INFECTION AND IMMUNITY, May 1993, p. 1657–1666
0019-9567/93/051657-10$02.00/0

Vol. 61, No. 5

Anaerobic Growth and Cytidine 5′-Monophospho-*N*-Acetylneuraminic Acid Act Synergistically To Induce High-Level Serum Resistance in *Neisseria gonorrhoeae*

JOSEPH V. FRANGIPANE AND RICHARD F. REST*

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

Received 5 October 1992/Accepted 27 January 1993

In vivo, gonococci encounter a myriad of conditions not present in vitro. At some stages of infection and disease, gonococci may grow anaerobically, probably by using sodium nitrite as a terminal electron acceptor. Also, gonococci sialylate their lipooligosaccharide (LOS) in vivo, by using low concentrations of cytidine 5′-monophospho-*N*-acetylneuraminic acid (CMP-NANA) present in host tissue. This sialylation is responsible for the acquired resistance of gonococci to both normal and immune human serum. Given that gonococci grown in the absence of oxygen or in the presence of CMP-NANA probably more closely resemble gonococci grown inside a human host, we studied the serum resistance of gonococci cultivated under these conditions. In the absence of CMP-NANA, anaerobically grown (anaerobic) gonococci were somewhat less sensitive to serum killing than were aerobically grown (aerobic) gonococci. However, anaerobic gonococci grown with 6 μg of CMP-NANA per ml exhibited almost complete serum resistance, while aerobic gonococci required 16-fold-higher CMP-NANA concentrations to achieve significant serum resistance. Anaerobic gonococci incubated in CMP-NANA converted to serum resistance two to three times faster than did similarly treated aerobic gonococci and incorporated up to six times as much sialic acid into their LOS. Gonococci can express several different LOS molecules. Anaerobic gonococci expressed the LOS molecule that acts as an acceptor for sialic acid from CMP-NANA in greater quantity than aerobic gonococci did. Finally, Triton X-100 extracts of anaerobic gonococci contained about four times more sialyltransferase activity than did extracts of aerobic gonococci. Sialyltransferase activity in these extracts was not inhibited by oxygen or enhanced by anaerobiosis. These data indicate that anaerobic conditions lead to altered LOS biosynthesis and to induction of sialyltransferase activity in gonococci. In vivo, where decreased oxygen levels and relevant concentrations of CMP-NANA are found, gonococci could readily become resistant to killing by normal and immune human serum.

Neisseria gonorrhoeae, the causative agent of gonorrhea, has been isolated in the presence of obligate anaerobes from the genitourinary tract (53) and from individuals suffering from pelvic inflammatory disease (4, 5). These primary isolates can survive and grow for long periods without oxygen (24, 51). However, after passage in vitro, gonococci grow anaerobically only if provided with millimolar concentrations of nitrite, which they use as a terminal electron acceptor (26). Nitrite is readily available in the human host from biological fluids such as blood, urine, and saliva (61) and as a result of the metabolism of normal anaerobic flora in the mouth, vagina, and rectum (15).

Anaerobic gonococci express at least three novel outer membrane proteins (PANs 1 to 3) and repress at least five aerobically induced outer membrane proteins (POXs 1 to 5) (6). Such induction and repression of outer membrane components could be crucial to colonization or pathogenesis at anaerobic sites of infection, but the function, if any, of these proteins remains unknown. Sera from patients recovering from gonorrhea contain antibodies that react strongly with PAN 1 on Western blots (immunoblots), while sera from healthy individuals do not, indicating that gonococci express PAN 1 at some point during infection, probably as a result of anaerobic growth (7). Thus, anaerobic cultivation of gonococci may more closely resemble in vivo conditions than the routine aerobic incubation performed in most laboratories.

Resistance to the bactericidal activity of normal human serum (NHS) is another aspect of pathogenesis to which gonococci react differently under in vitro and in vivo growth conditions (52). One major mechanism for NHS bactericidal activity apparently involves the binding of serum immunoglobulin M antibody to the Galβ1→4GlcNac epitope in the terminal lactoside of gonococcal lipooligosaccharide (LOS), thus activating the complement cascade and killing the gonococcus (2, 14, 18, 19, 29, 36, 44, 47, 49, 62, 63). Although gonococci taken directly from urethral exudates are generally resistant to killing by NHS, many strains become serum sensitive after just one passage on laboratory medium (52, 59). In addition, these serum-sensitive gonococci, after incubation in vitro in the presence of heat-inactivated NHS (30, 31, 58), genital secretions (32), erythrocytes (34, 38, 39, 42), or extracts of phagocytes (41) become serum resistant and simultaneously exhibit alteration of their LOS structure (56). This acquired form of serum resistance is due to the covalent transfer of sialic acid (*N*-acetylneuraminic acid [NANA]) from host-derived cytidine 5′-monophosphate-NANA (CMP-NANA) to the galactose in the terminal lactoside by using a gonococcal sialyltransferase. Sialylation of this LOS residue blocks binding of bactericidal immunoglobulin M and inhibits classical complement pathway-mediated killing (29, 34, 36, 38). Sialylation may also interfere with the deposition of complement

* Corresponding author.

from immune serum onto gonococci by the alternate complement pathway (60).

CMP-NANA is present in many types of human tissue, including cervical epithelial cells (50), and is used by cellular sialyltransferases to donate sialic acid to glycoproteins, carbohydrates, and gangliosides (3, 17). Recently, Apicella et al. (1) and Parsons et al. (37) observed sialylation of gonococci associated with urethral exudate neutrophils from males with gonorrhea, confirming that sialylation indeed occurs during disease. Thus, it appears that gonococci can interact with human serum quite differently, depending on whether they are grown in vitro or in vivo, and that growth in vitro in the presence of CMP-NANA may more closely approximate the phenotypic and pathogenic characteristics of gonococci grown in vivo.

In most studies reported in the literature, gonococci have been grown in CMP-NANA concentrations that were generally substantially higher (25 to 100 μg/ml) than those proposed to be present in human blood (40 ng/ml) (34). CMP-NANA concentrations in the genitourinary tract or in gonorrhea exudates are unknown. In preliminary experiments investigating the role of anaerobiosis and/or CMP-NANA on the pathogenesis of gonococci, we noticed that anaerobic gonococci required substantially lower concentrations of CMP-NANA to gain complete resistance to killing by NHS than were required by aerobic gonococci. This effect appears to be due to alteration of LOS biosynthesis and induction of gonococcal sialyltransferase activity by anaerobiosis.

MATERIALS AND METHODS

Gonococcal strains and growth conditions. Nonpiliated gonococci of strain F62 (obtained from P. Frederick Sparling, University of North Carolina) were used in these studies. Strain F62 is a cervical isolate and is very serum sensitive (25). Initially, experiments were performed with variants of strain F62 expressing either no Opa proteins or an Opa protein termed Opa4. These variants were clonally passaged daily on GC agar (Difco), according to the criteria of Swanson (54, 55) and Kellogg et al. (23, 25), for verification of opacity and lack of piliation. When Opa^+ and Opa^- gonococci were compared in preliminary experiments, identical results were obtained with regard to serum resistance. Therefore, subsequent experiments were conducted with Opa^- gonococci.

For experiments using aerobic bacteria exclusively, passaged gonococci were suspended in 10 ml of warm GC broth plus added supplements and incubated at 37°C with agitation for about 3 h (to mid-log phase). Bacteria were washed once in sterile warm Dulbecco's phosphate-buffered saline (PBS) with 0.1% (wt/vol) gelatin (PBSG) and 0.01% (wt/vol) each of $CaCl_2$ and $MgCl_2$, suspended to 2×10^8 CFU/ml (optical density at 550 nm, 0.18; Spectronic 20), and kept at room temperature for no longer than 15 min prior to use (45).

For experiments comparing anaerobic and aerobic gonococci, GC agar plates were streaked confluently with a suspension of gonococci at about 6×10^8 CFU/ml, and the plates were incubated anaerobically or aerobically for 15 to 16 h at 37°C in 5% CO_2. Anaerobic growth of gonococci was achieved by using the nitrite-disk method developed by Knapp and Clark (26), as modified by Frangipane and Rest (13), and was confirmed by the observation of PAN 1 expression via sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (13). Aerobic growth of gonococci was carried out under these same conditions to control for any nitrite-specific effects. Aerobic incubation was conducted inside a 5% CO_2 incubator (Forma Scientific), and anaerobic incubation was conducted inside an anaerobic jar containing a BBL GasPak Plus system or inside an anaerobic chamber. The chamber was set up and maintained as described previously (13). All liquid and agar media were prereduced in the chamber for 24 h before being used in experiments. After incubation, gonococci were swabbed from agar plates and suspended in sterile warm PBSG plus Ca^{2+} and Mg^{2+} as described above.

Gonococci were grown with CMP-NANA exactly as described above, except that various concentrations of CMP-NANA (indicated in Results) were added to broth or solidified agar media from a 1.25-mg/ml stock of CMP-NANA in water. Stock CMP-NANA was kept in small aliquots at −20°C and thawed only once. CMP-NANA does not affect viability, clumping, or Opa expression of gonococci (43).

Preparation of NHS. Between 60 and 120 ml of venous blood was collected from five healthy human donors having no history of neisserial infection and allowed to clot for 30 min at 37°C in sterile plastic tubes. Clots were dislodged from the sides of the tubes (rimmed), and the tubes were incubated for an additional 30 min at 37°C. The tubes were placed on ice for 2 h and then centrifuged at $1,000 \times g$ for 10 min at 4°C. The resulting supernatants were depleted of residual erythrocytes by centrifugation at $10,000 \times g$ for 10 min at 4°C, pooled, and frozen at −70°C in 1-ml aliquots. Pooled NHS was thawed only once and used only on the day it was thawed.

Serum bactericidal assay. Pooled NHS was diluted to various concentrations (described as percent serum) in 450 μl of sterile warm PBSG plus Ca^{2+} and Mg^{2+}. Gonococci (10^7 in 50 μl of buffer described above) were added to the mixture, which was incubated at 37°C without agitation. After 30 min, 10-μl samples were appropriately diluted in sterile buffer and plated in duplicate on GC agar for overnight incubation. After quantitation of colonies, values from duplicate plates were averaged. Results are expressed as percent viable gonococci, determined by the formula 100 × (CFU of NHS-treated gonococci at 30 min)/(CFU of untreated gonococci at 0 min).

Treatment of gonococci with sialidase. Anaerobic and aerobic gonococci (10^5), grown in the presence of 0 or 50 μg of CMP-NANA per ml, were mixed with sterile PBSG plus Ca^{2+} and Mg^{2+} containing 0 or 330 mU of sialidase (Sigma type V) per ml to a volume of 0.3 ml and incubated at 37°C. After 30 min, 0.2 ml of pooled NHS or sterile buffer was added, and a serum bactericidal assay was conducted. Small aliquots of stock sialidase (4 U/ml in water) were kept at −20°C and thawed only once. Sialidase did not alter the bactericidal properties of NHS, as determined in preliminary experiments by treating NHS with 0 or 330 mU of sialidase per ml for 30 min at 37°C before using it in bactericidal assays (data not shown).

Isolation and purification of LOS from whole gonococci. For analysis of LOS phenotypes, LOS was isolated directly from whole gonococci by the method of Hitchcock (20). Anaerobic or aerobic gonococci grown on agar for 16 h (2×10^8 CFU in 1.0 ml of PBSG plus Ca^{2+} and Mg^{2+}) were pelleted ($3,200 \times g$, 1 min), resuspended in 50 μl of a 0.5-mg/ml concentration of proteinase K in Laemmli sample buffer containing β-mercaptoethanol (27), and incubated at 60°C for 30 min to digest gonococcal proteins. Samples were incubated at 100°C for 3 min to stop the reaction. For other experiments, LOS was purified from ethanol-acetone-dried gonococci of strain F62 as described by Darveau and Han-

cock (9). Such preparations (about 10 mg/ml) are typically free of DNA, RNA, protein, and peptidoglycan.

SDS-PAGE. The gonococcal LOS structure was examined by using SDS-PAGE of either purified LOS or proteinase K-treated whole gonococci, as described by Lesse et al. (28), with 14% acrylamide and 2.67 M urea. Reagents were purchased from Bio-Rad Laboratories, and electrophoresis was conducted for approximately 4.5 h at 25 to 35 mA in a Bio-Rad Mini Protean II apparatus. Gels were fixed overnight in a 50% methanol solution and silver-stained as described by Tsai and Frasch (57). LOS phenotypes from strain F62 were identical in Opa^+ and Opa^- gonococci throughout these studies.

Incorporation of sialic acid into LOS from anaerobic and aerobic gonococci. Anaerobic and aerobic gonococci were grown on agar containing increasing concentrations of CMP-NANA (indicated in Results) mixed with CMP-[sialic-9-^{3}H] (0.1 mCi/ml; DuPont, NEN Research Products) to a specific activity of 5.25×10^{-3} μCi/μg. After 16 h of incubation at 37°C, anaerobic and aerobic gonococci were individually suspended in PBSG plus Ca^{2+} and Mg^{2+} at 4×10^8 CFU/ml, and 1.5-ml samples were centrifuged (3,200 × *g*, 1 min). Pellets were resuspended in 0.5 ml of 1% Triton X-100 and mixed with 5 ml of Liquiscint (DuPont NEN). Radioactivity (in counts per minute) was measured with a liquid scintillation counter (United Technologies). Pellets were also prepared from gonococci grown on agar containing 6.3 μg of CMP-NANA per ml mixed with CMP-[sialic-4,5,6,7,8,9-^{14}C] (0.02 mCi/ml, DuPont NEN) to a specific activity of 5.0×10^{-3} μCi/μg. These pellets were proteinase K treated, diluted, and run on SDS-polyacrylamide gels. Gels were silver stained, treated with En^{3}Hance (DuPont NEN), and dried. Autoradiography was conducted for 30 days at −70°C by using Kodak X-Omatic Film.

Detergent extracts of gonococci and measurement of sialyltransferase activity. Anaerobic or aerobic gonococci (2×10^{10} of each) were washed once in PBSG plus Ca^{2+} and Mg^{2+}, pelleted, and resuspended in 4 ml of 0.5% Triton X-100 (29). Suspensions were bath sonicated (Heat Systems Ultrasonics) twice at 100% intensity for 10 s each and centrifuged at 10,000 × *g* for 2 min. Aliquots of the supernatants were frozen at −20°C. Extracts contained about 1.0 mg of protein per ml as determined by the BCA assay (Pierce). To measure sialyltransferase activity, 40 μl of diluted extract (1:20 in 0.5% Triton X-100) was mixed with 20 μl of purified gonococcal LOS (0.33 mg/ml), 20 μl of CMP-NANA (1.25 mg/ml), and 20 μl of PBS. Mixtures were tumbled end-over-end at 37°C, and 10 μl was removed at 10-min intervals and mixed with 10 μl of proteinase K (2.5 mg/ml in Laemmli sample buffer). After incubation at 60°C for 30 min, the LOS content of the samples was analyzed by SDS-PAGE.

RESULTS

Decreased serum sensitivity of anaerobic gonococci. Initially, we compared the serum sensitivities of anaerobic and aerobic gonococci grown in the absence of CMP-NANA. Since no reliable method yet exists for growing anaerobic gonococci in broth, we grew serum-sensitive *N. gonorrhoeae* F62 on GC agar in the presence or absence of oxygen. Gonococci were suspended in sterile PBSG plus Ca^{2+} and Mg^{2+} and mixed with 0 to 9% pooled NHS for 30 min at 37°C. Serum resistance was measured as a function of the percent viable gonococci compared with that of inoculum as described in Materials and Methods. Aerobic gonococci were not killed by 2% NHS but were readily killed by NHS concentrations of ≥3% (Fig. 1). Anaerobic gonococci, on the other hand, remained between 128 and 84% viable after treatment with up to 6% NHS and were effectively killed only by ≥7% NHS. Similar results were obtained whether the experiments were performed in ambient air, as described above, or inside an anaerobic chamber (data not shown). Aerobic gonococci grown in the presence of nitrite were as serum sensitive as aerobic gonococci grown without nitrite, indicating that nitrite does not seem to affect gonococcal serum resistance. Thus, at NHS concentrations between 3 and 6%, anaerobic gonococci demonstrate a dramatic (1 to 2 orders of magnitude) decrease in serum sensitivity when compared with aerobic gonococci. The biological relevance of this difference, if any, remains to be determined.

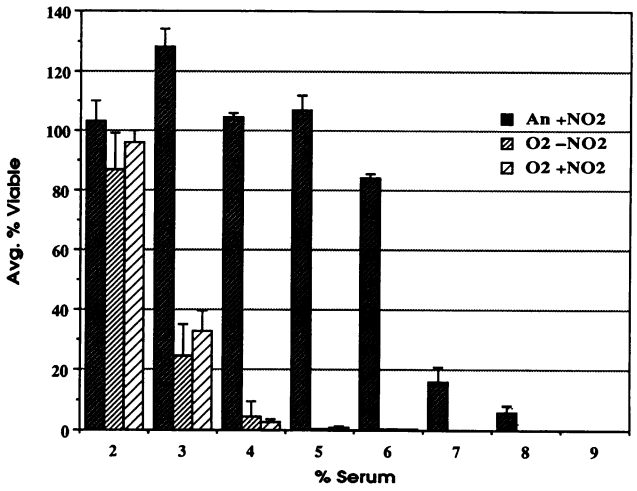

FIG. 1. Sensitivity of anaerobic and aerobic gonococci to killing by NHS. Gonococci of strain F62 were grown anaerobically (An) or aerobically (O_2) on GC agar in the presence (+) or absence (−) of sodium nitrite (NO_2), suspended in PBSG, and mixed with NHS. Serum killing was measured as described in Materials and Methods. Percent serum indicates the final concentration. Bars indicate 1 standard deviation (n = 3).

Serum resistance of aerobic gonococci grown in broth with CMP-NANA. To determine the concentration at which CMP-NANA effectively converts our strain F62 to serum resistance, we grew gonococci aerobically in GC broth containing 2.5 to 50.0 μg of CMP-NANA per ml, suspended them in sterile buffer, and mixed them with various dilutions of pooled NHS for 30 min at 37°C. Gonococci grown in the absence of CMP-NANA were 100% killed by a concentration of NHS as low as 8%. Growth of gonococci with increasing concentrations of CMP-NANA (12.5 to 50.0 μg/ml) resulted in a progressively greater level of serum resistance. In a bactericidal assay using 90% NHS, 12.5 μg of CMP-NANA per ml resulted in 70% viability, 25.0 μg/ml resulted in 97% viability, and 50.0 μg/ml resulted in 132% viability. Viability greater than 100% suggests that, in addition to surviving incubation in NHS, gonococci actually grew in NHS during the 30-min assay. Gonococci grown in lower concentrations of CMP-NANA (2.5 to 6.3 μg/ml) remained 50 to 67% viable in 15 to 20% NHS. These experiments support the findings of Nairn et al. and Parsons et al. (34, 38) by showing that growth of serum-sensitive gonococci in the presence of CMP-NANA results in significant resistance to NHS.

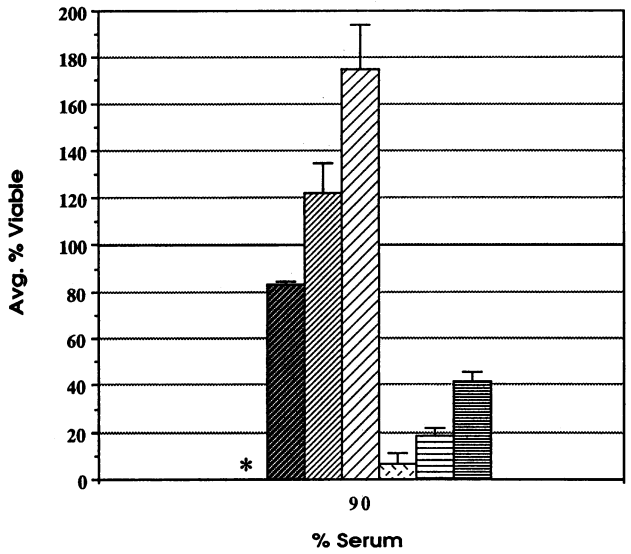

FIG. 2. Synergistic induction of high-level serum resistance in gonococci by anaerobic growth and CMP-NANA. Gonococci of strain F62 were grown anaerobically on GC agar containing 6.3 (▨), 12.5 (▨), or 25.0 (▨) μg of CMP-NANA per ml or aerobically on GC agar containing 25.0 (▨), 50.0 (▨), or 100.0 (▨) μg of CMP-NANA per ml, suspended in PBSG, and mixed with NHS. Serum killing was measured as described in Materials and Methods. The asterisk indicates that gonococci incubated without CMP-NANA were 100% killed by NHS. Percent serum indicates the final concentration. Bars indicate 1 standard deviation (n = 3).

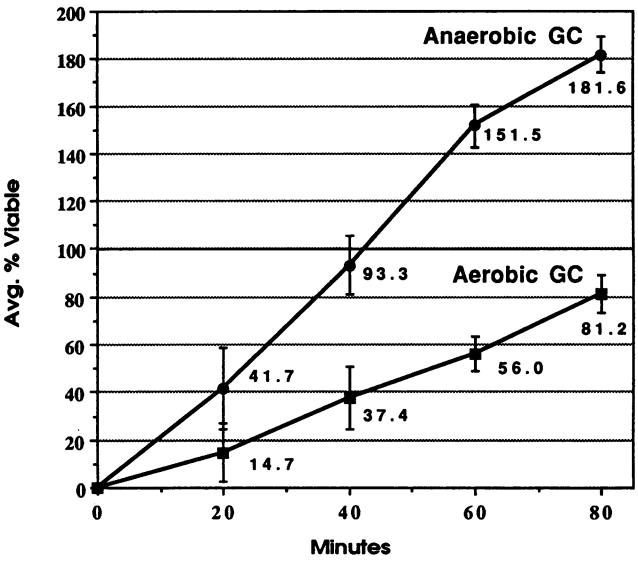

FIG. 3. Conversion to serum resistance of anaerobic and aerobic gonococci during incubation in CMP-NANA. Gonococci of strain F62 were grown anaerobically (●) or aerobically (■) on GC agar without CMP-NANA and then suspended in GC broth containing 10.0 μg of CMP-NANA per ml. Suspensions were incubated as described in Materials and Methods, and samples were removed at 20-min intervals for serum bactericidal assays. Bars indicate 1 standard deviation (n = 3).

Effect of anaerobiosis and CMP-NANA on gonococcal serum resistance. We then determined whether anaerobic and aerobic gonococci differed in their ability to become serum resistant when grown in the presence of CMP-NANA. We grew strain F62 gonococci anaerobically or aerobically on GC agar containing various concentrations of CMP-NANA, as described in Materials and Methods. After being suspended in sterile buffer, these gonococci were mixed with 45 or 90% NHS for 30 min at 37°C. Gonococci grown in the absence of CMP-NANA were 100% killed regardless of anaerobic or aerobic phenotype. In contrast to our experiments with aerobic gonococci grown in broth, aerobic gonococci grown on agar containing 25.0, 50.0, or 100.0 μg of CMP-NANA per ml remained only 6, 18, or 41% viable, respectively, in assays using 90% NHS (Fig. 2). Essentially identical results were obtained when 45% NHS was used (data not shown). Growth of aerobic gonococci with CMP-NANA concentrations of ≤12.5 μg/ml resulted in no serum resistance. Thus, broth-grown aerobic gonococci are much more readily converted to serum resistance by CMP-NANA than are agar-grown aerobic gonococci.

In contrast to the limited ability of CMP-NANA to convert aerobic agar-grown gonococci to serum resistance, anaerobic gonococci grown on agar containing 6.3, 12.5, or 25.0 μg of CMP-NANA per ml remained 83, 122, or 175% viable, respectively, in assays using 45 or 90% NHS (Fig. 2). Thus, anaerobic growth of gonococci in the presence of CMP-NANA synergistically enhances their resistance to NHS. This synergy was observed with CMP-NANA at concentrations as low as 3.0 μg/ml.

Similar results were obtained whether bactericidal assays were performed in ambient air, as described above, or inside an anaerobic chamber. The increased serum resistance provided by growth of anaerobic or aerobic gonococci with CMP-NANA was completely abrogated by the removal of sialic acid from gonococci by pretreatment with sialidase (data not shown).

Conversion of gonococci to serum resistance over time by growth in CMP-NANA. To further examine the differences in acquired serum resistance between anaerobic and aerobic gonococci, we compared the rates of conversion of anaerobic and aerobic gonococci to serum resistance during growth in the presence of CMP-NANA. Anaerobic and aerobic gonococci were grown for 16 h on GC agar without CMP-NANA and then suspended in 6.0 ml of GC broth plus supplements to 2×10^8 CFU/ml. CMP-NANA (10.0 μg/ml) was added to the suspension, and gonococci were incubated aerobically with agitation at 37°C. At 20-min intervals, samples of gonococci were removed and treated with 40% NHS for 30 min at 37°C.

At any given time interval, anaerobic gonococci were two to three times more serum resistant than aerobic gonococci (Fig. 3). Conversely, anaerobic gonococci took two to three-fold-less incubation time than did aerobic gonococci to reach nearly all levels of serum resistance. These results suggest that anaerobic gonococci convert to serum resistance two to three times more quickly and efficiently than aerobic gonococci do during growth in the presence of CMP-NANA.

Gonococci did not become serum resistant when incubated with CMP-NANA in GC broth if (i) supplements were not added to the broth, (ii) the temperature was reduced from 37 to 25°C, or (iii) gonococci were not aerated properly (i.e., not agitated) in the absence of nitrite. They also did not become serum resistant when incubated with agitation at 37°C with CMP-NANA diluted in PBSG instead of GC broth (data not shown). Furthermore, treatment of gonococci with chloramphenicol or neomycin inhibits acquired serum resistance (40). Since all of these conditions impair the growth of

TABLE 1. Incorporation of sialic acid into anaerobic and aerobic gonococci

CMP-NANA (μg/ml)	Avg cpm[a]		Ratio[b]
	Anaerobic gonococci	Aerobic gonococci	
0.0	12.1 ± 0.2	7.9 ± 0.0	
0.2	324.3 ± 20.0	54.0 ± 0.3	6.0
0.4	557.9 ± 98.7	93.2 ± 19.0	6.0
0.8	765.1 ± 86.8	160.6 ± 29.1	4.8
1.6	1,399.4 ± 100.6	299.2 ± 84.8	4.7
3.2	1,850.0 ± 73.7	454.8 ± 82.0	4.1
6.3	2,178.2 ± 101.3	847.1 ± 125.8	2.6
12.5	2,548.2 ± 107.3	1,436.7 ± 198.6	1.8
25.0	3,074.3 ± 162.2	2,081.3 ± 118.6	1.5

[a] Gonococci of strain F62 were grown anaerobically or aerobically on GC agar with increasing concentrations of tritiated CMP-NANA, solubilized in Triton X-100, and prepared for liquid scintillation counting as described in Materials and Methods. The average counts per minute ± standard deviation of two separate experiments was measured.

[b] The ratio was determined as (counts per minute measured in anaerobic gonococci)/(counts per minute measured in aerobic gonococci).

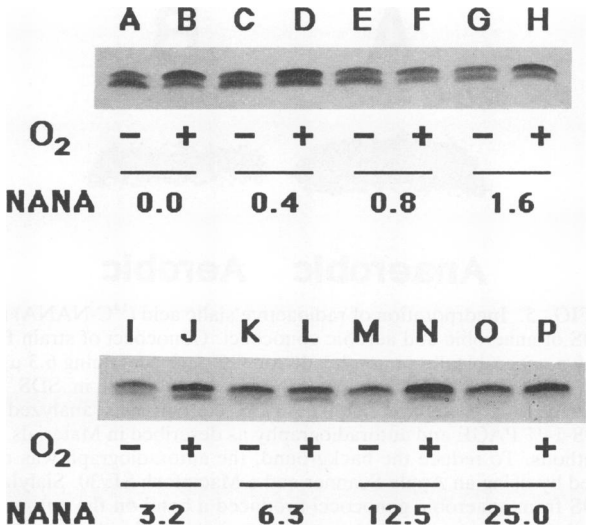

FIG. 4. Incorporation of sialic acid (NANA) into LOS of anaerobic and aerobic gonococci. Gonococci of strain F62, grown anaerobically (−) or aerobically (+) on GC agar containing 0.0 to 25.0 μg of CMP-NANA per ml, were solubilized in SDS and treated with proteinase K. The LOS contents of these samples were analyzed by SDS-14% PAGE and silver staining as described in Materials and Methods. LOS from anaerobic gonococci was completely sialylated at a concentration of 6.3 μg of CMP-NANA per ml (lane K), while LOS from aerobic gonococci was completely sialylated only at a concentration of 25.0 μg of CMP-NANA per ml (lane P).

gonococci, we conclude that CMP-NANA-mediated conversion of gonococci to serum resistance requires active growth.

Incorporation of sialic acid into anaerobic and aerobic gonococci. We hypothesized that anaerobic gonococci incorporated greater amounts of sialic acid into their LOS than did aerobic gonococci, thus increasing the rate and efficiency of their conversion to serum resistance in the presence of CMP-NANA. To test this idea, we grew gonococci anaerobically and aerobically on GC agar containing 0.2 to 25.0 μg of CMP-NANA per ml labeled with tritium in the sialic acid moiety. After overnight incubation, anaerobic and aerobic gonococci were individually suspended in sterile buffer to identical optical densities. Equal volumes of each suspension were pelleted and resuspended in 1.0% Triton X-100, and the amount of tritiated sialic acid incorporation was measured by liquid scintillation counting.

As we reduced the concentration of CMP-NANA in agar, the ratio of tritiated sialic acid incorporated by anaerobic gonococci to that incorporated by aerobic gonococci progressively increased to about 6:1 (Table 1). The concentrations of CMP-NANA at which we observed a five- to sixfold difference are in the range of 0.2 to 1.6 μg/ml and may more closely approximate the concentration of CMP-NANA at infection sites, which is assumed to be low (34). These results indicate that, at reduced concentrations of CMP-NANA, anaerobic gonococci incorporate up to six times more sialic acid than do aerobic gonococci and thus become resistant to NHS.

At 25.0 μg of CMP-NANA per ml, anaerobic gonococci incorporated 1.4 times more sialic acid than did aerobic gonococci (Table 1). Despite this small difference in apparent sialic acid incorporation, 25.0 μg of CMP-NANA per ml causes a 16-fold difference in serum resistance between anaerobic and aerobic gonococci (Fig. 2). This discrepancy remains unexplained.

Kinetics of sialylation of LOS from anaerobic and aerobic gonococci. We then determined whether the differential uptake of radiolabeled sialic acid by anaerobic and aerobic gonococci correlated with the sialylation of LOS molecules in the gonococcal outer membrane. Equal volumes of the suspensions prepared for radioincorporation assays, described immediately above, were pelleted, solubilized in SDS, and treated with proteinase K to destroy all gonococcal proteins. The remaining LOS was analyzed by SDS-14% PAGE and stained with periodate oxidation and silver. When grown in the absence of CMP-NANA, both anaerobic and aerobic gonococci of strain F62 expressed the same two major LOS molecules of 4.5 and 4.9 kDa (29), as seen by SDS-PAGE (Fig. 4, lanes A and B). As anaerobic and aerobic gonococci were incubated with increasing concentrations of CMP-NANA, sialic acid molecules were added to the LOS molecules constituting the 4.5-kDa band, causing them to shift up to the level of the 4.9-kDa band (lanes C to P). As can be seen in the gel, LOS from anaerobic gonococci was completely sialylated at 6.3 μg of CMP-NANA per ml (lane K), while LOS from aerobic gonococci was completely sialylated only at 25.0 μg of CMP-NANA per ml (lane P). Thus, anaerobic gonococci required four times less CMP-NANA not only to completely sialylate their LOS but also to achieve a level of tritiated sialic acid incorporation similar to the highest level achieved by aerobic gonococci (about 2,000 cpm; Table 1). These results indicate that the increased incorporation of sialic acid by anaerobic gonococci is due to enhanced sialylation of their LOS.

LOS from anaerobic and aerobic gonococci grown on GC agar containing 6.3 μg of radiolabeled CMP-NANA per ml was analyzed by SDS-PAGE, silver stain, and autoradiography. Radioactivity was detected only in the 4.9-kDa LOS band; sialylated LOS from anaerobic gonococci produced a band on the autoradiograph that was two to three times more intense than that produced by sialylated LOS from aerobic gonococci (Fig. 5, compare lanes A and B). These findings also support the hypothesis that the differential sialic acid incorporation by anaerobic gonococci occurs on their LOS.

Alteration of LOS expression induced by anaerobiosis. Enhanced sialylation of LOS of anaerobic gonococci could

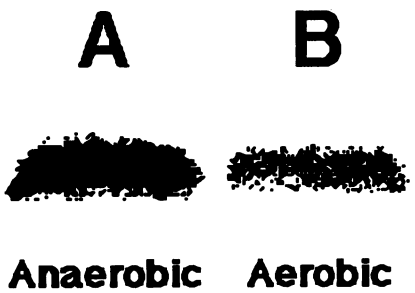

FIG. 5. Incorporation of radioactive sialic acid (^{14}C-NANA) into LOS of anaerobic and aerobic gonococci. Gonococci of strain F62, grown anaerobically or aerobically on GC agar containing 6.3 μg of radiolabeled CMP-NANA per ml, were solubilized in SDS and treated with proteinase K. The LOS content was analyzed by SDS-14% PAGE and autoradiography as described in Materials and Methods. To reduce the background, the autoradiograph was digitized by using an Apple Scanner and a Macintosh SE/30. Sialylated LOS from anaerobic gonococci produced a band on the autoradiograph that was two to three times more intense than that produced by sialylated LOS from aerobic gonococci (compare lanes A and B).

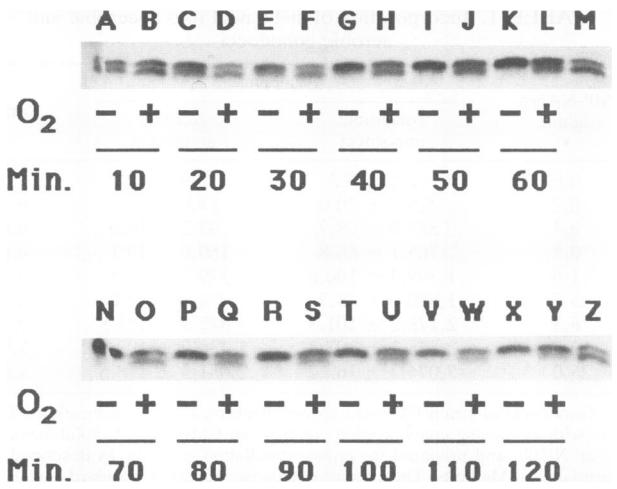

FIG. 6. Sialyltransferase activity in extracts of anaerobic and aerobic gonococci. Triton X-100 extracts of anaerobic (−) and aerobic (+) gonococci were prepared, mixed with CMP-NANA and purified gonococcal LOS, and incubated as described in Materials and Methods. Samples were removed at 10-min intervals, and their LOS contents were analyzed by SDS-14% PAGE and silver staining. The extract of anaerobic gonococci caused the complete sialylation of gonococcal LOS after only 40 min (lane G). In contrast, the extract of aerobic gonococci required 120 min to sialylate only about 90% of the same purified LOS (lane Y).

be due to a change in their LOS phenotype. To explore this possibility, we closely examined the LOS bands from anaerobic and aerobic gonococci grown on GC agar without CMP-NANA (Fig. 4, lanes A and B). We observed that, although anaerobic and aerobic gonococci appeared to possess the same two LOS bands, anaerobic gonococci expressed the 4.5-kDa band, which accepts sialic acid, in greater quantity than aerobic gonococci did. Conversely, aerobic gonococci expressed the higher band, which does not accept sialic acid, in greater quantity than anaerobic gonococci did. We have observed this difference in LOS expression by anaerobic and aerobic strain F62 gonococci repeatedly. Increased expression of the LOS acceptor molecule for sialic acid could result in uptake of greater amounts of sialic acid by anaerobic gonococci, resulting in enhanced resistance to NHS.

Sialyltransferase activity in extracts of anaerobic and aerobic gonococci. It is possible that, in addition to the change in LOS expression, anaerobic growth causes the expression of greater sialyltransferase activity by gonococci. To compare the relative sialyltransferase activity of anaerobic and aerobic gonococci, we prepared 0.5% Triton X-100 extracts by using identical amounts of both gonococcal phenotypes. Extracts were diluted in PBS containing an excess of CMP-NANA (250 μg/ml) and 66 μg of LOS per ml purified from aerobic gonococci and were incubated at 37°C. Samples were removed at 10-min intervals, solubilized in SDS, and treated with proteinase K. The remaining LOS was analyzed by SDS-14% PAGE and stained with periodate oxidation and silver. As evidenced by the upward shift in the 4.5-kDa LOS band, the extract of anaerobic gonococci caused the complete sialylation of gonococcal LOS after only 40 min (Fig. 6, lane G). In contrast, the extract of aerobic gonococci required 120 min to sialylate only about 90% of the same purified LOS (lane Y). Such a level of sialylation was achieved by the anaerobic extract after only 30 min of incubation with LOS and CMP-NANA (lane E). These results suggest that anaerobic gonococci possess up to four times as much sialyltransferase activity as aerobic gonococci do.

The fourfold difference in enzyme activity was observed when at least four different preparations of anaerobic and aerobic gonococcal extracts prepared on different days were used. The same results were also observed when the assay was performed inside an anaerobic chamber (data not shown), demonstrating that sialyltransferase activity is not modulated by the oxidation state of the enzyme. Also, extracts of anaerobic gonococci prepared and assayed under either anaerobic or aerobic conditions possessed equal sialyltransferase activity, indicating that the enzyme is not irreversibly damaged by oxidation. Assays performed using 0.5% Triton X-100 instead of extracts yielded no sialylation (lanes M and Z).

DISCUSSION

In this report, we studied the serum resistance of gonococci grown in the presence or absence of oxygen and in the presence or absence of CMP-NANA. We discovered that without CMP-NANA and at low concentrations of NHS (3 to 6%), strain F62 gonococci grown anaerobically were less serum sensitive than gonococci grown aerobically were. At higher concentrations of NHS (≥7%), anaerobic and aerobic gonococci were equally serum sensitive. In addition, although both anaerobic and aerobic gonococci became serum resistant when grown on agar containing CMP-NANA, anaerobic gonococci exhibited a significantly greater increase in serum resistance, at lower concentrations of CMP-NANA, than did similarly grown aerobic gonococci. Anaerobic gonococci demonstrated a two- to threefold-higher rate and level of conversion to serum resistance during growth in CMP-NANA. Also, at lower concentrations of CMP-NANA, anaerobic gonococci incorporated up to six times as much radioactive sialic acid into their LOS than aerobic gonococci did. When grown without CMP-NANA, both anaerobic and aerobic gonococci expressed the same two LOS molecules, as seen by SDS-PAGE. However, the 4.5-kDa band, which binds sialic acid, was expressed in

greater quantity by anaerobic gonococci, and the 4.9-kDa band, which does not bind sialic acid, was expressed in greater quantity by aerobic gonococci. Finally, Triton X-100 extracts of anaerobic gonococci, containing sialyltransferase activity, transferred sialic acid into purified gonococcal LOS four times faster than did extracts of aerobic gonococci.

We observed a dramatic decrease in the serum sensitivity of anaerobic gonococci compared with that of aerobic gonococci grown in the absence of CMP-NANA. A possible explanation for this decrease may be the decreased expression of the 4.9-kDa LOS molecule by anaerobic gonococci. Since this molecule contains the binding site for bactericidal immunoglobulin M present in NHS (18, 19, 29, 36, 46), gonococci possessing less of it would conceivably be less susceptible to killing by NHS. Also, undetected antigenic differences (such as carbohydrate substitution on the OS chains) may be responsible for the decrease in serum sensitivity of anaerobic gonococci. Furthermore, it is possible that, by using the ability to more quickly and efficiently sialylate their LOS (observed in this report), anaerobic gonococci may have used the minute amount of CMP-NANA present in NHS to become moderately serum resistant within a relatively short period of time.

Dilution of CMP-NANA in agar was less effective than dilution in broth at converting aerobic gonococci to serum resistance. Since CMP-NANA was added to solidified agar, it is not possible that this effect was due to the heat inactivation of the substance. Perhaps gonococci growing on the surface of an agar plate do not have full access to the CMP-NANA distributed throughout the agar, as do broth-grown gonococci. It is also possible that GC agar contains a substance that degrades CMP-NANA or inhibits sialylation of LOS or that gonococci grown in suspension express more sialyltransferase activity than do gonococci grown on a solid substrate. There is strong evidence that gonococci exhibit changes in LOS composition and serum resistance when their growth rates and conditions are altered (33).

The serum resistance acquired by anaerobic and aerobic gonococci appeared to be due to sialylation of LOS on the basis of the following: (i) SDS-PAGE LOS profiles changed after growth with CMP-NANA, consistent with the addition of sialic acid; (ii) sialidase treatment of CMP-NANA-grown gonococci abrogated all serum resistance; and (iii) as observed in previous work (43), a stable LOS variant that was not sialylated after growth in 50 μg of CMP-NANA per ml failed to become serum resistant. It is important to note that the effects of CMP-NANA on serum resistance and sialylation of gonococci were dose dependent. Therefore, sialylation of gonococcal LOS is not an all-or-nothing phenomenon.

Although anaerobic gonococci required 16-fold-less CMP-NANA (6.3 μg/ml) than aerobic gonococci did to achieve significant sialylation and serum resistance, this amount may be higher than the concentration of CMP-NANA at infection sites, which is assumed to be low (34). However, there is evidence suggesting that, when lower numbers of organisms such as those present during infection are used, physiological concentrations of CMP-NANA may effectively convert gonococci to serum resistance (60). Also, there are other donors of sialic acid present in vivo in addition to CMP-NANA, such as the high-M_r serum resistance inducing factor present in human blood (34). Constantinidou et al. have discovered that this factor has a synergistic effect on gonococcal sialylation in vitro in the presence of CMP-NANA (8).

The sialylation of purified LOS by detergent extracts of gonococci in our laboratory and those of others (29) indicates that gonococci possess one or more sialyltransferases which mediate this activity. Despite the apparently simple enzymatic nature of this sialylation mechanism, we (this report) and others (16, 30, 40, 58) have observed that conversion to serum resistance by growth in CMP-NANA requires active growth of gonococci. This requirement for active growth might indicate that (i) only newly synthesized LOS can be sialylated by whole gonococci, (ii) the sialyltransferase is most active or abundant in log-phase gonococci, or (iii) the sialyltransferase has a short half-life (and perhaps is even degraded by a gonococcal protease) and gonococci must constantly transport more enzyme to the outer membrane (its proposed site of action) to sialylate LOS.

When incubated with CMP-NANA, anaerobic gonococci converted to serum resistance at two to three times the rate and extent of similarly grown aerobic gonococci. More rapid and complete sialylation of anaerobic gonococci in vivo could allow them to more effectively escape killing by NHS. The experiments measuring conversion to serum resistance were carried out in broth under aerobic conditions. It is interesting to note that during the first 60 min of the assay, anaerobic gonococci converted to serum resistance about 2.8 times faster than did aerobic gonococci (as measured by the average slope in Fig. 3). However, after 80 min, the conversion rate of anaerobic gonococci was only about 1.2 times higher than that of aerobic gonococci. Since the average doubling time of gonococci is about 60 min, it is likely that anaerobic gonococci had begun to convert to the aerobic phenotype at this time, and their conversion rate began to approximate that of aerobic gonococci.

The increased efficiency with which anaerobic gonococci sialylate themselves can be partially explained by their enhanced expression (usually two- to threefold) of the LOS molecule that accepts sialic acid (Fig. 4). It is important to note that in vitro expression of this molecule by strain F62 populations is a stable phenomenon (48) and did not change over the 2 years represented by this research. Schneider et al. (46) have observed in vivo selection of gonococcal strains expressing LOS epitopes that resemble human glycosphingolipids and bind sialic acid. Perhaps anaerobiosis is one of the factors causing this in vivo selection. Enhanced sialylation of anaerobic gonococci can also be explained by the fourfold increase in their sialyltransferase activity (Fig. 6). It is possible that anaerobic gonococci produce one or more additional sialyltransferases than aerobic gonococci do or that the genetic expression of a single enzyme is upregulated in anaerobic gonococci. Alternatively, the sialyltransferase may be less labile in, or degraded more slowly by, anaerobic gonococci. Also, aerobic gonococci may produce an inhibitory substance that prevents complete enzyme activity or may lack an anaerobically expressed cofactor required for complete enzyme activity. These possibilities are not mutually exclusive.

Can the considerable difference in the serum resistance by sialylated anaerobic versus sialylated aerobic gonococci be explained solely by the changes observed in LOS and sialyltransferase activity of anaerobic gonococci? It is possible that as-yet-undiscovered factors might be partially responsible for this dramatic synergy as well. Perhaps anaerobic gonococci also sialylate outer membrane proteins that are important during serum killing, although there is no evidence that aerobic gonococci sialylate such protein components (10, 36, 40). Our data indicate that, at CMP-NANA concentrations that appear to completely sialylate LOS from

either anaerobic (6.3 μg/ml) or aerobic (25.0 μg/ml) gonococci (as determined by SDS-PAGE in Fig. 4), equal amounts of tritiated sialic acid were bound to both gonococcal phenotypes (about 2,000 cpm; Table 1), suggesting that only LOS was being sialylated.

Another factor possibly contributing to synergy involves the amplification of serum killing of gonococci by the alternative complement pathway. When strain F62 and many other gonococcal strains are treated with NHS, killing by the classical complement pathway can be significantly augmented by the alternative pathway (18, 21, 44). Sialic acid is known to inhibit activation of the alternative pathway by meningococci (22) and sheep erythrocytes (12, 35). Recently, Wetzler et al. (60) observed that sialylation of gonococcal LOS causes antibody-independent resistance to immune sera, and Elkins et al. demonstrated that such sialylation inhibits the deposition of complement components C3 and C9 (11). These findings suggest that the acquired serum resistance of gonococci is due in part to the negative effect of sialylation on complement deposition. Thus, in addition to abrogating the classical pathway by blocking the binding of bactericidal antibody (36), increased sialylation of LOS on anaerobic gonococci probably also inhibits the alternative pathway, creating a dramatic increase in serum resistance.

In summary, anaerobic gonococci grown with CMP-NANA became substantially more serum resistant than similarly grown aerobic gonococci did. Also, anaerobic gonococci gained considerable serum resistance after growth with CMP-NANA concentrations that caused no serum resistance in aerobic gonococci. It appears that the effects of gonococcal growth in anaerobiosis and in the presence of CMP-NANA on serum resistance are not merely additive but are synergistic. The effects appear to be due to alteration of LOS biosynthesis and induction of gonococcal sialyltransferase activity by anaerobiosis. These results suggest that, at anaerobic infection sites, serum-sensitive gonococci can use available CMP-NANA more efficiently and significantly increase their resistance to NHS.

ACKNOWLEDGMENTS

This work was supported in part by grant AI20897 from the National Institute of Allergy and Infectious Diseases.

We thank Margaret Emily Hickman for her helpful critique and assistance in typing this manuscript. We also thank Michael Apicella for his suggestion to try growing gonococci anaerobically in the presence of CMP-NANA and for subsequent discussions.

REFERENCES

1. **Apicella, M. A., R. E. Mandrell, M. Shero, M. E. Wilson, J. M. Griffiss, G. F. Brooks, C. Lammel, J. F. Breen, and P. A. Rice.** 1990. Modification by sialic acid of *Neisseria gonorrhoeae* lipooligosaccharide epitope expression in human urethral exudates: an immunoelectron microscopic analysis. J. Infect. Dis. **162:**506–512.
2. **Apicella, M. A., J. Westerink, S. A. Morse, H. Schneider, P. A. Rice, and J. M. Griffiss.** 1986. Bactericidal antibody response of normal human serum to the lipooligosaccharide of *Neisseria gonorrhoeae*. J. Infect. Dis. **153:**520–526.
3. **Brackmann, T. R., S. Geldmeyer, R. Jahn, and H. D. Soling.** 1983. Radioenzymatic determination of CMP-*N*-acetylsialic acid and free *N*-acetylsialic acid in biological material. Anal. Biochem. **130:**369–375.
4. **Burnakis, T. G., and N. B. Hildebrandt.** 1986. Pelvic inflammatory disease: a review with emphasis on antimicrobial therapy. Rev. Infect. Dis. **8:**86–116.
5. **Chow, A. W., K. L. Malkasian, J. R. Marshall, and L. B. Guze.** 1975. The bacteriology of acute pelvic inflammatory disease. Am. J. Obstet. Gynecol. **122:**876–879.
6. **Clark, V. L., L. A. Campbell, D. A. Palermo, T. M. Evans, and K. W. Klimpel.** 1987. Induction and repression of outer membrane proteins by anaerobic growth of *Neisseria gonorrhoeae*. Infect. Immun. **55:**1359–1364.
7. **Clark, V. L., J. S. Knapp, S. Thompson, and K. W. Klimpel.** 1988. Presence of antibodies to the major anaerobically induced gonococcal outer membrane protein in sera from patients with gonococcal infections. Microb. Pathog. **5:**381–390.
8. **Constantinidou, C., D. Beadle, X. H. Zhou, N. J. Parsons, C. C. Sammons, J. A. Cole, and H. Smith.** 1992. A high Mr factor in human blood which confers serum resistance on gonococci: some properties and synergism with CMP-NANA. Microb. Pathog. **12:**421–432.
9. **Darveau, R. P., and R. E. W. Hancock.** 1983. Procedure for isolation of bacterial lipopolysaccharides from both smooth and rough *Pseudomonas aeruginosa* and *Salmonella typhimurium* strains. J. Bacteriol. **155:**831–838.
10. **Demarco de Hormaeche, R., R. van Crevel, and C. E. Hormaeche.** 1991. *Neisseria gonorrhoeae* LPS variation, serum resistance and its induction by cytidine 5′-monophospho-*N*-acetylneuraminic acid. Microb. Pathog. **10:**323–332.
11. **Elkins, C., N. H. Carbonetti, V. A. Varela, D. Stirewalt, D. G. Klapper, and P. F. Sparling.** 1992. Antibodies to *N*-terminal peptides of gonococcal porin are bactericidal when gonococcal lipopolysaccharide is not sialylated. Mol. Microbiol. **6:**2617–2628.
12. **Fearon, D. T.** 1978. Regulation by membrane sialic acid of β1H-dependent decay dissociation of amplification C3 convertase of the alternative complement pathway. Proc. Natl. Acad. Sci. USA **75:**1971–1975.
13. **Frangipane, J. V., and R. F. Rest.** 1992. Anaerobic growth of gonococci does not alter their Opa-mediated interactions with human neutrophils. Infect. Immun. **60:**1793–1799.
14. **Glynn, A. A., and M. E. Ward.** 1970. Nature and heterogeneity of the antigens of *Neisseria gonorrhoeae* involved in the serum bactericidal reaction. Infect. Immun. **2:**162–168.
15. **Goaz, P. W., and H. A. Biswell.** 1961. Nitrate reduction in whole saliva. J. Dent. Res. **40:**355–365.
16. **Goldner, M., C. W. Penn, S. C. Sanyal, D. R. Veale, and H. Smith.** 1979. Phenotypically determined resistance of *Neisseria gonorrhoeae* to normal human serum: environmental factors in subcutaneous chambers in guinea pigs. J. Gen. Microbiol. **114:**169–177.
17. **Gottschalk, A.** 1972. Glycoproteins. Their composition, structure and function. Elsevier/North Holland Publishing Co., Amsterdam.
18. **Griffiss, J. M., G. A. Jarvis, J. P. O'Brien, M. M. Eads, and H. Schneider.** 1991. Lysis of *Neisseria gonorrhoeae* initiated by binding of normal human IgM to a hexosamine-containing lipooligosaccharide epitope(s) is augmented by strain specific, properdin-binding-dependent alternative complement pathway activation. J. Immunol. **147:**298–305.
19. **Griffiss, J. M., J. P. O'Brien, R. Yamasaki, G. D. Williams, P. A. Rice, and H. Schneider.** 1987. Physical heterogeneity of neisserial lipooligosaccharides reflects oligosaccharides that differ in apparent molecular weight, chemical composition, and antigenic expression. Infect. Immun. **55:**1792–1800.
20. **Hitchcock, P. J.** 1984. Analyses of gonococcal lipopolysaccharide in whole-cell lysates by sodium dodecyl sulfate-polyacrylamide gel electrophoresis: stable association of lipopolysaccharide with the major outer membrane protein (protein I) of *Neisseria gonorrhoeae*. Infect. Immun. **46:**202–212.
21. **Jarvis, G. A., H. Schneider, M. K. Albertson, and J. M. Griffiss.** 1988. Strain-specific direct binding of properdin accounts for variable lysis of *Neisseria gonorrhoeae*, p. 717–722. *In* J. T. Poolman et al. (ed.), Gonococci and meningococci. Kluwer Academic Publishers, Dordrecht, The Netherlands.
22. **Jarvis, G. A., and N. A. Vedros.** 1987. Sialic acid of group B *Neisseria meningitidis* regulates alternative complement pathway activation. Infect. Immun. **55:**174–180.

23. **Kellogg, D. S., Jr., I. R. Cohen, L. C. Norins, A. L. Schroeter, and G. Reising.** 1968. *Neisseria gonorrhoeae*. II. Colonial variation and pathogenicity during 35 months in vitro. J. Bacteriol. **96:**596–605.
24. **Kellogg, D. S., Jr., J. A. Crawford, and C. S. Calloway.** 1983. Cultivation of *Neisseria gonorrhoeae* under low-oxygen conditions. J. Clin. Microbiol. **18:**178–184.
25. **Kellogg, D. S., Jr., W. L. Peacock, Jr., L. Brown, and C. I. Pirkle.** 1963. *Neisseria gonorrhoeae*. I. Virulence genetically linked to colonial variation. J. Bacteriol. **85:**1274–1279.
26. **Knapp, J. S., and V. L. Clark.** 1984. Anaerobic growth of *Neisseria gonorrhoeae* coupled to nitrite reduction. Infect. Immun. **46:**176–181.
27. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227:**680–685.
28. **Lesse, A. J., A. A. Campagnari, W. E. Bittner, and M. A. Apicella.** 1990. Increased resolution of lipopolysaccharides and lipooligosaccharides utilizing tricine-sodium dodecyl sulfate-polyacrylamide gel electrophoresis. J. Immunol. Methods **126:** 109–117.
29. **Mandrell, R. E., A. J. Lesse, J. V. Sugai, M. Shero, J. M. Griffiss, J. A. Cole, N. J. Parsons, H. Smith, S. A. Morse, and M. A. Apicella.** 1990. *In vitro* and *in vivo* modification of *Neisseria gonorrhoeae* lipooligosaccharide epitope structure by sialylation. J. Exp. Med. **171:**1649–1664.
30. **Martin, P. M. V., P. V. Patel, N. J. Parsons, and H. Smith.** 1981. Induction of phenotypically determined resistance of *Neisseria gonorrhoeae* to human serum by factors in human serum. J. Gen. Microbiol. **127:**213–217.
31. **Martin, P. M. V., P. V. Patel, N. J. Parsons, and H. Smith.** 1982. Induction of phenotypically determined resistance of *Neisseria gonorrhoeae* to human serum by sera from patients with gonorrhea. Br. J. Vener. Dis. **58:**302–304.
32. **Martin, P. M. V., P. V. Patel, N. J. Parsons, and H. Smith.** 1982. Induction in gonococci of phenotypic resistance to killing by human serum by human genital secretions. Br. J. Vener. Dis. **58:**363–365.
33. **Morse, S. A., C. S. Mintz, S. K. Sarafian, L. Bartenstein, M. Bertram, and M. A. Apicella.** 1983. Effect of dilution rate on lipopolysaccharide and serum resistance of *Neisseria gonorrhoeae* grown in continuous culture. Infect. Immun. **41:**74–82.
34. **Nairn, C. A., J. A. Cole, P. V. Patel, N. J. Parsons, J. E. Fox, and H. Smith.** 1988. Cytidine 5′-monophospho-*N*-acetylneuraminic acid or a related compound is the low Mr factor from human red blood cells which induces gonococcal resistance to killing by human serum. J. Gen. Microbiol. **134:**3295–3306.
35. **Okada, N., T. Yasuda, and H. Okada.** 1982. Restriction of alternative complement pathway activation by sialosylglycolipids. Nature (London) **299:**261–263.
36. **Parsons, N. J., J. R. C. Andrade, P. V. Patel, J. A. Cole, and H. Smith.** 1989. Sialylation of lipopolysaccharide and loss of absorption of bactericidal antibody during conversion of gonococci to serum resistance by cytidine 5′-monophospho-*N*-acetylneuraminic acid. Microb. Pathog. **7:**63–72.
37. **Parsons, N. J., J. A. Cole, and H. Smith.** 1990. Resistance to human serum of gonococci in urethral exudates is reduced by neuraminidase. Proc. R. Soc. Lond. Ser. B Biol. Sci. **241:**3–5.
38. **Parsons, N. J., P. V. Patel, E. L. Tan, J. R. C. Andrade, C. A. Nairn, M. Goldner, J. A. Cole, and H. Smith.** 1988. Cytidine 5′-monophospho-*N*-acetylneuraminic acid and a low molecular weight factor from human blood cells induce lipopolysaccharide alteration in gonococci when conferring resistance to killing by human serum. Microb. Pathog. **5:**303–309.
39. **Patel, P. V., P. M. V. Martin, M. Goldner, N. J. Parsons, and H. Smith.** 1984. Red blood cells, a source of factors which induce *Neisseria gonorrhoeae* to resistance to complement-mediated killing by human serum. J. Gen. Microbiol. **130:**2767–2770.
40. **Patel, P. V., P. M. V. Martin, E. L. Tan, C. A. Nairn, N. J. Parsons, M. Goldner, and H. Smith.** 1988. Protein changes associated with induced resistance of *Neisseria gonorrhoeae* to killing by human serum are relatively minor. J. Gen. Microbiol. **134:**499–507.
41. **Patel, P. V., N. J. Parsons, J. R. C. Andrade, C. A. Nairn, E. L. Tan, M. Goldner, J. A. Cole, and H. Smith.** 1988. White blood cells including polymorphonuclear phagocytes contain a factor which induces gonococcal resistance to complement-mediated serum killing. FEMS Microbiol. Lett. **50:**173–176.
42. **Patel, P. V., D. R. Veale, J. E. Fox, P. M. V. Martin, N. J. Parsons, and H. Smith.** 1984. Fractionation of guinea pig serum for an inducer of gonococcal resistance to killing by human serum: active fractions containing glucopeptides similar to those from human red blood cells. J. Gen. Microbiol. **130:**2757–2766.
43. **Rest, R. F., and J. V. Frangipane.** 1992. Growth of *Neisseria gonorrhoeae* in CMP-*N*-acetylneuraminic acid inhibits nonopsonic (opacity-associated outer membrane protein-mediated) interactions with human neutrophils. Infect. Immun. **60:**989–997.
44. **Rice, P. A.** 1989. Molecular basis for serum resistance in *Neisseria gonorrhoeae*. Clin. Microbiol. Rev. **2:**S112–S117.
45. **Rock, J. P., and R. F. Rest.** 1988. Rapid damage to membranes of *Neisseria gonorrhoeae* caused by human neutrophil granule extracts. J. Gen. Microbiol. **134:**509–519.
46. **Schneider, H., J. M. Griffiss, J. W. Boslego, P. J. Hitchcock, K. M. Zahos, and M. A. Apicella.** 1991. Expression of paragloboside-like lipooligosaccharides may be a necessary component of gonococcal pathogenesis in men. J. Exp. Med. **174:**1601–1605.
47. **Schneider, H., J. M. Griffiss, G. D. Williams, and G. B. Pier.** 1982. Immunological basis of serum resistance of *Neisseria gonorrhoeae*. J. Gen. Microbiol. **128:**13–22.
48. **Schneider, H., C. A. Hammack, B. A. Schuman, and J. M. Griffiss.** 1986. Stability of expression of *Neisseria gonorrhoeae* lipooligosaccharides. Infect. Immun. **54:**924–927.
49. **Schoolnik, G. K., H. D. Ochs, and T. M. Buchanan.** 1979. Immunoglobulin class responsible for gonococcal bactericidal activity of normal human sera. J. Immunol. **122:**1771–1779.
50. **Scudder, P. R., and E. N. Chantler.** 1981. Glycosyltransferases of the human cervical epithelium. II. Characteristics of a CMP-*N*-acetylneuraminate, galactosyl glycoprotein sialyltransferase. Biochim. Biophys. Acta **660:**136–141.
51. **Short, H. B., V. L. Clark, D. S. Kellogg, and F. E. Young.** 1982. Anaerobic survival of clinical isolates and laboratory strains of *Neisseria gonorrhoeae*: use in transfer and storage. J. Clin. Microbiol. **15:**915–919.
52. **Smith, H.** 1990. Pathogenicity and the microbe *in vivo*. J. Gen. Microbiol. **136:**377–393.
53. **Smith, L. D. S.** 1975. The pathogenic anaerobic bacteria, 2nd ed. Charles C Thomas, Publisher, Springfield, Ill.
54. **Swanson, J.** 1978. Studies on gonococcus infection. XII. Colony color and opacity variants of gonococci. Infect. Immun. **19:**320–331.
55. **Swanson, J.** 1982. Colony opacity and protein II composition of gonococci. Infect. Immun. **37:**359–368.
56. **Tan, E. L., P. V. Patel, N. J. Parsons, P. M. V. Martin, and H. Smith.** 1986. Lipopolysaccharide alteration is associated with induced resistance of *Neisseria gonorrhoeae* to killing by human serum. J. Gen. Microbiol. **132:**1407–1413.
57. **Tsai, C., and C. E. Frasch.** 1982. A sensitive silver stain for detecting lipopolysaccharides in polyacrylamide gels. Anal. Biochem. **119:**115–119.
58. **Veale, D. R., C. W. Penn, and H. Smith.** 1981. Factors affecting the induction of phenotypically determined serum resistance of *Neisseria gonorrhoeae* grown in media containing serum or its diffusible components. J. Gen. Microbiol. **122:**235–245.
59. **Ward, M. E., P. J. Watt, and A. A. Glynn.** 1970. Gonococci in urethral exudates possess a virulence factor lost on subculture. Nature (London) **227:**382–384.
60. **Wetzler, L. M., K. Barry, M. S. Blake, and E. C. Gotschlich.** 1992. Gonococcal lipooligosaccharide sialylation prevents complement-dependent killing by immune sera. Infect. Immun. **60:**39–43.

61. **Whelan, M.** 1930. A colorimetric method for the quantitative determination of nitrates and nitrites in biological fluids. J. Biol. Chem. **86:**189–197.
62. **Yamasaki, R., B. E. Bacon, W. Nasholds, H. Schneider, and J. M. Griffiss.** 1991. Structural determination of oligosaccharides derived from lipooligosaccharide of *Neisseria gonorrhoeae* F62 by chemical, enzymatic, and two-dimensional NMR methods. Biochemistry **30:**10566–10575.
63. **Yamasaki, R., W. Nasholds, H. Schneider, and M. A. Apicella.** 1991. Epitope expression and partial structural characterization of F62 lipooligosaccharide (LOS) of *Neisseria gonorrhoeae*: IgM monoclonal antibodies (3F11 and 1-1-M) recognize nonreducing termini of the LOS components. Mol. Immunol. **28:** 1233–1242.