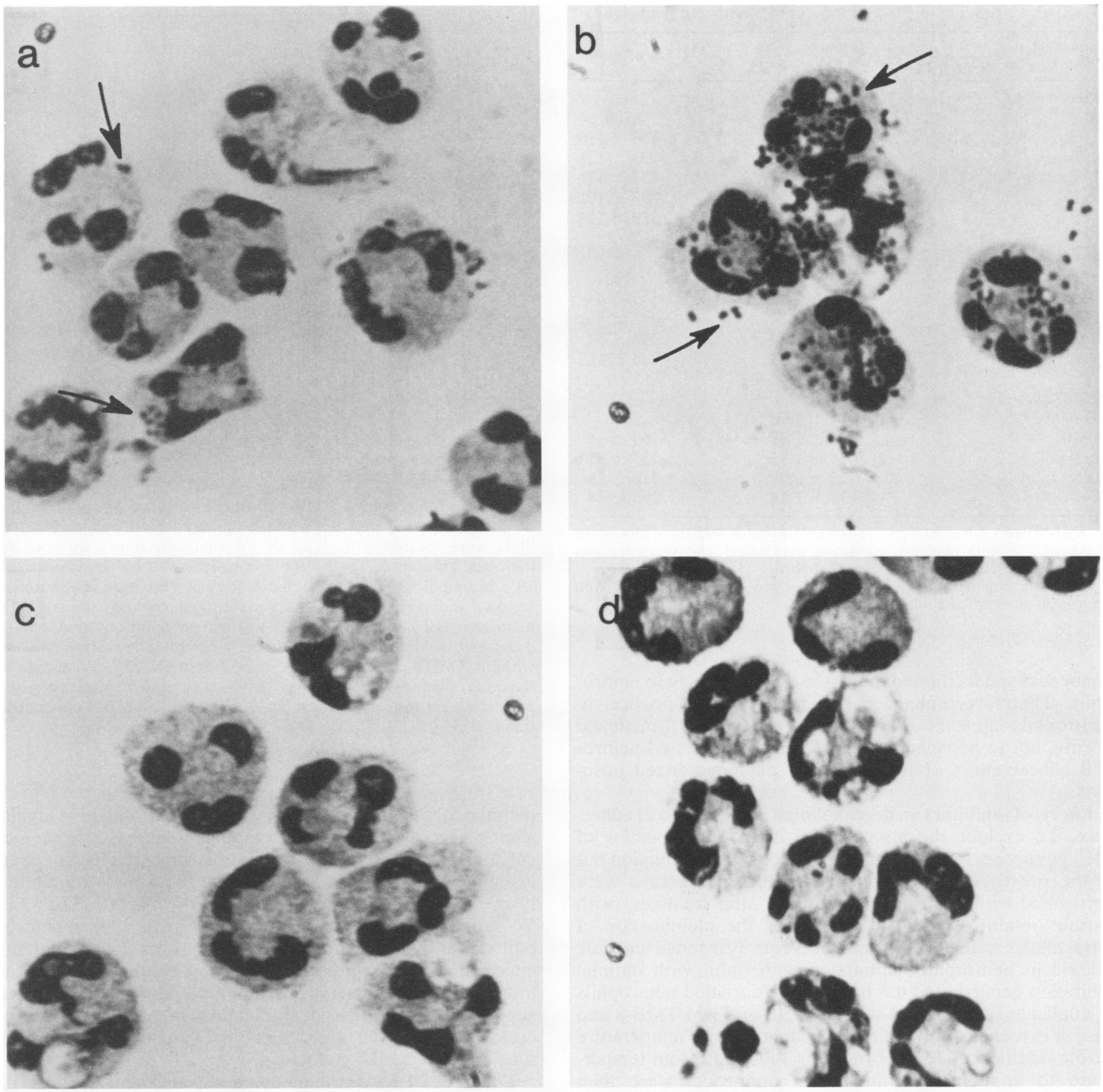

FIG. 1. Adherence of PII+ (a and b) and PII− (c and d) gonococci to unstimulated (a and c) and PMA (10 ng/ml)-stimulated (b and d) neutrophils. Photographs were from a typical adherence experiment, at an initial magnification of ×1,000. Arrows indicate gonococci. Diplococci were counted as a unit.

FA1090 and F62, gonococcal strains responsible for disseminated and localized in vivo infections, respectively, bound to neutrophils to similar degrees.

Effects of secretagogues on gonococcal adherence to neutrophils. To test, in a more controlled manner, whether activated neutrophils bound greater numbers of gonococci than did resting or unstimulated neutrophils, we treated neutrophils in suspension with three different secretagogues and measured their ability to bind PII+ or PII− gonococci. A23187, fmlp, and PMA all caused an increase in adherence of PII+ gonococci to neutrophils in a dose-responsive manner (Table 1 and Fig. 1). PII− gonococci adhered only slightly better to stimulated than to unstimulated neutrophils (less than one diplococcus per neutrophil, Fig. 1). The slight increase in adherence of PII− gonococci to stimulated neutrophils was probably due to the few PII+ gonococci always present in a PII− culture (6, 47). Essentially identical results to those presented in Table 1 were observed when neutrophils stimulated with 10^{-6} M PMA or 10^{-7} M fmlp were washed prior to their exposure to gonococci. This indicated that increased adherence of gonococci to neutrophils was not due to a secreted opsonin that initially bound to the

TABLE 1. Binding of PII$^+$ gonococci to stimulated neutrophils

Stimulant and concn (log M)[a]	Gonococci/ PMN	% of control
PMA (n = 6)[b]		
0	2.84 ± 1.65	100
−9	5.34 ± 3.59	188 ± 126
−8	6.34 ± 3.46	223 ± 121
−7	7.99 ± 3.17	281 ± 111
−6	8.51 ± 2.92	297 ± 101
−5	10.78 ± 3.81	389 ± 133
fmlp (n = 4)		
0	4.16 ± 2.28	100
−9	6.02 ± 1.83	145 ± 44
−8	6.89 ± 2.12	166 ± 51
−7	7.98 ± 2.44	192 ± 59
−6	9.98 ± 4.89	240 ± 117
A23187 (n = 3)		
0	3.44 ± 1.96	100
−10	3.10 ± 1.45	90 ± 34
−9	4.76 ± 1.93	138 ± 55
−8	7.23 ± 0.98	210 ± 28
−7	10.14 ± 1.18	295 ± 34

[a] Cytochalasin B-treated neutrophils were incubated with the stimulant for 10 min at 37°C with tumbling, gonococci were added, and adherence was measured as described in Materials and Methods.

[b] n = the number of experiments performed on different days with neutrophils from different donors.

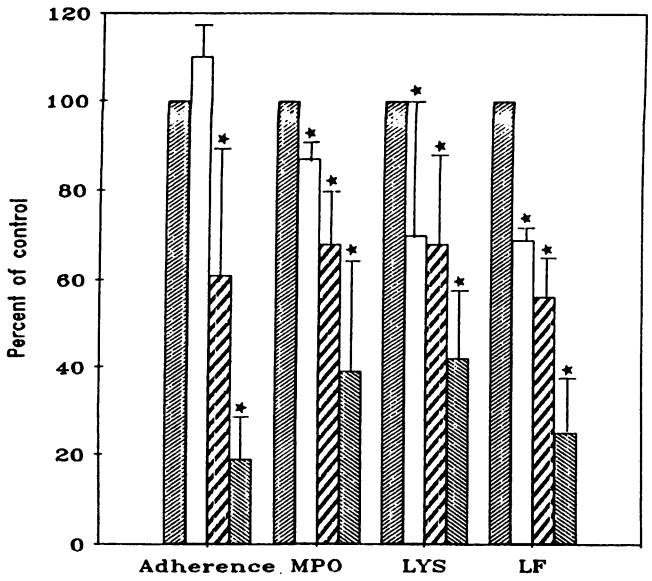

FIG. 2. Inhibition by TMB-8 of fmlp-induced degranulation and of gonococcal adherence to fmlp-stimulated neutrophils. Neutrophils were treated with 5 μg of cytochalasin B per ml and the indicated amount of TMB-8 for 5 min, followed by treatment with 10^{-7} M fmlp for 10 min. Treated neutrophils were used in adherence assays, and degranulation was quantitated by measuring MPO, lactoferrin (LF), and lysozyme (LYS) in neutrophil supernatants, as described in Materials and Methods. Control cells were incubated without TMB-8. Results are expressed as the mean percentage of the control value ± standard deviation, calculated from experiments performed on four separate days. ★, $P \leq 0.05$. TMB-8 concentrations: ▨, 0; □, 5 μM; ▨, 50 μM; ▧, 500 μM.

gonococci and facilitated their subsequent binding to neutrophils. Therefore, enhanced binding of PII$^+$ gonococci to neutrophils appears to be due to specific PII-mediated events, not to nonspecific events such as increased neutrophil adhesiveness or the release of uncharacterized opsonins.

Effects of inhibitors on degranulation and gonococcal adherence. To exclude the possibility that increased binding of PII$^+$ gonococci occurred in the absence of degranulation but in the presence of secretagogues, adherence assays were performed with neutrophils stimulated after treatment with various inhibitors. TMB-8 diminishes the mobilization of intracellular calcium (28) and prevents lysosomal enzyme release in neutrophils stimulated with fmlp, with optimal inhibition occurring at 0.5 mM (41). We treated neutrophils in duplicate sets of tubes with 500, 50, or 5 μM TMB-8 and 5 μg of cytochalasin B per ml for 10 min at room temperature before addition of 10^{-7} M fmlp for 10 min at room temperature to stimulate degranulation. Gonococci were then added to one set of tubes to measure adherence. The supernatants from the other set of tubes were obtained (250 × g, 10 min, 37°C) and used to quantitate degranulation. Both degranulation and gonococcal adherence were inhibited by TMB-8, and enzyme release from neutrophils paralleled levels of adherence by gonococci (Fig. 2).

To confirm that inhibition of neutrophil degranulation paralleled inhibition of gonococcal adherence to neutrophils, we used the inhibitors TFP and W-7. TFP and W-7 prevent the interaction of calmodulin and calcium within cells (27, 41). Neutrophils require alteration in cytoplasmic calcium to undergo optimal degranulation (18, 40). Pretreatment of neutrophils with 5 μg of cytochalasin B per ml and 50 μM TFP (Fig. 3) or 100 μM W-7 (Fig. 4) inhibited binding of PII$^+$ gonococci to neutrophils by 89 and 83%, respectively. The effects of TFP and W-7 were dose responsive (Fig. 3 and 4). Supernatants isolated from neutrophils treated in the same manner contained MPO, lysozyme, and lactoferrin at significantly decreased levels that paralleled decreased gonococcal adherence (Fig. 3 and 4). Fifty micromolar TFP inhibited lysozyme release by 54.3%, whereas 100 μM W-7 inhibited lysozyme release by 78.8%. None of the TMB-8, TFP, and W-7 concentrations used in our experiments altered neutrophil viability as measured by trypan blue exclusion. Adherence values in the presence of an inhibitor(s) were never lower than those observed with untreated cells. These data support the idea that degranulation is necessary for increased expression (up-regulation) of neutrophil receptors for nonpiliated PII$^+$ gonococci.

Effects of PT on degranulation and gonococcal binding. To verify that neutrophil degranulation was necessary for increased gonococcal adherence, neutrophils were incubated with PT before adherence assays were performed. PT inhibits fmlp-induced inositol lipid hydrolysis, mobilization of intracellular calcium, and neutrophil secretion and aggregation (24) by inhibiting the G protein–phosphatidyl inositol-4,5-bisphosphate pathway through which fmlp functions. PT does not affect degranulation induced by PMA, which acts via direct binding to protein kinase C (31). We treated neutrophils consecutively with 100 ng of PT per ml in PBSG for 2 h, 5 mg of cytochalasin B per ml for 5 min, and finally with either fmlp (10^{-7} M) or PMA (10 ng/ml) for 10 min. All incubations were at 37°C with gentle mixing. Gonococci were then added to the neutrophils, and adherence was measured. PT (100 ng/ml) decreased gonococcal binding by 63% while decreasing degranulation to the same or a slightly greater degree (Table 2). On the other hand, PT-treated,

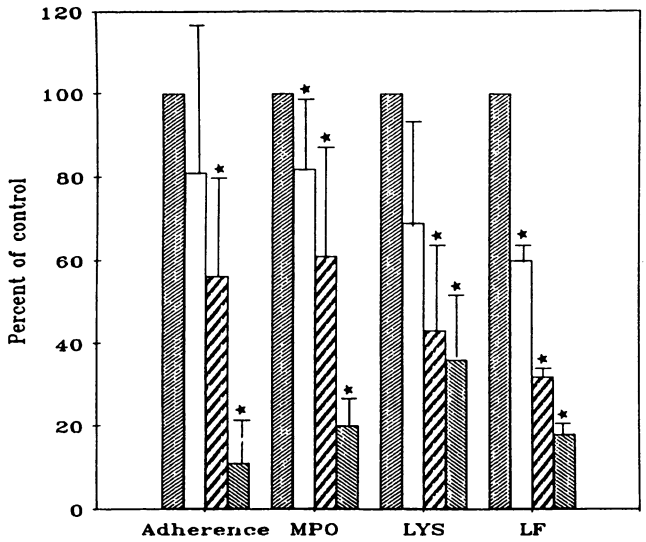

FIG. 3. Inhibition by TFP of fmlp-induced degranulation and of gonococcal adherence to fmlp-stimulated neutrophils. Assays were performed as described in the legend to Fig. 2. Control cells were incubated without TFP. Results are expressed as the mean percentage of the control value ± standard deviation, calculated from experiments performed on four separate days. ⋆, $P \leq 0.05$. TFP concentrations: ▨, 0; □, 0.5 μM; ▨, 5 μM; ▧, 50 μM.

PMA-stimulated neutrophils secreted MPO and lactoferrin at or above control values and bound gonococci to an equal or slightly greater extent than non-PT-treated, PMA-stimulated neutrophils (Table 2). As above, these data support the concept that neutrophil degranulation is required for increased binding of nonpiliated PII+ gonococci to stimulated human neutrophils.

Adherence of gonococci to HL-60 cells. Data obtained thus

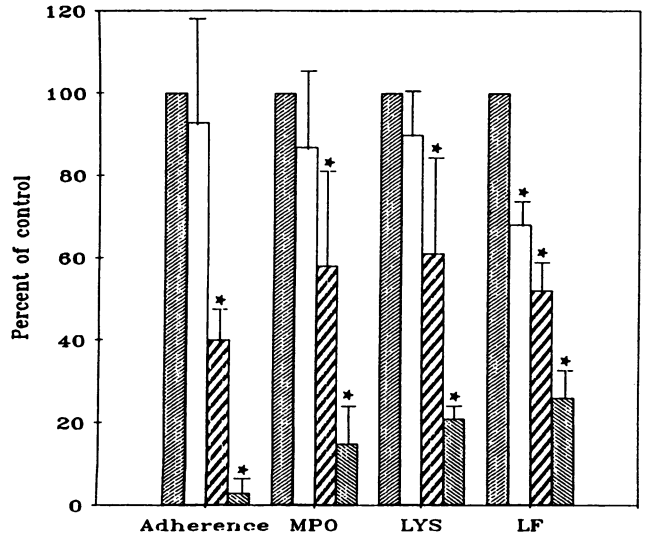

FIG. 4. Inhibition by W-7 of fmlp-induced degranulation and of gonococcal adherence to fmlp-stimulated neutrophils. Assays were performed as described in the legend to Fig. 2. Control cells were incubated without W-7. Results are expressed as the mean percentage of the control value ± standard deviation, calculated from experiments performed on four separate days. ⋆, $P \leq 0.05$. W-7 concentrations: ▨, 0; □, 1 μM; ▨, 10 μM; ▧, 100 μM.

TABLE 2. Effects of PT on fmlp- or PMA-stimulated neutrophil degranulation and gonococcal adherence[a]

Measurement	% of control[b]	
	PT + fmlp	PT + PMA
Adherence (n = 3)	32 ± 1[c]	96 ± 2
MPO secretion (n = 4)	20 ± 5[c]	190 ± 43[c]
Lysozyme secretion (n = 4)	21 ± 3[c]	72 ± 9[c]
Lactoferrin secretion (n = 4)	30 ± 18[c]	116 ± 47

[a] Neutrophils were incubated with or without 100 ng of PT per ml in buffer (PBSG) for 2 h with tumbling at 37°C. Cytochalasin B (5 μg/ml) and then fmlp (10^{-7} M) or PMA (10^{-9} M) were added for 10 min, and PMN degranulation and adherence of gonococci to neutrophils were measured, as described in Materials and Methods.

[b] PBSG-treated PMNs stimulated with fmlp or PMA were assigned 100% adherence and degranulation values in each experiment. The percentage of the control value was calculated for individual experiments. Results are the mean percentage of the control value ± the standard deviation from experiments performed on n number of days with neutrophils from n different donors.

[c] $P \leq 0.05$.

far suggested that neutrophil receptors for PII+ gonococci were stored within a granule population(s) within neutrophils. To probe this phenomenon further, we examined the interaction of gonococci with HL-60 cells, a promyelocytic leukemia cell line deficient in secondary granules that can be induced to differentiate into more mature monocytes or neutrophils (8, 30, 32).

We examined binding of gonococci (PII+ and PII−) to undifferentiated HL-60 cells, as well as to HL-60 cells induced to differentiate with 1.25% DMSO for 8 days. DMSO-differentiated HL-60 cells are capable of reducing cytochrome *c* upon stimulation with PMA (indicating their ability to produce superoxide through activation of their NADPH oxidase) and more closely resemble mature neutrophils. Gonococcal binding was also examined with HL-60 cells treated with PMA, fmlp, or A23187. Neither undifferentiated, differentiated, nor stimulated HL-60 cells bound significant numbers of gonococci (Table 3). HL-60 cells induced to differentiate into monocytes in the presence of 5×10^{-8} M PMA for 8 days were able to reduce cytochrome *c*, as described previously (50), but remained unable to bind gonococci (Table 3). In summary, negligible levels of adherence occurred with HL-60 cells regardless of their treatment. Therefore, it appears that HL-60 cells lack receptors for PII+ gonococci.

Degranulation and gonococcal adherence after PMA treatment. Since HL-60 cells have defective or nonexistent secondary granules (30), the HL-60 experiments suggested that secondary or tertiary granule degranulation was necessary for gonococcal adherence to be enhanced. Therefore, we treated PMNs with increasing doses of PMA, a chemical that causes preferential degranulation of secondary granules, and we monitored adherence of PII+ gonococci and release of lactoferrin and MPO. Adherence of PII+ gonococci to neutrophils began to increase at 0.1 ng of PMA per ml, as did lactoferrin secretion (Fig. 5). MPO release was not detected until 1 ng of PMA per ml was used. As the amount of PMA increased above 10 ng/ml, a significant amount of MPO was released by neutrophils; however, trypan blue staining of the neutrophils showed that some cell damage also occurred above 10 ng of PMA per ml. Thus, increased gonococcal adherence paralleled lactoferrin release (Fig. 5). These data again imply that the component responsible for binding PII+ gonococci comes from granule populations other than primary granules, most likely secondary or tertiary.

TABLE 3. Gonococcal adherence to neutrophils and HL-60 cells[a]

Cells and treatment	Stimulant[b]	GC/cell	Absorbance (cytochrome c reduction[c])
PMNs	None	3.30	
	PMA	8.85	0.880
HL-60	None	0.02	
	PMA	0.03	0.004
	fmlp	0.02	
	A23187	0.19	
HL-60 + DMSO for 8 days	None	0.21	
	PMA	0.16	0.980
	fmlp	0.19	
	A23187	0.74	

[a] Data are from one representative experiment of three. All gonococci used were PII$^+$.

[b] Stimulants were used at the following final concentrations: PMA, 100 ng/ml; fmlp, 10^{-7} M; A23187, 5×10^{-7} M.

[c] Superoxide dismutase-inhibitable cytochrome c reduction was measured in a continuous rate assay as described previously (50). Results are expressed as the change in optical density at 550 nm per minute per 10^5 cells.

Effects of antineutrophil MAbs on gonococcal adherence. We investigated whether a known granulocyte and/or up-regulatable neutrophil surface protein(s) might be involved in the interactions of neutrophils with PII$^+$ gonococci. Several such MAbs were preincubated with neutrophils at dilutions ranging from 1:5 to 1:1,000 prior to the addition of PII$^+$ or PII$^-$ gonococci (Table 4). None of the antibodies inhibited gonococcal adherence to neutrophils. It appears that the up-regulatable granulocyte cytoplasmic membrane proteins, such as CR1, CR3, or CR4 or those recognized by the MAbs NCD-1 and -2, all of which are involved in various neutrophil functions, are not involved in the interaction of neutrophils with nonpiliated, PII$^+$ gonococci. These data support our observations that nonpiliated gonococci do not adhere to other blood cells which share some or all of these membrane proteins.

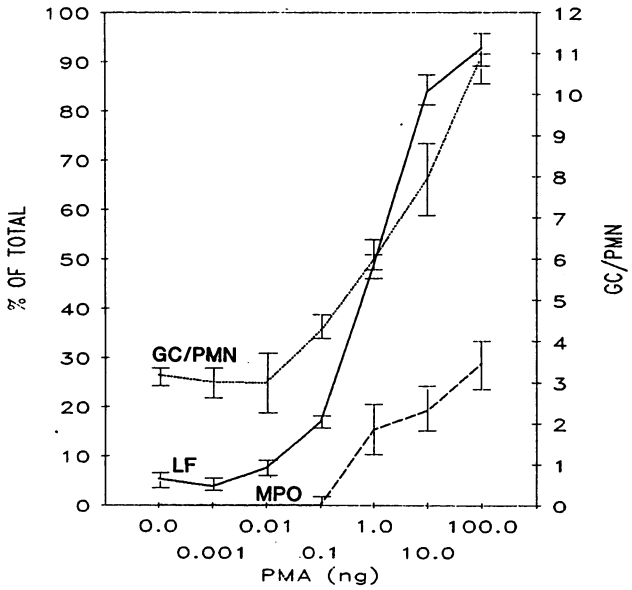

FIG. 5. Effect of PMA on neutrophil degranulation and gonococcal adherence. PMNs were pretreated with cytochalasin B and incubated with the PMA amounts shown, as stated in Materials and Methods. Some of the PMNs were used in adherence assays, while the rest were spun down and their supernatants were used to measure degranulation. The MPO and lactoferrin (LF) values were compared with total protein from Triton (0.2%)-lysed PMNs and are presented as percentages of the total secreted. Purified lactoferrin and MPO were used as standards. Gonococcal/PMN ratios (GC/PMN) were quantitated from the number of gonococci adhering to 100 PMNs. Results are from assays done in duplicate on three separate days.

TABLE 4. MAbs to neutrophil surface antigens that do not inhibit gonococcal adherence[a]

MAb	Human PMN epitope bound by antibody
60.3	Human PMN LFA family, beta chain (CD18) (31)
TS1/18	Human PMN LFA family, beta chain (CD18) (42)
NCD-3	C3bi receptor (CR3), alpha chain (CD11b) (42)
Anti-Leu M5	gp95,150 (CR4), alpha chain (CD11c)[b]
Anti-C3b receptor	C3b receptor (CR1)[c]
Anti-CR1	C3b receptor (CR1)[d]
NCD-1	Inhibits chemotaxis and lysozyme secretion (10)
NCD-2	Epitope involved in phagocytosis (9)

[a] Results are representative of two or three separate assays for each MAb.

[b] Obtained from Becton-Dickinson and Co., catalog no. 7630.

[c] Obtained from DAKO Scientific, catalog no. M710.

[d] Obtained from Becton-Dickinson and Co., catalog no. 7600.

DISCUSSION

As a rule, nonpiliated, PII$^+$ gonococci bind to human neutrophils in the absence of serum, whereas nonpiliated, PII$^-$ gonococci do not (14, 52). Although pili are certainly implicated in the adherence of gonococci to various cell types, they do not seem to play a major role in adherence of gonococci to human neutrophils. Rather, it is PII that appears to be the primary factor in gonococcus-neutrophil interactions (39, 49, 52). We are interested in characterizing the neutrophil receptor(s) for PII$^+$ gonococci. It appears that we have found a useful tool for the study of neutrophil-gonococcus interactions in that pretreatment of neutrophils with the stimulant PMA, fmlp, or A23187 strongly enhanced binding of PII$^+$ gonococci. Each of these reagents, via different molecular and cellular mechanisms, provokes the neutrophil to undergo a respiratory burst and to degranulate (3, 12, 55). Cytochalasin B was used in all of our assays, initially to assure that we were observing adherent and not internalized gonococci. Cytochalasin B also amplifies the degranulation and up-regulation phenomena since it causes the subcellular granules to merge with the cell membrane and release their contents onto the neutrophil surface and into the extracellular environment (for a review, see reference 1). In addition, for still unknown reasons, fmlp requires pretreatment with cytochalasin B in order to exert maximal stimulatory effects.

Our data show that when neutrophils were stimulated to degranulate, they concurrently bound higher numbers of gonococci (about 3 times more on average but 5 to 10 times more depending on the experiment and the donor). Conversely, if neutrophil degranulation was inhibited by TFP, W-7, or TMB-8, enhanced binding of gonococci was abrogated (Fig. 2 to 4). Similarly, PT treatment of neutrophils blocked fmlp-inducible stimulation and degranulation but not PMA-elicited events. When degranulation was thus inhibited, so was gonococcal adherence. Thus, some component of the granules seems necessary, if not sufficient, for gonococcal adherence. Although peak adherence of gonococci occurred at concentrations which are higher than those known to induce chemotactic phenomena, concentrations as

low as 1 ng/ml still caused higher numbers of PII+ gonococci to adhere to PMNs. Whether the granule constituent that binds to gonococci is contained within a granule or is a component of a granule membrane is not known. Increased attachment is not a nonspecific event, since PII− gonococci never or negligibly adhered to stimulated or unstimulated neutrophils. Also, since increased attachment occurred regardless of the gonococcal strain, it appears that the same neutrophil receptor binds PII from gonococci able to cause either a disseminated or localized infection.

Knowing that degranulation increased gonococcal attachment to neutrophils, we investigated whether receptors existed in only one of the known granule populations. For instance, relatively low concentrations of PMA induce predominantly secondary (specific) and tertiary granule release, as opposed to primary (azurophil) granule release (55). Even at a very low concentration (1 ng/ml) of PMA, increases in gonococcal binding were still significant (Table 1, Fig. 5), suggesting that the receptors we are observing are not located in primary granules. In the same vein, we examined adherence to both differentiated and undifferentiated HL-60 cells. HL-60-derived granulocytes have defective or nonexistent secondary (i.e., lactoferrin-containing) granules (30). HL-60-derived granulocytes bound little or no PII+ (or PII−) gonococci in the presence or absence of secretagogues. These results indicate that the component of mature neutrophil plasma membranes and granules that binds gonococci is absent in HL-60 cells and that the receptors appear to reside in a granule population other than the primary granules.

We also investigated whether some known, up-regulatable neutrophil receptor could be the receptor for PII+ gonococci. There are several characterized receptors on granulocytes, monocytes, and lymphocytes. CR1, the C3b receptor, is a glycoprotein on erythrocytes, neutrophils, monocytes, and B lymphocytes and is up-regulated in stimulated neutrophils (4). However, PII+ gonococci do not adhere to erythrocytes, monocytes, or B lymphocytes, suggesting that CR1 is not involved in gonococcal adherence. In addition, neither of two MAbs recognizing CR1 inhibited adherence of gonococci to neutrophils (Table 4). CR3, the receptor for C3bi, which is shared by neutrophils and monocytes (4), also appears not to be the receptor involved in our observations since PII+ gonococci do not bind to monocytes or monocyte-derived macrophages and since an anti-CR3 MAb (NCD-3) did not inhibit gonococcal attachment to neutrophils (Table 4). gp150,95, another member of the human leukocyte LFA family (2, 4), is found on granulocytes and monocytes, is thought to bind C3 degradation products (i.e., is CR4), and like CR3, is up-regulated; however, MAbs 60.3 and anti-Leu M5 did not decrease adherence by PII+ gonococci. It may be that the molecule we are searching for could be similar to the neutrophil BH2-C6 antigen, which is only expressed in mature, human neutrophils (34). However, this antigen appears on differentiated HL-60 cells, unlike the neutrophil receptor we hope to identify.

Some of our experiments have been attempted with purified PII, but purified PII does not bind well to neutrophils. However, further experiments dealing with PII, rather than whole gonococci, are being done and will be discussed in a future publication.

If we in fact are observing a receptor for gonococci that is up-regulated in human neutrophils, this would be an unusual phenomenon. As stated above, there are several complement receptors that are up-regulated from the subcellular granules, but very few up-regulated receptors for pathogens have been identified. *E. coli* type 1 fimbriae bind to the up-regulatable gp150,95 on neutrophils (38) and to the CR3, LFA, and gp150,95 on human macrophages via their lipopolysaccharides (56). As for other pathogens, CR3 has been identified as the receptor for *Leishmania donovani* in human monocytes (54); experiments using a MAb against CR3 inhibited binding by the organism. Human monocyte complement receptors mediate binding of *Legionella pneumophila* (33). However, we appear to have found a receptor which has not been characterized.

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