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Effect of Dilution Rate on Lipopolysaccharide and Serum Resistance of *Neisseria gonorrhoeae* Grown in Continuous Culture

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Growth of *Neisseria gonorrhoeae* strain FA171 in continuous culture under glucose-limiting conditions resulted in a growth-rate-dependent change in the lipopolysaccharide (LPS). The evidence for this change is an alteration in the mobility of purified alkali-treated LPS on sodium dodecyl sulfate-polyacrylamide gels and a quantitative difference in the amount of the LPS serotype antigen. The LPS from cells grown at a low dilution rate (0.12 h^{-1}) contained ca. eightfold less serotype antigen than the LPS from cells grown at a high dilution rate (0.56 h^{-1}). The decrease in LPS serotype antigen was associated with an increase in sensitivity to the bactericidal activity of normal human serum and an increase in cell surface hydrophobicity. An increase in the amount of serotype antigen was associated with a reduction in the accessibility of a monoclonal antibody to a core LPS determinant, an increase in resistance to normal human serum, and a decrease in cell surface hydrophobicity. The microheterogeneity of gonococcal LPS with respect to the content of serotype antigen may result from an alteration in the metabolism of glucose.

Gonococci are obligate human pathogens that can produce asymptomatic or symptomatic infections at many body sites. At any of these sites, invading gonococci must deal with variable environmental conditions such as pH, oxygen saturation, nutrient supply, inhibitory agents, and the resident competing microbial flora. It is well known that the chemical composition of microorganisms is markedly influenced by their environment. One way to study these changes in cell composition is through the use of a chemostat in which the growth rate is controlled by the rate at which a growth-limiting substance is added to the culture vessel (48).

Resistance to the complement-mediated bactericidal action of serum has been associated with the ability of gonococci to cause disseminated disease in persons with a functionally intact complement pathway (23, 42). Although the molecular basis of gonococcal resistance to normal human serum (NHS) has not been fully defined, the available evidence suggests that certain cell surface structures are able to protect gonococci from the action of the serum bactericidal system (4). Studies of the gonococcal cell surface in relation to pathogenicity have frequently utilized cells grown in batch culture under conditions of nutrient excess and, consequently, with a short generation time. There are no available data concerning the generation time of gonococci in vivo. Nevertheless, with other microorganisms, the measurement of true division rates in vivo has indicated that their growth rate in mammalian tissues is very slow in comparison with the maximum growth rate (26, 28, 38), suggesting that, in vivo, the growth rate is limited by the availability of substrates. Therefore, this study was undertaken to determine the effect of a controlled growth environment on the lipopolysaccharide (LPS) composition and serum resistance of *Neisseria gonorrhoeae*. The gonococci were maintained at a constant physiological state by use of a chemostat so that specific cell surface parameters could be studied more effectively.

MATERIALS AND METHODS

Organism. *N. gonorrhoeae* strain FA171 (*mtr-2 sac-1 sac-3*) was kindly provided by P. F. Sparling (University of North Carolina, Chapel Hill). This strain is an isogenic derivative of strain FA19 (wild type envelope phenotype *sac-1 sac-3*) which was originally isolated from an uncomplicated genital infection. Strain FA19 exhibits the serum resistance and penicillin susceptibility properties of DGI strains. Serological

testing by coagglutination indicated that strain FA171 belonged to protein serogroup WI (34). The auxotype of this strain is zero (prototrophic) (6), and the LPS possessed the serotype 5 antigen. Cells from nonpiliated (P^-), transparent colonies (Op^-) were used in all experiments.

Medium and growth conditions. A modification of the chemically defined medium described by Morse and Bartenstein (32) was used for continuous culture experiments. The modified medium contained 10 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid and 10 mM glucose as the growth rate-limiting nutrient. The $NaHCO_3$ was omitted. The medium was prepared in 10-liter amounts and sterilized by membrane filtration (0.45 μm; Millipore Corp., Bedford, Mass.). The final pH of the uninoculated medium was 7.4. A solid medium consisting of GC agar (Difco Laboratories, Detroit, Mich.) containing a growth factor supplement (31) was used where indicated.

For continuous culture experiments, gonococci were grown in a model C-30 chemostat (New Brunswick Scientific Co., New Brunswick, N.J.) equipped with a 350-ml volume culture vessel. Dissolved oxygen (as percent saturation) was monitored with a 900 series dissolved oxygen electrode (New Brunswick Scientific Co.) and a model DO-50 dissolved O_2 analyzer with recorder (New Brunswick Scientific Co.). Before inoculation, the medium was saturated with dissolved oxygen by adjusting the air flow to 400 ml/min and the impeller speed to 700 to 800 rpm. During the experiments the dissolved oxygen remained between 80 and 100% of saturation.

Inocula were prepared by removing the growth from two GC agar plates that were incubated overnight at 37°C in an atmosphere of 4% CO_2 and suspending the cells in 5 to 10 ml of chemically defined medium. This cell suspension was added to the chemostat vessel and incubated for 10 h (air flow, 400 ml/min; impeller speed, 500 rpm) at 37°C. After 10 h, the nutrient pump was started, and the impeller speed was increased to 700 to 800 rpm. The purity of the culture was checked periodically by both Gram staining and by streaking GC agar plates. Foaming was controlled by the aseptic addition of 0.5 ml of a sterile 1:3 dilution of Antifoam C emulsion (Sigma Chemical Co., St. Louis, Mo.) in distilled water. At the concentration of Antifoam C emulsion used, there was no effect upon the growth or viability of *N. gonorrhoeae* strain FA171. To insure the attainment of a steady state, gonococci were allowed to remain at each dilution rate until at least 2 volumes of medium (660 ml) had passed through the culture vessel. To reduce the possibility that long-term cultivation in continuous culture would select for mutants, we alternated the low and high dilution rates. During continuous culture, the pH of the medium was 7.2 ± 0.2. The methods of Veldkamp (48) and Evans et al. (8) were used to confirm that glucose was actually limiting growth.

Analytical procedures. Total protein and total carbohydrate were determined on washed cell suspensions. Samples were removed from the chemostat, harvested by centrifugation, and washed twice with 0.85% NaCl. The cell pellet was suspended to its original volume in distilled water. Total protein was measured by the Folin reaction (24) after heating the cell suspensions at 90°C in 1 N NaOH for 10 min to obtain complete solubilization (15). The protein standard (bovine serum albumin) was treated in a similar fashion. Total carbohydrate was measured by the anthrone reaction (21). Dry weights were determined as previously described (35). At least two dry weight determinations were made at each dilution rate.

For glucose and acetate determinations, samples were removed from the chemostat and filtered through a 0.45-μm membrane filter. The filtrates were stored frozen until assayed. Glucose concentrations in the reservoir and vessel medium were determined enzymatically with the Statzyme reagent obtained from Worthington Diagnostics (Freehold, N.J.). Acetate in the vessel medium was estimated with acetate kinase as described by Hempfling and Mainzer (17).

Measurement of resistance to killing by NHS. Cells grown at various dilution rates were removed from the chemostat and diluted in defined medium to ca. 3×10^4 CFU/ml. The serum resistance of these cells was determined after incubation for 40 min in a 1:4 dilution of pooled NHS as previously described (30). A portion of the diluted serum was treated at 56°C for 30 min and served as a control. Viable counts were determined after the plates had been incubated for 48 h at 37°C in an atmosphere of 4% CO_2. The average count of viable organisms in the experimental samples that contained fresh human serum, which was expressed as a percentage of that in the control sample containing heated serum, was considered to be the measure of serum resistance. There was no significant increase or decrease in the viable counts in the control samples over the 40-min incubation period.

Extraction and purification of lipopolysaccharide. Cells grown at constant dilution rates were collected continuously from the effluent port of the chemostat vessel. To minimize changes in cell composition, 37% formaldehyde was continuously added to the product reservoir so that the final formaldehyde concentration was maintained at 2% (1). After 10 liters of medium was collected, the formalinized cells were harvested by centrifugation, washed with distilled water, and lyophilized. Previous studies (7) indicated that formaldehyde treatment did not affect LPS structure.

Gonococcal LPS was prepared by a modification of the phenol-water method of Perry et al. (37). The aqueous phase was collected, dialyzed until it was free of phenol, and concentrated by precipitation with acetone. The precipitate was washed three times in distilled water and digested overnight in 0.05 N NaOH at 37°C. The neutralized digest was treated with 20 μg of pronase (Calbiochem Behring Corp., La Jolla, Calif.) per ml at 37°C for 4 h, boiled for 5 min to destroy pronase activity, dialyzed overnight against distilled water, and lyophilized. The LPS-derived gonococcal polysaccharide was prepared by DEAE-Sepharose (Pharmacia Fine Chemicals, Piscataway, N.J.) as previously described (30).

Comparative studies demonstrated that the treatment of LPS from strain FA171 with 0.05 N NaOH had no effect on the expression of the gonococcal LPS serotype antigen. With LPS prepared from cells grown in batch culture, the concentration of alkali-treated FA171 LPS necessary to achieve 50% inhibition of the serotype enzyme-linked immunosorbent assay (ELISA) system was 9.5 μg/ml (95% confidence limits, 6.41 to 14.1 μg/ml). The concentration of native FA171 LPS necessary for 50% inhibition was 11.6 μg/ml (95% confidence limits, 7.8 to 16.6 μg/ml).

Antigenic analysis of gonococcal LPS. The common and serotype antigens of gonococcal LPS were quantitated by an ELISA inhibition assay. The specific details of this system have been recently described (30). For the determination of serotype antigen, the wells were coated with specific serotype antigen, and the antiserum used was absorbed to make it specific for the serotype determinant. For the analysis of the common determinant, the wells were coated with strain JW-31R LPS (lacks serotype and variable determinants) (30), and unadsorbed homologous antiserum (serotype 5 antiserum) was used. Ligand inhibition curves were calculated as a percentage of control values with the programmed linear regression function of a programmable calculator (model TI-59; Texas Instruments, Lubbock, Tex.). Confidence limits around the calculated lines were determined as described by Steel and Torrie (45).

SDS-polyacrylamide gel electrophoresis. Gonococcal LPS was analyzed by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis by a modified version of the method of Laemmli (22). In this modification, riboflavin (0.0018%) rather than ammonium persulfate was used as a catalyst. Linear gradient gels (1.5 mm in thickness) containing 8 to 20% acrylamide with bisacrylamide as a cross-linking agent were allowed to polymerize overnight before use. The stacking gel contained 5% acrylamide. The acrylamide/bisacrylamide ratio in both the separating and stacking gels was 30:0.8. LPS samples were suspended in a buffer (pH 6.8) composed of 0.025 M Tris-hydrochloride, 1.0% SDS, 6% glycerol, 5% 2-mercaptoethanol, and 0.002% bromphenol blue added as a tracking dye. The samples were solubilized by heating at 100°C for 5 min. Electrophoresis was carried out in the cold (4°C) at constant power with an initial power setting of 3 W. Once the dye front entered the separating gel, the power was increased to 4 W and maintained at this setting until the completion of the run. After electrophoresis, the gels were fixed and stained for carbohydrate by the method of Tsai and Frasch (46).

Monoclonal antibody binding. The binding of ^{125}I-monoclonal antibody 3F11 to cells of strain FA171 was determined by a filtration assay. The production and selection of the hybrid clone producing this LPS-specific monoclonal antibody has been previously described (2, 30). Monoclonal antibody 3F11 is a *k*-chain immunoglobulin G (IgG) that recognizes a core LPS determinant having the chemical structure D-galactosamine-*O*-D-galactopyranosyl-(1-4)-D-glucopyranose. ^{125}I was coupled to the monoclonal antibody with 1,3,4,6-tetrachloro-3α,6α-diphenylglycoluril (9). Cells for binding studies were removed from the chemostat and treated with formaldehyde (2%, vol/vol) (1). The formaldehyde-treated cells were washed twice by centrifugation and suspended to their original volume in defined medium containing 0.025% (wt/vol) sodium azide and 1% (wt/vol) bovine serum albumin. These cells were stored at 4°C until assayed. The results from preliminary experiments indicated that a linear relationship existed between cell concentration and binding of the ^{125}I-monoclonal antibody. For the binding assays, the formaldehyde-treated cell suspensions were diluted 1:8 in defined medium containing 1% bovine serum albumin. ^{125}I-monoclonal antibody (5 μl) was added to 0.1 ml of the diluted cell suspension, and the samples were incubated for 40 min at 37°C. The cells were collected by filtration under vacuum onto 0.45-μm membrane filters (Schleicher & Schuell Co., Keene, N.H.). The filters were previously soaked in defined medium containing 1% bovine serum albumin to reduce the nonspecific binding of ^{125}I-antibody. After filtration, the filters were washed with 20 ml of defined medium containing 1% (wt/vol) bovine serum albumin and counted in a gamma counter (Beckman Instruments, Inc., Fullerton, Calif.). All samples were run in duplicate. A nonspecific binding control consisted of ^{125}I-monoclonal antibody plus diluent. Results were corrected for background and for nonspecific binding and are expressed as counts per minute of ^{125}I bound per milligram (dry weight) of cells.

Adherence to hexadecane and autolysis. Adherence to hexadecane was measured by the method of Rosenberg et al. (40). Rates of autolysis were determined as previously described (49).

RESULTS

Growth in continuous culture. Glucose was selected as the growth rate-limiting nutrient because it is a key intermediate in the synthesis of many important cellular constituents. Also, it is the only carbohydrate that can be utilized by *N. gonorrhoeae* (29). The defined medium used in these experiments did not support the growth of strain FA171 unless glucose was added. In batch culture experiments, the maximum cell yield was proportional to the concentration of glucose in the medium. During batch culture in defined medium containing excess glucose, strain FA171 exhibited a mean generation time of 74.6 ± 1.4 min.

N. gonorrhoeae strain FA171 (P^- Op^-) was grown in continuous culture under glucose-limiting conditions. A steady state was attained at various dilution rates, and the dry weights at each of these dilution rates were determined (Fig. 1). Washouts occurred at dilution rates greater than 0.75 h^{-1}. Viable and total cell counts were also determined over the same range of dilution rates. Cell viability was ≥84% at each

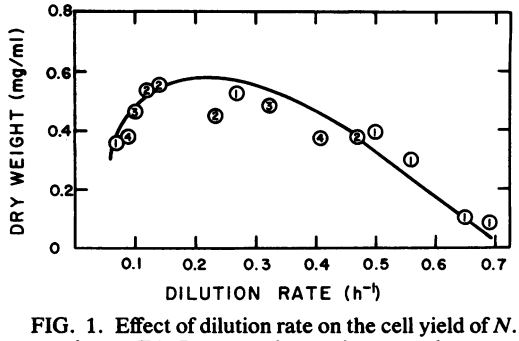

FIG. 1. Effect of dilution rate on the cell yield of *N. gonorrhoeae* FA171 grown in continuous culture under glucose-limited conditions. The number in the symbol represents the number of separate experiments conducted at the indicated dilution rate.

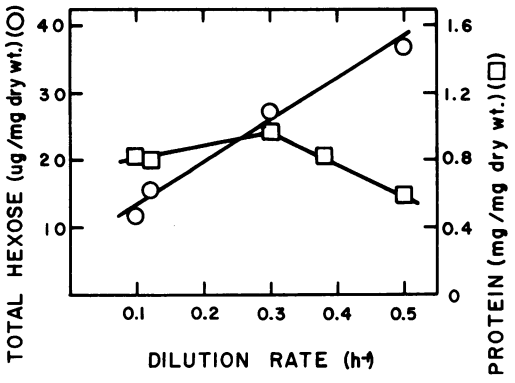

FIG. 2. Effect of dilution rate on the total hexose (○) and total protein (□) content of *N. gonorrhoeae* FA171 grown under glucose-limited conditions in continuous culture.

dilution rate examined. Examination of the colonies under a dissecting microscope indicated that opaque (Op^+) colonies comprised <5% of the total population. The dilution rate had no significant effect upon the frequency of Op^+ colonies.

Total protein and carbohydrate. The protein and carbohydrate content was determined for cells growing at various dilution rates. Figure 2 shows that the protein content of the biomass varied somewhat, but only decreased (ca. 25%) at higher dilution rates. Of more significance was that the carbohydrate content of the cells increased three- to fourfold as the dilution rate increased from 0.07 to 0.5 h^{-1}.

The effect of dilution rate on the metabolism of glucose was examined further to explain this increase in carbohydrate content. Radiorespirometric (35) experiments with cells grown in batch culture indicated that ca. 78% of the glucose was catabolized via the Entner-Doudoroff pathway. The remainder was dissimilated via the pentose phosphate pathway and was used primarily for pentose synthesis. Acetate was the only nongaseous end product of glucose metabolism produced by gonococci growing under aerobic conditions (35). The acetate accumulated in the medium and was not oxidized until glucose was depleted. Thus, 2 mol of acetate would be produced and accumulate in the medium from each mol of glucose catabolized via the Entner-Doudoroff pathway. For our purposes in assessing glucose catabolism in continuous culture, we have assumed that the Entner-Doudoroff pathway was utilized to the same degree as during batch culture growth, and that acetate was the only nongaseous end product formed from glucose. This assumption was tested by measuring the glucose and acetate levels in the chemostat culture medium (Fig. 3). There was no residual glucose remaining at dilution rates of <0.3 h^{-1}. At dilution rates of >0.3 h^{-1}, the residual glucose in the growth medium increased and was accompanied by a concomitant decrease in acetate. There was good agreement between the expected and observed levels of acetate (Table 1). At dilution rates of ≤0.23 h^{-1}, there were 2 mol of acetate produced per mol of glucose. However, at dilution rates of ≥0.33 h^{-1}, there was less acetate produced than was expected. When compared with the expected values, there

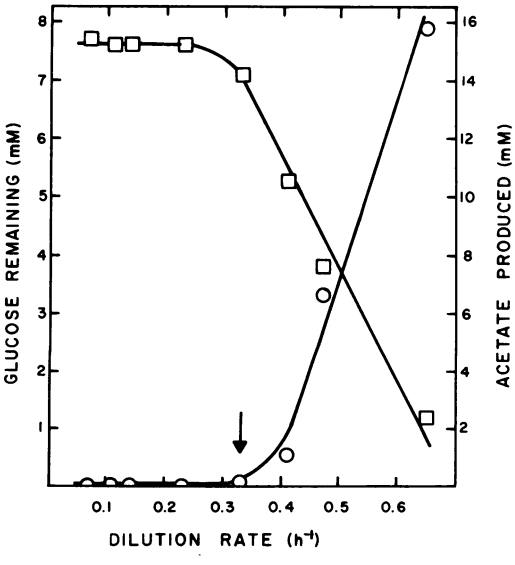

FIG. 3. Effect of dilution rate on glucose and acetate levels. The arrow indicates the point at which cells become completely serum resistant (see Fig. 4).

TABLE 1. Effect of dilution rate on the catabolism of glucose by *N. gonorrhoeae* FA171

Dilution rate (h^{-1})	Glucose catabolized via ED pathway (mM)[a]	Acetate (mM)		
		Expected[b]	Observed	E − O[c]
0.07	7.72	15.4	15.4	0
0.11	7.72	15.4	15.2	0.2
0.14	7.72	15.4	15.2	0.2
0.23	7.72	15.4	15.2	0.2
0.33	7.68	15.4	14.2	1.2
0.47	5.12	10.2	7.6	2.6
0.65	1.57	3.1	2.3	0.8

[a] Based upon radiorespirometric data (29) suggesting that ca. 78% of glucose is catabolized via the Entner-Doudoroff (ED) pathway (9.91 × 0.78 = 7.72 mM).

[b] Based upon the assumption that 2 mol of acetate are produced per mol of glucose consumed.

[c] Expected acetate concentration minus the observed concentration.

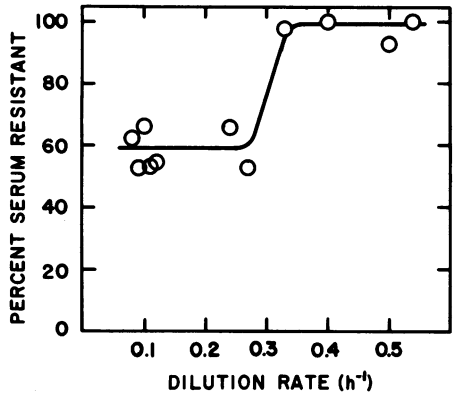

FIG. 4. Effect of dilution rate on the serum sensitivity of *N. gonorrhoeae* FA171 grown under glucose-limited conditions in continuous culture. Each point represents the mean of four determinations. The standard deviation about these means was ±<10%.

was 8% less acetate produced at a dilution rate of 0.33 h^{-1}, and there was 26% less acetate produced at dilution rates of 0.47 and 0.65 h^{-1}.

Serum resistance. The results from preliminary experiments suggested that the kinetics of killing of gonococci were first order during the 40-min incubation period and that the survivors did not represent a serum-resistant subpopulation of organisms. The resistance of chemostat-grown cells to killing by NHS was determined over a range of dilution rates. The results (Fig. 4) show that 40 to 50% of cells grown at dilution rates of 0.07 to 0.29 h^{-1} were killed during a 40-min incubation with 25% NHS. At dilution rates of >0.3 h^{-1}, the cells were completely resistant to the bactericidal activity of 25% NHS. Complement was not limiting under the assay conditions used. It is interesting to note that this shift in serum resistance coincided with a shift in glucose metabolism (Fig. 3).

LPS. LPS is an important antigen in the sensitivity of gonococci to NHS (11). LPS was extracted from both serum-sensitive (dilution rate, 0.12 h^{-1}) and serum-resistant (dilution rate, 0.56 h^{-1}) cells as described above. The extracted, alkali-treated LPSs were compared by SDS-polyacrylamide gel electrophoresis. Figure 5 shows that the LPS from serum-sensitive cells migrated as a single band on electrophoresis. The LPS from serum-resistant cells exhibited a common band with that of serum-sensitive cells as well as two additional bands with decreased mobilities.

The LPS from serum-sensitive (dilution rate, 0.12 h^{-1}) and serum-resistant (dilution rate, 0.56 h^{-1}) cells were examined serologically to determine whether there were any differences in the structure of the polysaccharide component. The common and serotype antigenic determinants were quantitated by an ELISA inhibition test. By comparing the concentrations LPS required to inhibit the ELISA by 50% and the 95% confidence limits of these values, it was possible to assess the relative amounts of the serotype and common antigenic determinants. The 50% inhibitory concentrations for the common antigen were 260 μg (95% confidence limits, 41.8 to 1617.9 μg) of serum-sensitive LPS per ml and 123.8 μg (95% confidence limits, 55.1 to 277.8 μg) of serum-resistant LPS per ml. The overlapping confidence limits suggest that the LPS from both serum-resistant and serum-sensitive cells contained similar amounts of the common antigenic determinant. The 50% inhibitory concentrations for the serotype 5 antigen were 18.68 μg (95% confidence limits, 10.52 to 29.71 μg) of serum-sensitive LPS per ml and 2.3 μg (95% confidence limits, 1.26 to 4.23 μg) of serum-resistant LPS per ml. The nonoverlapping confidence limits and the concentration of LPS required to inhibit the ELISA by 50% suggest that the LPS from serum-resistant cells contained >8 times more of the serotype antigenic determinant than the LPS from serum-sensitive cells (Fig. 6).

The presence of LPS containing the serotype antigen on the cell surface may decrease the accessibility of LPS core determinants to antibody. This possibility was examined by measuring the binding of monoclonal antibody 3F11 to chemostat-grown cells. Monoclonal antibody 3F11 recognizes an antigenic determinant found in the core region of gonococcal LPS. The results of these experiments (Table 2) show that the binding of monoclonal 3F11 was reduced by 95% in serum-resistant cells. It is not likely that

FIG. 5. SDS-polyacrylamide gel electrophoresis of purified, alkali-treated LPS from cells grown at (A) low (0.12 h^{-1}) and (B) high (0.56 h^{-1}) dilution rates. Approximately 20 μg of LPS was added to each slot.

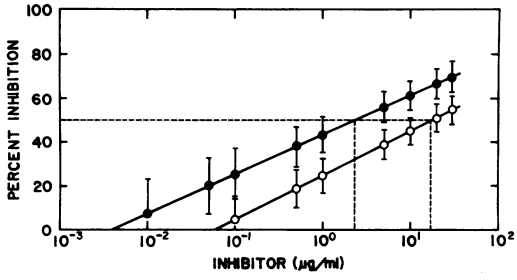

FIG. 6. Inhibition of an ELISA with antiserum to GC_5 by untreated (native) LPSs from *N. gonorrhoeae* FA171 grown at low and high dilution rates. The wells were coated with GC_5 antigen. Symbols: ○, LPS from cells grown at a low (0.12 h^{-1}) dilution rate; ●, LPS from cells grown at a high (0.56 h^{-1}) dilution rate.

this reduction in binding represents an alteration in the structure of the antigenic determinant since the LPS from serum-sensitive and serum-resistant cells possessed similar amounts of the core antigenic determinant.

Hexadecane binding. Gonococci grown at several dilution rates were tested for their ability to adhere to hexadecane. The results (Table 3) show that cells grown at low dilution rates ($\leq$0.30 h^{-1}) exhibited a 61% increase in their binding to hexadecane when compared with cells grown at higher dilution rates ($\geq$0.46 h^{-1}). This difference is significant at $P < 0.001$. Thus, serum-resistant cells (e.g., those with an LPS containing increased serotype antigen) were less hydrophobic than serum-sensitive cells (e.g., those with an LPS containing decreased serotype antigen).

DISCUSSION

The results of this study demonstrate that gonococci can be grown in continuous culture in a chemically defined medium under glucose-limiting conditions. There has been only one other report (25) concerning continuous cultivation of gonococci under similar conditions.

TABLE 2. Effect of dilution rate on the binding of ^{125}I-monoclonal antibody 3F11 to the core LPS of glucose-limited cells[a]

Dilution rate (h^{-1})	^{125}I-Antibody (cpm/mg [dry wt])[b]	Ratio[c]
0.12	107,927	1.00
0.24	11,894	0.11
0.56	5,032	0.05

[a] Monoclonal antibody 3F11 is specific for a D-galactosamine-D-galactopyranosyl-D-glucose moiety (30).

[b] Binding was determined after 40 min of incubation at 37°C.

[c] Ratio relative to a dilution rate of 0.12 h^{-1}.

However, the results of the present study differ somewhat from those previously reported. The maximum cell yield we obtained (ca. 0.6 mg/ml) was approximately 25% of that reported by Manchee et al. (25). This difference may be due to their use of a defined medium containing 41.6 mM glucose as compared with the 10 mM glucose used in the present study. In addition, pyruvate (17.5 mM) was also present in their defined medium. Pyruvate is readily catabolized by *N. gonorrhoeae*. Holten (19) observed that the carboxyl carbon of pyruvate was rapidly converted to CO_2, whereas the carbonyl and methyl carbons accumulated in the medium and were not readily converted to CO_2. Also, pyruvate can be used as an energy source for the growth of *N. gonorrhoeae* (49). Perhaps another important difference is that Manchee et al. (25) utilized a defined medium containing only 9 amino acids, whereas 21 amino acids were included in the medium used in the present study; the presence of only 9 amino acids would result in the cell diverting a portion of the energy and carbon derived from glucose and pyruvate for use in amino acid biosynthesis.

Glucose-limited growth of *N. gonorrhoeae* strain FA171 results in growth rate-dependent changes in the LPS. The change in LPS occurs with increasing dilution rate and is accompanied by a three- to fourfold increase in the total carbohydrate content of the cell. Several lines of evidence suggest that LPS structure is markedly affected when the growth rate is controlled by glucose limitation. Electrophoretic analysis of purified alkali-treated LPS indicated the presence of multiple bands with LPS from cells grown at a high dilution rate; only a single band was present with LPS from cells grown at a low dilution rate. Serological analysis of these LPSs revealed that the LPS from cells grown at a high dilution rate contained eight- to ninefold more serotype antigen than did the LPS from cells grown at a low dilution rate. Although this serotype antigen appears to be immunologically analogous to *O*-polysaccharide (30), we were unable to observe the multiple banding pattern on SDS gels that is characteristic of the long *O*-polysaccharide chains of LPS from smooth strains of enteric bacteria (46). The variation in

TABLE 3. Effect of dilution rate on adherence of *N. gonorrhoeae* FA171 to hexadecane

Dilution rate (h^{-1})	% Adherence[a]
0.11	28.3 ± 3.8
0.29	28.6 ± 3.4
0.46	18.7 ± 3.4
0.56	16.8 ± 3.8

[a] Values represent the mean of three determinations ± standard deviations.

the number of repeating of O antigenic units on the LPS from smooth strains of enteric bacteria is indicative of microheterogeneity in the molecule (12, 36). The serotype antigenic determinants on gonococcal LPS may be modifications of the core structure or a single repeat unit. The differences in the amount of serotype antigen on the LPS from cells grown at low and high dilution rates may be analogous to the microheterogeneity seen with enteric LPS. With *N. gonorrhoeae*, the microheterogeneity is affected by the physiological state of the cell. A similar phenomenon has been observed with *Neisseria sicca*. McDonald and Adams (27) reported differences in the amount and chemical composition of LPS from *N. sicca* grown at different growth rates in batch culture. Thus, environmental factors can affect the composition of LPS from *Neisseria* spp. With whole gonococci, the binding of a monoclonal antibody specific for a core LPS determinant decreased with increasing dilution rate and varied inversely with the content of the serotype antigen on purified LPS. This suggests that the presence of increased amounts of LPS serotype antigen may reduce the accessibility to or mask the core antigenic determinant recognized by monoclonal antibody 3F11. The former possibility is more likely as LPS from cells grown at both low and high dilution rates had equivalent amounts of core antigen.

The change in LPS structure may be related to a shift in glucose metabolism. An inventory of the nongaseous end products of glucose metabolism indicated that, at low dilution rates, all of the glucose catabolized via the Entner-Doudoroff pathway could be recovered as acetate. At high dilution rates, there was less acetate recovered than was expected. The increased carbohydrate content of the cells lends support to the possibility that an increasing portion of the glucose was utilized for biosynthetic reactions at these higher dilution rates. Alternatively, some of the acetate may be oxidized via a functional tricarboxylic acid cycle (16). This would result in an increase in the amount of ATP produced per mole of glucose and may allow the cell to utilize more glucose for biosynthetic reactions. Although we have no data with continuous cultures to support this possibility, data with batch cultures grown at neutral pH (16, 35) indicated that acetate oxidation was only initiated after the depletion of glucose from the medium or when the cells entered the stationary phase of growth. In batch culture, acetate oxidation did occur concomitantly with glucose metabolism when cells were grown at pH 6.0 (33). Since the pH of the chemostat cultures was ca. 7.2, concomitant acetate oxidation may not occur.

An alternative explanation for the observed change in LPS structure is that, at low dilution rates, gonococci produce increased amounts of an enzyme(s) that is capable of degrading the serotype antigen of the LPS. Apicella et al. (3) observed an enzyme with this activity in cell extracts of *N. gonorrhoeae* strain 1291. This enzyme may have to be excreted or released via autolysis to be effective against LPS in the outer membrane. However, we observed no difference in the rates of autolysis of cells grown at either low or high dilution rates (data not shown). Nevertheless, we cannot exclude this possibility.

The decrease in the amount of LPS serotype antigen was associated with an increase in the susceptibility to killing by NHS. A similar relationship between loss of LPS structure and serum susceptibility has been noted previously (13, 30). Morse and Apicella (30) isolated a pyocin-resistant mutant (strain JW-31R) of the serum-resistant *N. gonorrhoeae* strain JW-31. The pyocin-resistant mutant was serum sensitive and was found to lack both the serotype and variable LPS determinants. Guymon et al. (13) isolated several pyocin-resistant mutants of *N. gonorrhoeae* strain FA19 (a strain isogenic with strain FA171). Two classes of these mutants exhibited marked alterations in the LPS composition coupled with an increased susceptibility to killing by NHS. Neither Morse and Apicella (30) nor Guymon et al. (13) observed an alteration in outer membrane protein composition that accompanied the loss of LPS determinants. There is an important difference between these studies and the present investigation. The previous reports (13, 30) utilized LPS mutants that exhibited increased serum sensitivity when compared with the parent strains. The present study demonstrates that the LPS structure, and hence serum sensitivity, may vary phenotypically depending upon specific growth conditions. However, all of these investigations serve to point out that LPS is an important factor in the resistance of gonococci to NHS.

The bactericidal activity of NHS is mediated, in part, by IgM antibodies that recognize determinants on the gonococcal LPS (11). Recent studies have determined that these determinants are located on the core oligosaccharide of gonococcal LPS (M. A. J. Westerink, M. A. Apicella, and J. M. Griffiss, Program Abstr. Intersci. Conf. Antimicrob. Agents Chemother. 22nd, Miami Beach, Fla., abstr. no. 545, 1982). Affinity-purified anti-LPS IgM antibody did not kill the serum-resistant strain JW-31, but was bactericidal for an isogenic LPS mutant (JW-31R) that lacks the gonococcal serotype antigen. These data suggest that the presence of serotype antigen may sterically hinder the binding of IgM to core determinants. In a similar fashion, the

binding of a monoclonal antibody to an LPS core determinant of strain FA171 decreased as the serotype antigen content of the LPS increased.

Gonococci can activate either the classical or alternative complement pathways (41). The results of the present study do not distinguish between these two possibilities. However, future studies will determine whether growth conditions can influence the pathway of complement activation by *N. gonorrhoeae*.

The molecular basis of serum resistance of *N. gonorrhoeae* has not been fully defined. A number of reports have suggested that changes in the LPS (13, 14, 30), protein I (18, 20), and colony opacity-related protein II species (20) contribute to the sensitivity of gonococci to NHS. Recently, three genetic loci have been described which govern the level of serum resistance of *N. gonorrhoeae*. These loci have been designated *sac-1* (5), *sac-2* (44), and *sac-3* (43). Shafer et al. (43) postulated that the expression of these loci modified the cell envelope structure in some fashion that enabled the gonococcus to evade the bactericidal activity of NHS. The gene products of these loci have not been identified. The strain used in the present investigation (FA171) has both the *sac-1* and *sac-3* loci. Thus, it is not known whether the phenotypic modification of serum resistance observed with organisms grown in continuous culture has resulted from changes in the expression of these loci or is due to some as yet unidentified locus.

As mentioned above, cells grown at low dilution rates produced reduced amounts of the LPS serotype determinant. These cells exhibited a 61% increase in their adherence to hexadecane. The adherence of cells to hexadecane is a measurement of cell surface hydrophobicity (39). Cells that adhere to a greater extent have a more hydrophobic cell surface than do those that adhere less well. A similar phenomenon occurs with LPS-deficient mutants of enteric bacteria as core mutants have a more hydrophobic cell surface than do bacteria with a wild-type LPS (39). An increase in cell-surface hydrophobicity has been correlated with increased adherence to epithelial cells (40) and with increased attachment to phagocytes (47).

We have no information concerning the nature of the LPS on gonococci in vivo. However, it is likely that the cells are growing under nutrient-limited conditions. Thus, the gonococcus may be more serum sensitive and have a more hydrophobic cell surface when grown under some in vivo conditions than when grown under optimum conditions in vitro. Gibbs and Roberts (10), using egg-grown organisms, observed that log-phase P^+ gonococci resisted phagocytosis by human neutrophils, whereas P^+ stationary-phase gonococci did not. These differences may be related to changes in the cell surface that occur between log-phase (nutrient-excess) and stationary-phase (nutrient-limited) growth.

In summary, growth of gonococci in continuous culture under glucose-limiting conditions results in an alteration in the LPS. The evidence for an altered LPS is as follows: (i) a change in the banding pattern seen on SDS-polyacrylamide gels; (ii) quantitative differences in the amount of LPS serotype antigen; and (iii) changes in cell surface hydrophobicity. The change in LPS alters the serum resistance of the cell and may ultimately affect the in vivo survival and virulence of the organism. Studies to correlate these in vitro changes with in vivo correlates of virulence such as adherence to epithelial cells and phagocytosis are in progress.

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