

## Growth of *Neisseria gonorrhoeae* in CMP-*N*-Acetylneuraminic Acid Inhibits Nonopsonic (Opacity-Associated Outer Membrane Protein-Mediated) Interactions with Human Neutrophils

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**Gonococci possessing certain opacity-associated (Opa) outer membrane proteins adhere to and are phagocytosed by human neutrophils in the absence of serum. Recently, it has been shown that serum-sensitive strains of *Neisseria gonorrhoeae* possessing the appropriate lipooligosaccharide phenotype become serum resistant when grown in the presence of CMP-*N*-acetylneuraminic acid (CMP-NANA) because of sialylation of their lipooligosaccharide. We investigated whether such sialylation affects nonopsonic (antibody- and complement-independent) interactions of gonococci with human neutrophils in vitro. We grew Opa<sup>+</sup> gonococci in the presence of up to 50 µg of CMP-NANA per ml, incubated them with neutrophils in vitro, and measured their abilities to adhere to neutrophils, stimulate neutrophil luminol-dependent chemiluminescence (LDCL), and be phagocytically killed by neutrophils. Growth in CMP-NANA dramatically inhibited (in a dose-dependent manner) the ability of Opa<sup>+</sup> gonococci to adhere to neutrophils and stimulate neutrophil LDCL. Growth of Opa<sup>+</sup> gonococci in 50 µg of CMP-NANA per ml appeared to delay, but did not inhibit, their killing by neutrophils. Sialidase treatment of sialylated Opa<sup>+</sup> gonococci, i.e., gonococci grown with CMP-NANA, totally restored their abilities to adhere to neutrophils and stimulate neutrophil LDCL. Opa<sup>-</sup> gonococci grown in the presence of 50 µg of CMP-NANA per ml and opsonized with fresh human serum bound to neutrophils only about 30% less efficiently than did Opa<sup>-</sup> gonococci grown without CMP-NANA and opsonized. The results of our studies show that sialylated Opa<sup>+</sup> gonococci have dramatically reduced nonopsonic interactions with neutrophils. Some gonococcal strains may resist killing by human neutrophils in vivo by such a mechanism.**

Gonorrhea is characterized by a cervical or urethral purulent exudate comprising tremendous numbers of neutrophils and free and cell-associated *Neisseria gonorrhoeae* organisms (4, 10, 16, 35, 36, 61, 62). Gonococci can interact with human neutrophils by nonopsonic (antibody- and complement-independent) mechanisms if they possess one or more of a family of heat-modifiable outer membrane proteins termed opacity-associated (Opa) proteins (previously called PII proteins) (13, 17, 23-26, 45, 47, 50, 51, 52, 55, 59, 60, 64). Opa proteins are one of several gonococcal outer membrane components that undergo frequent antigenic and/or phase variation (8, 15, 32, 49). An individual gonococcus can possess about a dozen Opa genes and can express zero to three or more Opa proteins at a time (2, 3, 7, 8, 15). Most Opa<sup>+</sup> gonococci are phagocytized and killed by human neutrophils and induce a neutrophil oxidative burst (13, 47, 59).

Most studies of the interactions of gonococci with human neutrophils have been performed in vitro, with gonococci grown in vitro, by using isolated peripheral blood neutrophils. A few studies, however, most notably, a series of investigations by Harry Smith and Nicholas Parsons and their colleagues, investigated the interactions of human neutrophils and gonococci by studying urethral exudates from males with gonorrhea (5, 6, 39-42, 57, 61, 65). The studies suggest that a substantial percentage of in vivo-grown gonococci remain viable after prolonged exposure to exudate neutrophils. Such results are in stark contrast to results of the in vitro studies cited above, in which gonococci

were killed readily by neutrophils over a relatively short period, generally <3 h (13, 47, 59). Thus, gonococci grown in vivo appear to be different from gonococci grown in vitro in regard to their interaction with human neutrophils.

Yet another elegant and detailed set of experiments by Harry Smith and Nicholas Parsons and their coworkers and collaborators culminated in an exciting and unique set of observations (reviewed by Smith [48]). Whereas most strains of gonococci taken directly from urethral exudates are resistant to serum killing, the same gonococci passaged at least once on laboratory medium become serum sensitive (63). Conversely, serum-sensitive gonococci grown in vitro in the presence of human serum, genital secretions or erythrocytes, or their extracts, become serum resistant (29-31, 43, 44, 54, 58). This serum resistance is due to covalent transfer of sialic acid (*N*-acetylneuraminic acid [NANA]) by a sialyltransferase from CMP-NANA to gonococcal lipooligosaccharide (LOS) (28, 34, 37, 43). Such sialylation apparently prevents binding of bactericidal anti-LOS antibody to the gonococcus (37, 38). Thus, most strains of gonococci grown in vitro interact with human serum differently than do gonococci grown in vivo.

Apicella et al. have recently shown that gonococci associated with neutrophils in urethral exudates from males with gonorrhea are sialylated, indicating that sialylation indeed occurs in vivo (1, 28). Thus, gonococci grown in vivo interact with serum or neutrophils differently than do gonococci grown in vitro. In the studies presented below, we determined that sialylation of gonococcal LOS in vitro, by growth in the presence of CMP-NANA, altered the nonopsonic, i.e., Opa-mediated, association of Opa<sup>+</sup> gonococci with human peripheral blood neutrophils in vitro.

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## MATERIALS AND METHODS

*N. gonorrhoeae*. Two nonpiliated Opa variants of strain F62, obtained from P. Frederick Sparling, University of North Carolina, in 1977, were used in these studies and have been used extensively in our laboratory (47). Strain F62 is a cervical isolate and is very serum sensitive (22). Of the two nonpiliated Opa variants, one had no Opa proteins (Opa<sup>-</sup>) and one had an Opa protein termed Opa4 (9). These were clonally passaged every evening on GC agar (Difco) in accordance with the criteria of Swanson (50, 51) and Kellogg et al. (21, 22) for identification of piliation and opacity, i.e., possession of an Opa protein(s). For daily use, passaged gonococci were either (i) suspended in warm Dulbecco's phosphate-buffered saline with 0.1% (wt/vol) gelatin (PBSG; pH 7) to an  $A_{550}$  of 0.18 (Spectronic 20; Bausch & Lomb; equal to  $2 \times 10^8$  to  $4 \times 10^8$  gonococci per ml) and kept at room temperature or 37°C until used or (ii) suspended in 5 or 10 ml of warm GC broth plus added supplements, grown for about 3 h to mid-log phase, washed once in PBSG, and suspended as described above, as previously described (46). For long-term storage, selected colony types from GC agar plates were suspended in 50% (vol/vol) heat-inactivated horse serum in GC broth and stored at -70°C. Fresh cultures were struck from frozen stocks about once a month to ensure strain continuity.

Gonococci were grown with CMP-NANA exactly as described above, except that various concentrations of CMP-NANA (indicated elsewhere in the text) were added from a 1,250- $\mu$ g/ml stock of CMP-NANA in water. The CMP-NANA stocks were kept in small aliquots in a -20°C, non-frost-free freezer and thawed only once. Growth in CMP-NANA did not alter Opa expression, as determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (in more than a dozen observations), nor did it affect gonococcal viability or clumping as determined by quantitative viability determinations and by Gram stain (data not shown).

**Neutrophils.** Neutrophils were isolated from heparinized fresh human blood by single-step separation through a mixture of Ficoll and Hypaque as described by Ferrante and Thong (12). Erythrocytes were lysed by one 12-s treatment with distilled water followed by restoration of isotonicity with 3.6% (wt/vol) NaCl. Neutrophils were suspended to  $1 \times 10^7$ /ml or  $2 \times 10^7$ /ml in PBSG without calcium or magnesium and kept on ice until use. Neutrophil purity was  $\geq 95\%$  as determined by examining Wright-stained cytocentrifuge preparations. Neutrophil viability was  $\geq 98\%$  as determined by exclusion of 0.25% trypan blue in PBSG.

**Adherence assay.** Neutrophils ( $10^6$ , treated with  $3 \times 10^{-7}$  M formylmethionylleucylphenylalanine for 5 min at 37°C to upregulate neutrophil Opa receptors) were mixed with  $5 \times 10^7$  gonococci in a total volume of 1 ml of PBSG and tumbled end over end in 1.5-ml snap cap conical microcentrifuge tubes for 20 min at 37°C as previously described (11). Aliquots (150  $\mu$ l) were removed, cytocentrifuged (Shandon Southern), stained with Wright stain, and examined by light microscopy ( $\times 1,000$  magnification). One hundred consecutive neutrophils were counted, and the number of adherent diplococci was quantitated. Identifying marks on slides were covered with opaque tape, and the slides were rearranged before being counted to increase the objectivity of the counts.

**Neutrophil oxidative burst measured as luminol-dependent chemiluminescence (LDCL).** Neutrophils ( $10^6$ ),  $10^{-8}$  mol of luminol, and  $3 \times 10^7$  gonococci were mixed in a final volume

of 1 ml of PBSG, and LDCL was measured, with mixing at 37°C in a Pharmacia LKB Bio-Orbit 1251 Luminometer. LDCL, measured as millivolts over time, was recorded and the data were digitally stored.

**Phagocytic-killing assay.** To measure phagocytic killing,  $10^7$  neutrophils and  $10^7$  Opa4 gonococci were tumbled end over end (12 rpm) in 1 ml of PBSG in 1.5-ml conical snap cap tubes at 37°C. At 0, 45, 90, and 135 min, 10- $\mu$ l aliquots were removed, appropriately diluted in warm PBSG, and plated on GC agar plates. Viability was quantitated by counting CFU after 20 to 24 h of incubation. This assay measures intracellular killing of gonococci (13, 45). Results are expressed as percent control and computed as [(CFU at sampling time)/(input CFU at zero time)]  $\times 100$ .

**SDS-PAGE.** Identification of LOS was determined by tricine SDS-PAGE of proteinase K-treated whole gonococci (19), performed as described by Lesse et al., with 14% acrylamide and 2.67 M urea (27). SDS-PAGE gels were silver stained as described by Tsai and Frasch (56). All electrophoresis reagents were from Bio-Rad Laboratories (Richmond, Calif.). LOS phenotypes were the same in Opa4 and Opa<sup>-</sup> gonococci throughout these studies (data not shown).

**Treatment of gonococci with sialidase.** Gonococci grown to the log phase with or without CMP-NANA were centrifuged (1 min, 3,200  $\times g$ , room temperature), suspended in PBSG to an  $A_{550}$  of 0.18, and treated with sialidase for 1 h at 37°C at the concentrations indicated in figures and figure legends. They were then centrifuged (5,000  $\times g$ , 1 min), suspended in PBSG to an  $A_{550}$  of 0.18, and used in the various functional assays and to determine whether sialidase treatment alters the mobility of LOS on SDS-PAGE gels. Small aliquots of stock sialidase (4 U/ml in water) were kept at -20°C in a non-frost-free freezer and thawed only once.

**Reagents.** All reagents were from Sigma Chemical Company (St. Louis, Mo.) or Fisher Scientific (Malvern, Pa.). On one occasion, CMP-NANA was obtained from Boehringer Mannheim Corporation (Indianapolis, Ind.); similar results were obtained. Sialidase was Sigma type V purified from *Clostridium perfringens*.

## RESULTS

**Growth of Opa4 gonococci in the presence of CMP-NANA decreases their ability to adhere to neutrophils.** *N. gonorrhoeae* F62 possessing an adherence-promoting Opa protein (Opa4) was grown in the presence of increasing concentrations of CMP-NANA (0 to 50  $\mu$ g/ml) and mixed with neutrophils, and gonococcal adherence to neutrophils was quantitated. CMP-NANA used in this fashion caused dose-dependent inhibition of gonococcal adherence to human neutrophils in vitro, i.e., up to about 86% of the control value (Fig. 1). The differences were striking when viewed by light microscopy of Wright-stained cells (compare Fig. 2A and B). Gonococci possessing no Opa proteins (Opa<sup>-</sup> gonococci), which do not adhere to neutrophils in the absence of serum (47), remained nonadherent after growth in CMP-NANA ( $0.39 \pm 0.05$  gonococci per neutrophil with CMP-NANA versus  $0.32 \pm 0.03$  gonococci per neutrophil without CMP-NANA [ $n = 4$ ]), showing that growth in the presence of CMP-NANA did not nonspecifically affect association of Opa<sup>-</sup> gonococci with neutrophils.

Several experimental approaches were taken to determine whether decreased adherence to neutrophils of Opa4 gonococci grown with CMP-NANA was due to sialylation of gonococcal LOS (1, 28). (i) We observed that gonococci

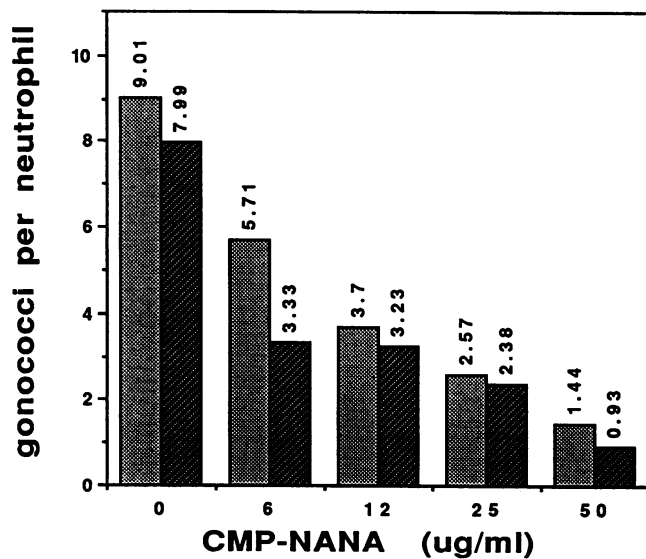


FIG. 1. Growth of Opa4 gonococci in CMP-NANA inhibits their adherence to human neutrophils. Opa4 gonococci grown with 0 to 50 µg of CMP-NANA per ml were tumbled with neutrophils, and their adherence was quantitated as described in Materials and Methods. The two different bar shadings represent two independent experiments, each done in duplicate.

grown in the presence of CMP-NANA expressed an altered LOS phenotype, as shown by subjecting proteinase K-treated whole gonococci to SDS-PAGE and subsequently identifying LOS bands by silver staining (Fig. 3). The faster migrating of the two LOS bands of our nonsialylated strain F62 showed decreased mobility, reflecting its increased molecular weight, presumably because of addition of at least one sialic acid (309 daltons) to a terminal LOS galactose (Fig. 3, compare lanes C and D) (28).

(ii) We inadvertently, upon clonal subpassage, isolated a naturally occurring, stable LOS variant of strain F62, designated F62 $\Sigma$ , which possessed an LOS phenotype that lacked the faster-migrating LOS band (the one that was sialylated in strain F62). The LOS of F62 $\Sigma$  was not sialylated when it was grown in the presence of up to 50 µg of CMP-NANA per ml. This was determined by showing that after growth in CMP-NANA the stable variant expressed no discernible alteration in LOS phenotype (Fig. 3, lanes E and F), and it retained exquisite serum sensitivity (the strain grown in the presence or absence of 50 µg CMP-NANA per ml was killed >99% after incubation with 25% fresh human serum at 37°C for 20 min [ $n = 4$ ]). An Opa<sup>+</sup> variant of this naturally occurring, stable LOS variant, which possessed the same apparent Opa as did the parent, F62, adhered to neutrophils to the same degree whether grown in the presence or absence of 50 µg of CMP-NANA per ml ( $12.3 \pm 1.7$  and  $11.8 \pm 1.3$  gonococci per neutrophil, respectively [ $n = 3$ ]). Thus, if LOS could not be sialylated, Opa<sup>+</sup> gonococci remained able to adhere to neutrophils, even after being grown in the presence of CMP-NANA.

By deduction, the results of experiments with the stable LOS variant F62 $\Sigma$  indicate that growth of F62 Opa4 gonococci in CMP-NANA caused their decreased adherence to neutrophils because of sialylation of their LOS, not some cryptic mechanism, such as a charge effect due to the mere presence of CMP-NANA, production of an inhibitory met-

abolic product of CMP-NANA, or contamination of CMP-NANA with some unknown inhibitor.

(iii) Neutrophil adherence assays were performed with Opa<sup>+</sup> gonococci that were grown in the presence of 50 µg of CMP-NANA per ml and then treated with increasing concentrations of sialidase. If gonococci grown in the presence of CMP-NANA adhered less well to neutrophils because they were sialylated, then sialidase should remove sialic acid and restore (increase) their adherence to the level of adherence seen for control gonococci grown in the absence of CMP-NANA. Indeed, sialidase treatment completely restored the ability of Opa<sup>+</sup> gonococci grown in the presence of CMP-NANA to adhere to neutrophils (Fig. 4). The effects of sialidase were very obvious upon viewing of Wright-stained smears of adherence assays performed with gonococci grown in CMP-NANA and treated without or with 0.05 U of sialidase per ml (compare Fig. 2B and D). Opa4 gonococci grown in the absence of CMP-NANA and subsequently treated with 0.05 U of sialidase per ml adhered to neutrophils to only a slightly higher degree than did untreated control Opa4 gonococci ( $10.85 \pm 1.44$  versus  $9.46 \pm 0.63$ , respectively [ $n = 6$ ]) (compare Fig. 2C and D). This indicated that sialidase treatment had minimal nonspecific effects on adherence of nonsialylated (control) Opa4 gonococci to neutrophils. LOS profiles of gonococci grown in CMP-NANA and treated with sialidase matched those of the nonsialylated (control) LOS phenotype (Fig. 3, compare lanes B and D). Treatment of control gonococci (grown in the absence of CMP-NANA) with sialidase did not alter the apparent mobility of nonsialylated LOS as observed by SDS-PAGE (Fig. 3, compare lanes A and D).

**Growth of Opa4 gonococci in the presence of CMP-NANA decreases their ability to stimulate the neutrophil oxidative burst.** Gonococci containing most Opa proteins stimulate the neutrophil oxidative burst in the absence of serum (33, 47). We assumed that if gonococci grown with CMP-NANA did not adhere to neutrophils, they would not induce an oxidative burst. Indeed, growth of Opa4 gonococci in the presence of 50 µg of CMP-NANA per ml inhibited their ability to stimulate neutrophil LDCL by about 50% (Fig. 5, compare open circles [control gonococci grown in the absence of CMP-NANA] with open squares [gonococci grown in the presence of 50 µg of CMP-NANA per ml]).

The same controls used for the adherence studies were used for the LDCL studies. Thus, after growth in 50 µg of CMP-NANA per ml, strain F62 showed an altered LOS phenotype, whereas F62 $\Sigma$  (the nonsialylatable variant) stimulated LDCL to the same extent as did F62 $\Sigma$  grown in the absence of CMP-NANA (data not shown). In addition, sialidase, in a dose-dependent manner, restored the ability of sialylated Opa4 gonococci to induce LDCL (Fig. 5). Sialidase at the highest concentration used in these assays (0.066 U/ml) did not induce neutrophil LDCL in the absence of gonococci (Fig. 5, open triangles).

High concentrations of sialidase did have some additional effect on the ability of gonococci to induce LDCL, since gonococci treated with the highest concentration of sialidase (0.066 U/ml) (Fig. 5, closed triangles) induced more LDCL than did control (untreated) gonococci (Fig. 5, open circles). This effect remains uninvestigated.

**Phagocytic killing of gonococci grown in the presence of CMP-NANA.** Gonococci possessing most Opa proteins are phagocytically killed by human neutrophils in the absence of serum (47). To determine whether sialylation of gonococci can affect this activity, phagocytic-killing assays were performed with Opa4 gonococci grown in the presence or

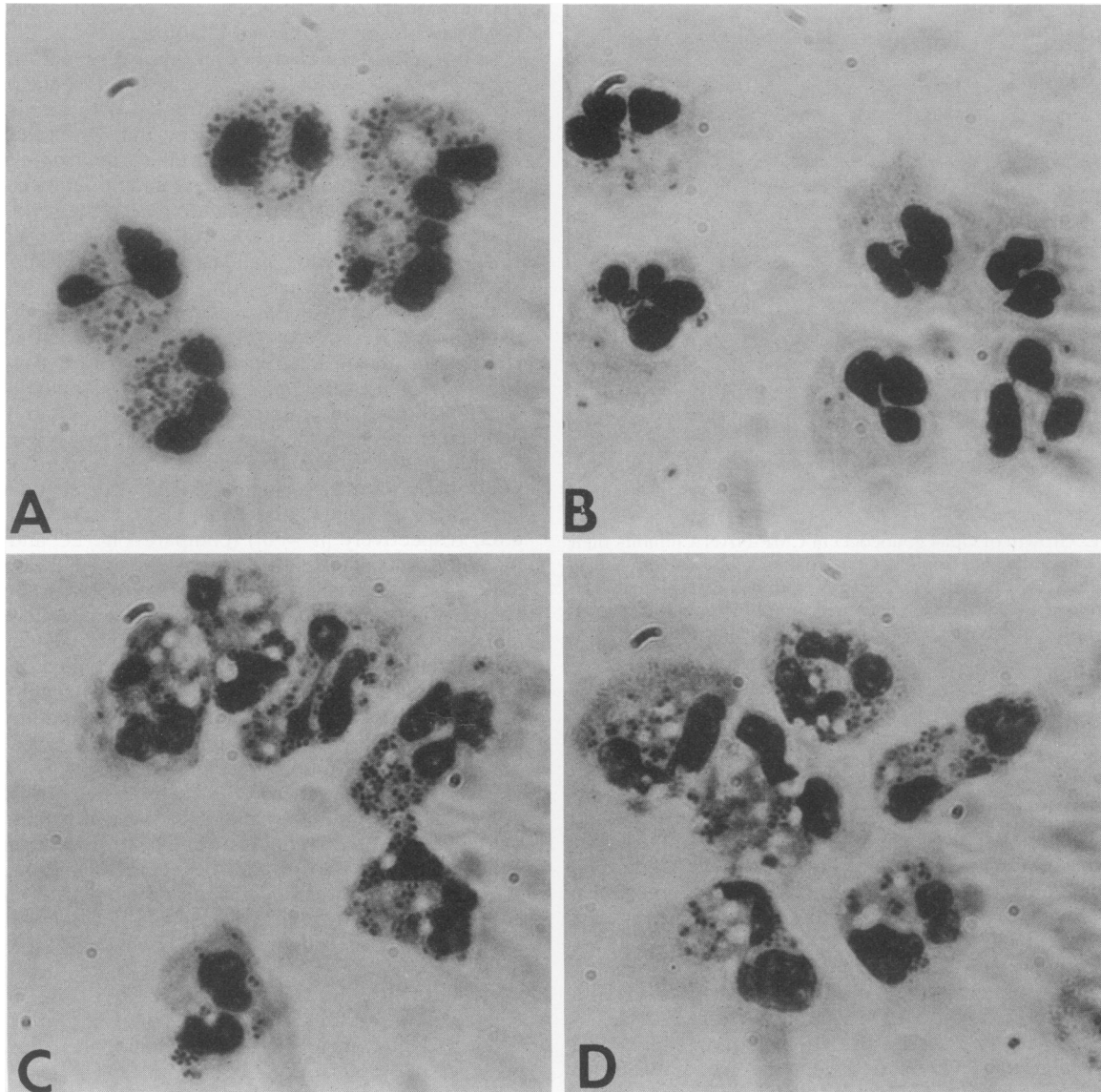


FIG. 2. Effects of CMP-NANA and sialidase on adherence of Opa4 gonococci to human neutrophils. Adherence assays were performed with neutrophils and gonococci that were grown with no CMP-NANA (A), grown with 50  $\mu\text{g}$  of CMP-NANA per ml (B), grown with no CMP-NANA and subsequently treated with 0.05 U of sialidase (C), or grown with 50  $\mu\text{g}$  of CMP-NANA per ml and subsequently treated with 0.05 U of sialidase (D). These oil immersion light micrographs (magnification,  $\times 1,000$ ) are representative fields of Wright-stained smears of aliquots from adherence assays.

absence of 50  $\mu\text{g}$  of CMP-NANA per ml. Slightly variant results were obtained at the four times these experiments were performed. On two occasions, neutrophils killed sialylated gonococci to the same degree and at the same rate as control (nonsialylated) gonococci, and on two other occasions, sialylated gonococci were killed to the same degree but at a slightly slower rate than were control gonococci (Fig. 6). Thus, when these sets of data are combined for presentation in Fig. 6, the standard deviations seen at 45 min are rather large, whereas they are much smaller for the data presented at 90 and 135 min.

We interpreted the results of our phagocytic-killing assays to indicate that neutrophil-endogenous sialidase, or perhaps other glycosidases, slowly removed sialic acid from gonococcal LOS, allowing gonococci to adhere and be subse-

quently killed over the relatively long (135-min) assay. Alternatively, sialylated LOS was gradually turned over on the gonococcal surface, allowing nonsialylated LOS to dilute out the sialylated LOS. Thus, whereas growth in GC broth in the presence of 50  $\mu\text{g}$  of CMP-NANA per ml is sufficient to sialylate gonococci and prevent their nonopsonic adherence to neutrophils, it appears that long-term (2- to 3-h) incubation of gonococci with neutrophils in vitro results in loss of sialylation.

To address this question, we performed adherence assays for 90 min, instead of the standard 20 min, to see whether gonococci grown with 50  $\mu\text{g}$  of CMP-NANA per ml would regain the ability to adhere to neutrophils over time. If they did, we could deduce that they lost or diluted out surface sialic acid. Indeed, Opa4 gonococci grown in the presence of

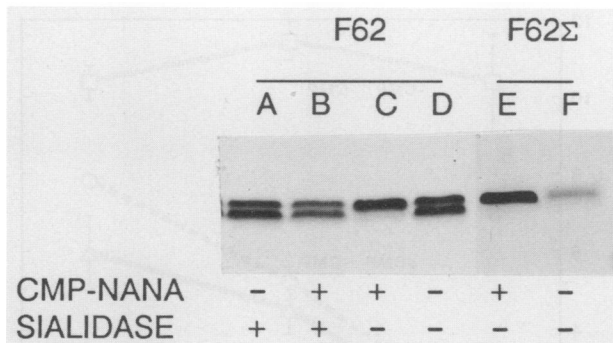


FIG. 3. LOS phenotypes of strain F62 (lanes A to D) and F62Σ (lanes E and F) gonococci grown with or without 50 μg of CMP-NANA per ml and subsequently treated with or without 0.05 U of sialidase, as indicated. After the various treatments, gonococci were washed, treated with proteinase K, run on SDS-PAGE, and silver stained, as described in Materials and Methods. After growth in CMP-NANA, the LOS of strain F62 gonococci was sialylated (compare lanes C and D) whereas the LOS of mutant F62Σ gonococci was not (compare lanes E and F). Sialidase removed sialic acid from the LOS of F62 gonococci grown in CMP-NANA (compare lanes B and C), whereas it did not alter the LOS phenotype of control gonococci grown without CMP-NANA (compare lanes A and B).

50 μg of CMP-NANA per ml gradually regained the ability to adhere to neutrophils over the 90-min adherence assay (Fig. 7). The presence of 50 μg of CMP-NANA per ml in these assays slightly slowed the increased adherence over time, suggesting that there was a small degree of sialic acid incorporation in the presence of CMP-NANA in buffer (PBSG).

**Opsonization of Opa<sup>-</sup> gonococci grown in the presence of CMP-NANA.** Unlike most Opa<sup>+</sup> gonococci, Opa<sup>-</sup> gonococci

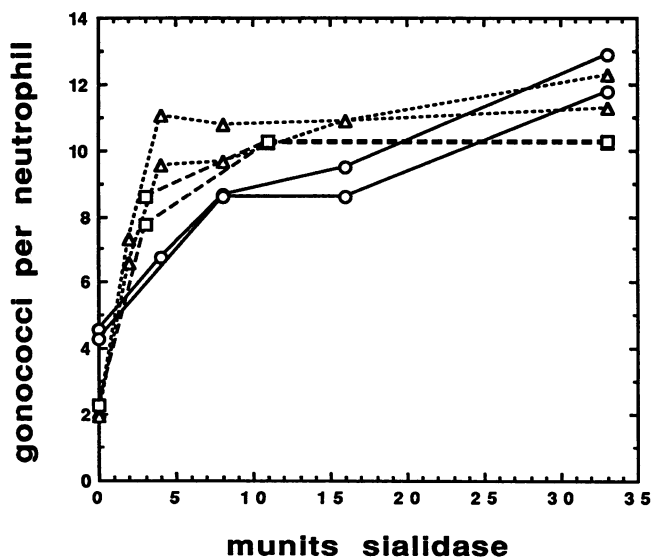


FIG. 4. Sialidase restores the ability of Opa4 gonococci grown in CMP-NANA to adhere to human neutrophils. Opa4 gonococci were grown with 50 μg of CMP-NANA per ml and treated with 0 to 33 mU of sialidase for 1 h, and their adherence to neutrophils was quantitated as described in Materials and Methods. The triangles, circles, and squares represent three independent experiments performed in duplicate.

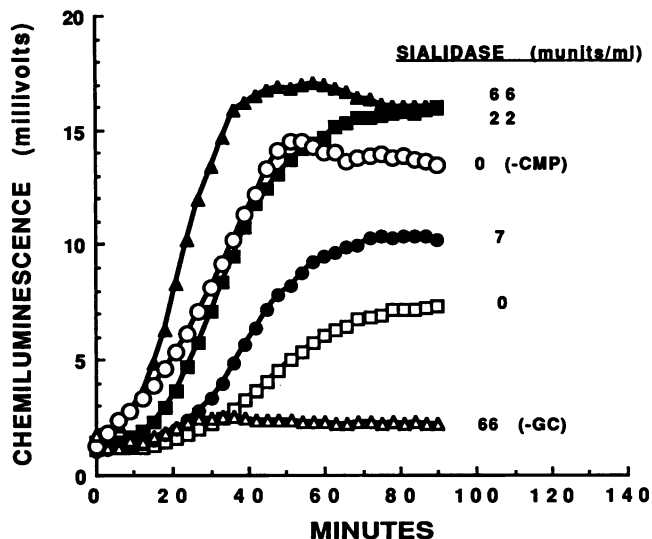


FIG. 5. Effects of CMP-NANA and sialidase on the ability of Opa4 gonococci (GC) to stimulate neutrophil LDCL. Opa4 gonococci were grown in the presence of 50 μg of CMP-NANA per ml; treated with 0 (□), 7 (●), 22 (■), or 66 (▲) mU of sialidase for 1 h at 37°C; and used to stimulate neutrophil LDCL. Other symbols: ○, LDCL stimulated by control gonococci grown in the absence of CMP-NANA and not treated with sialidase; △, neutrophil LDCL in the presence of 66 mU of sialidase per ml in the absence of gonococci. This is one of two experiments done in duplicate. Variability between the two experiments in regard to maximal LDCL was about 25%, which is why the results of one representative experiment are shown.

do not adhere to, and are not phagocytically killed by, human neutrophils in the absence of serum (47). However, when opsonized with fresh normal human serum, Opa<sup>-</sup> gonococci are phagocytically killed by human neutrophils to the same degree as are nonopsonized Opa<sup>+</sup> gonococci (13, 45, 59). To see whether sialylation of gonococci affects their ability to be opsonized, we grew Opa<sup>-</sup> gonococci in the presence or absence of 50 μg of CMP-NANA per ml, opsonized them with 5% fresh normal human serum in PBSG for 30 min at 37°C, and measured their ability to adhere to neutrophils as described in Materials and Methods. Growth in CMP-NANA decreased the ability of gonococci to be opsonized by fresh human serum by about 30% compared with that of gonococci grown without CMP-NANA. The mean number of gonococci per neutrophil (± the standard deviation) without CMP-NANA or serum was  $0.45 \pm 0.28$  ( $n = 4$ ), that without CMP-NANA but with serum was  $9.45 \pm 1.54$  ( $n = 4$ ), that with CMP-NANA without serum was  $0.28 \pm 0.30$  ( $n = 2$ ), and that with CMP-NANA and serum was  $6.45 \pm 0.63$  ( $n = 4$ ). This was substantially less inhibition than that seen for nonopsonic adherence. Whether such a limited effect on opsonization is biologically relevant in regard to gonococcal infection remains to be determined.

## DISCUSSION

We showed that Opa4 gonococci grown in the presence of increasing concentrations of CMP-NANA (up to 50 μg/ml) adhered to neutrophils in the absence of serum significantly less than did Opa4 gonococci grown without CMP-NANA. On the other hand, growth in CMP-NANA did not lead to a significant decrease in the ability of Opa<sup>-</sup> gonococci to be

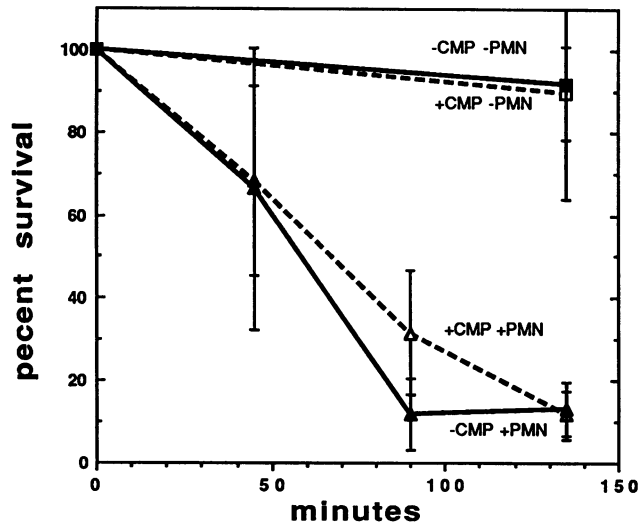


FIG. 6. Opa4 gonococci grown with CMP-NANA are phagocytically killed by human neutrophils. Opa4 gonococci grown in the absence (-CMP) or presence (+CMP) of 50  $\mu$ g of CMP-NANA per ml were washed and suspended in PBSG, and their ability to be killed by human neutrophils (PMN) was quantitated as described in Materials and Methods. Control experiments performed without neutrophils (-PMN) indicated survival of gonococci at the end of the 150-min assay. Bars, 1 standard deviation ( $n = 8$ ; four experiments were done in duplicate).

opsonized by fresh normal human serum. All of the effects we observed appeared to be due to sialylation of LOS, since (i) SDS-PAGE LOS profiles changed after growth in CMP-NANA, consistent with the addition of sialic acid; (ii) sialidase treatment of CMP-NANA-grown gonococci reversed all effects; and (iii) a stable LOS variant that was not sialylated when grown in the presence of up to 50  $\mu$ g of CMP-NANA per ml adhered to the same degree as gonococci grown without CMP-NANA. The mechanisms by which sialylation of LOS affects the functions of Opa proteins remain to be elucidated.

It is interesting that there is a dichotomy between the effects of CMP-NANA on gonococcal interactions with host humoral and cellular defenses. On the one hand, Opa4 gonococci grown in the presence of 50  $\mu$ g of CMP-NANA per ml had a dramatically decreased association with neutrophils and were totally resistant to the bactericidal action of serum. On the other hand, CMP-NANA had only minimal effects on the ability of Opa<sup>-</sup> gonococci to be opsonized by the serum towards which they were resistant. Thus, whereas sialylated gonococci resist the bactericidal activity of normal human serum (by avoiding recognition by antibody [37], or perhaps by other mechanisms), they do have the capacity to be coated with opsonic complement. The various mechanisms behind these observations remain to be investigated.

Most isolates from gonorrhea urethral exudates contain more opaque or Opa<sup>+</sup> gonococci than Opa<sup>-</sup> gonococci (20, 47, 53). Could this be due to their differential association with (and killing by) neutrophils? We do not think so, since it does not appear that Opa<sup>-</sup> gonococci are more readily opsonized or phagocytically killed than are Opa<sup>+</sup> gonococci in the presence of serum. Rest et al. showed that opsonized Opa<sup>+</sup> gonococci are killed by human neutrophils to the same extent as are nonopsonized Opa<sup>+</sup> gonococci or opsonized Opa<sup>-</sup> gonococci (45). Thus, taken together, our results

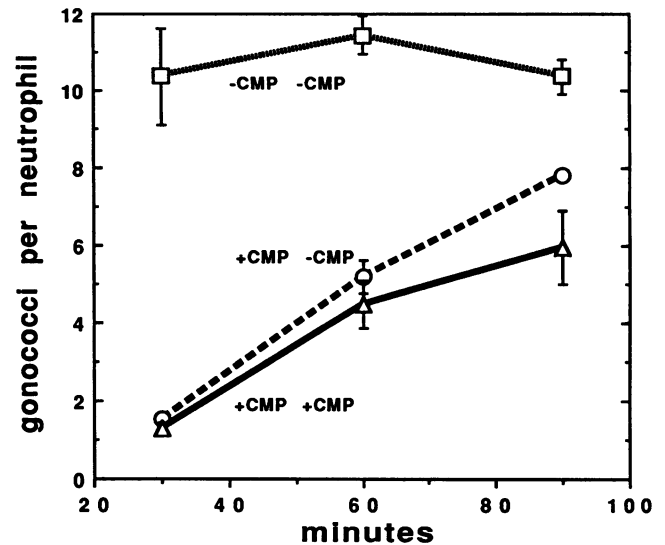


FIG. 7. Effect of CMP-NANA on adherence of Opa4 gonococci to neutrophils over time. Opa4 gonococci were grown in the absence or presence of 50  $\mu$ g of CMP-NANA per ml, and their adherence to neutrophils was quantitated in the absence or presence of 50  $\mu$ g of CMP-NANA per ml. Symbols:  $\square$ , gonococci grown without CMP-NANA adherent to neutrophils in the absence of CMP-NANA (-CMP -CNP);  $\circ$ , gonococci grown with CMP-NANA adherent to neutrophils in the absence of CMP-NANA (+CMP -CNP);  $\Delta$ , gonococci grown with CMP-NANA adherent to neutrophils in the presence of CMP-NANA (+CMP +CNP). Bars, 1 standard deviation ( $n = 4$ ). Where error bars are not present, they are equal to or smaller than the symbols.

suggest that once inside neutrophils, gonococci appear to be killed to the same degree, be they Opa<sup>+</sup> or Opa<sup>-</sup>.

Apicella et al. and Mandrell et al. have shown that gonococci are sialylated within neutrophils in urethral pus from males with gonorrhea (1, 28). Our observations in this report indicate that such intracellular sialylated gonococci must be opsonized prior to their ingestion; if they were not, they would not be able to associate with neutrophils. These combined observations also suggest that the concentration of complement components in the urethra is by definition functional and sufficient for opsonization.

In regard to the opsonization experiments, it can be questioned whether 5% fresh human serum is optimal for opsonization. Such a relatively low concentration of serum was used because F62 gonococci (grown without CMP-NANA) are very serum sensitive. Previous studies in our laboratory showed that 5% serum is an optimal compromise at which significant opsonization occurs in the absence of serum killing (45). Such opsonization and killing are complement dependent, since serum heated at 56°C for 30 min neither kills nor opsonizes Opa<sup>-</sup> F62 gonococci (45).

In the adherence studies, but not in the phagocytic-killing or LDCL studies, we used neutrophils stimulated with the chemotactic peptide formylmethionylleucylphenylalanine. Farrell and Rest showed that human peripheral blood neutrophils bind substantially more Opa<sup>+</sup> gonococci after incubation with any of a number of potent neutrophil-stimulating agents, including formylmethionylleucylphenylalanine, the calcium ionophore A23187, and the cocarcinogen phorbol myristate acetate (11). We concluded from these observations that (unidentified) Opa receptors are located within the membranes of specific granules and brought to the cytoplas-

mic membrane surface upon activation of the neutrophils (11). Exudate neutrophils, e.g., neutrophils found in the urethra or cervix during gonorrhea, are naturally activated in that, during diaporesis and chemotaxis, they are induced to degranulate (release) some or all of their specific granule contents (66). Therefore, the use of activated neutrophils in our adherence assays probably more accurately reflects gonococcus-neutrophil interactions that occur during gonorrhea, i.e., within the urethra or cervix, than does use of resting (unstimulated) neutrophils.

Stimulated neutrophils were not used in LDCL assays, since formylmethionylleucylphenylalanine induces a rapid, large LDCL peak. Induction of a secondary LDCL response by gonococci would lead to results that would be very difficult to interpret properly.

Whereas growth in the presence of 50 µg of CMP-NANA per ml inhibited adherence of gonococci to neutrophils by about 86%, it inhibited gonococcal stimulation of neutrophil LDCL by only about 50%. The lesser inhibition seen in the LDCL assays could be due to the fact that the LDCL assays were performed for 90 min, whereas the adherence assays were performed for 20 min. We hypothesize that gonococci become desialylated during the assay because of growth and dilution of sialylated LOS on the gonococcal surface or release of sialidase-like enzymes from the neutrophils. These observations were supported by those of the 135-min phagocytic-killing assays (Fig. 6), in which gonococci grown with CMP-NANA were killed to the same degree as controls, albeit somewhat slower, and by the adherence assays over time (Fig. 7).

A very simple, but very important, observation is that the CMP-NANA effects we observed in our studies were dose dependent. Thus, sialylation of gonococcal LOS is not an all-or-nothing phenomenon. Even in our *in vitro* assays, the longer sialylated gonococci were incubated without CMP-NANA, the more the effects of CMP-NANA diminished. We assume that the 50-µg/ml CMP-NANA that we used to confer maximal sialylation, and maximally inhibit biological functions, was much higher than the amounts of sialic acid actually found in gonorrhea pus (which are not known). What may occur in the microenvironment of the urethra or cervix during acute gonorrhea is competition among the number of gonococci, the gonococcal growth rate, limited availability of CMP-NANA, release of sialidase-like enzymes by neutrophils, limited availability of oxygen (14), and other conditions not studied. All of these things, to one degree or another, have an effect on the abilities of gonococci to be sialylated by CMP-NANA and killed by serum components and neutrophils.

How could sialylation of LOS affect the function of Opa proteins so dramatically? Sialic acid may cause a change in the overall charge of the microenvironment of individual Opa proteins, causing the affinity of Opa proteins for their receptor(s) to be decreased. Such observations have been made before (18, 64). Or, perhaps the change in charge caused by addition of sialic acid causes a conformational change in the surface-exposed portions of Opa proteins, thus decreasing or abrogating their ability to bind their receptor(s). It may also be that the fully sialylated LOS acts as a type of capsule or negatively charged halo surrounding the gonococcus, preventing its close association with the negatively charged neutrophil surface. Perhaps sialylation of LOS alters LOS structure, which in turn interacts with Opa proteins so as to decrease their receptor affinity. Certainly, it is possible that gonococcal outer membrane components other than LOS are affected by growth in CMP-NANA but

have not been detected. None of these possibilities are mutually exclusive. Regardless of which mechanism actually functions *in vivo*, our idea of how Opa proteins function as virulence factors in gonorrhea must be reassessed.

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