Characterization of Gonococcal Antigens Responsible for Induction of Bactericidal Antibody in Disseminated Infection

THE ROLE OF GONOCOCCAL ENDOTOXINS

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ABSTRACT The role of gonococcal antigens in serum bactericidal activity was determined for an isolate of *Neisseria gonorrhoeae* from a patient with disseminated gonococcal infection (DGI). Gonococcal outer membranes were purified by differential ultracentrifugation of sheared organisms treated with EDTA. The membranes were solubilized in an endotoxin-disaggregating buffer, and the proteins were separated from the endotoxin by molecular sieve chromatography. Chemical characterization of the endotoxin from the DGI strain revealed the presence of heptose (7.8% of carbohydrate composition) and 2-keto-3-deoxyoctonate (6.1%, wt/wt) in concentrations similar to rough endotoxins of gram-negative enteric bacteria. Dermal Schwartzman reactions were positive for this endotoxin (4 μg) and the corresponding outer membrane (139 μg), but negative for the protein fraction (>500 μg). The patient's whole serum or the IgG fraction, each with complement, reduced the number of the infecting organisms by greater than 1 $\log_{10}$ in a bactericidal assay. Outer membrane and its protein and endotoxin fractions (0.8–500 μg) from the DGI strain were separately preincubated with the patient's convalescent serum and specific inhibition of bactericidal activity occurred with the endotoxin fraction (25 μg) and the outer membrane (100 μg); the protein (500 μg) exhibited no inhibition. Similar results were obtained by inhibiting the bactericidal activity of rabbit antiserum, prepared by intravenous inoculation of an isolate from a patient with pelvic inflammatory disease, with antigen purified from that strain. That this was specific immune inhibition and not anticomplementary activity was shown by the failure of these antigens to inhibit other complement-dependent bactericidal systems.

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INTRODUCTION

The search for immunologically important and distinctive cellular antigens of the species *Neisseria gonorrhoeae* has continued to be elusive. Growth characteristics (1) and auxotrophic markers (2) of certain strains that cause disseminated infection have enabled limited microbiologic differentiation, and clonal typing has shown that T_1 and T_2 colony types are isolated from fresh clinical isolates, indicating that pili are important markers in clinical infection (3). Despite these advances in gonococcal technology, specific antigens that induce protective immunity and provide complete and type-specific classification of *N. gonorrhoeae* still remain unidentified.

The outer membrane of gram-negative bacteria is an immunologically important structure because of its accessibility to host defense mechanisms. *N. gonorrhoeae* outer membranes contain protein, lipopolysaccharide (LPS),[1] and loosely bound lipids (4, 5). Antibodies directed against surface proteins and lipopolysaccharides of certain strains of *N. gonorrhoeae* (6) have been used to demonstrate serologic differences in gonorrhoeae strains (6, 7). The identification of protein

[1] *Abbreviations used in this paper*: DGI, disseminated gonococcal infection; GLC, gas-liquid chromatography; KDO, 2-keto-3-deoxyoctonate; LPS, lipopolysaccharide; PAGE, polyacrylamide-gel electrophoresis; SDS, sodium dodecyl sulfate.

variations in gonococcal outer membrane has provided a serotyping system based on these differences (8). Polysaccharide antigens, possibly originating from the LPS of *N. gonorrhoeae*, may serve as important determinants in still another gonococcal serotyping system (9). Lipopolysaccharides have also served as major serotyping determinants for other bacterial species (10).

To evaluate the role of various gonococcal antigens in natural immunity, we have chosen to use an immunologic assay system that detects functioning antibodies which might have a host-protective effect against the organism, the complement-mediated bactericidal system. Earlier we have described such an assay for the detection of bactericidal antibody in sera obtained from humans with gonococcal infection (11). Here we report a technique for the separation of pure outer membrane from whole organisms that can be fractionated into high molecular weight protein aggregates and LPS (endotoxin), relatively free of protein. This LPS preparation and not the protein fraction was the major antigenic constituent responsible for the development of bactericidal antibody in a rabbit immunized with live organisms intravenously, and in a patient who suffered disseminated infection.

Case history. A 26-yr-old man developed a painful right ankle without swelling 2 wk before seeking medical assistance. During this period his right foot and left knee had become similarly involved; at the time he was first examined, his right wrist, the interphalangeal joint of his left thumb, and his left knee had become warm and painfully swollen with marked limitation of motion. He had no skin lesions. He denied having a urethral discharge, dysuria, or a sore throat. He refused to permit aspiration of the swollen joints; however, a swab of his throat cultured on Thayer-Martin media grew *N. gonorrhoeae*; cultures of blood and urethral swabs failed to grow the organism. Identification of the throat isolate was confirmed by oxidase testing and sugar fermentation patterns. He was treated with high doses of aqueous penicillin, had prompt resolution of pain and swelling in the affected joints, and recovered full range of motion.

METHODS

Gonococcal strains

The two strains of *N. gonorrhoeae* examined included an isolate obtained from the cervix of a patient with pelvic inflammatory disease and the strain isolated from the throat of the patient described above.

Sera

Antisera were prepared in rabbits (New Zealand white) by intravenous inoculation of 10^8 viable colonial types T_3 and T_4 gonococci, three times a week for 2 wk, and a boosting dose in the third week. Sera were obtained before inoculation and 1 wk after the last dose.

Serum specimens were obtained from the patient 18, 23, 37, and 61 days after onset of symptoms. He gave signed permission for the use of his blood.

Media and growth conditions

Gonococcal strains were stored frozen at −70°C in trypticase soy broth:glycerol (4:1, vol/vol). One loopful of thawed organisms (colonial types T_3 and T_4) was transferred to each of several chocolate agar plates and grown overnight in a candle extinction jar (37°C). Each plate was swabbed with a moistened cotton-tipped applicator and inoculated into 40 ml of a proteose peptone broth supplemented with Isovitalex BBL, Div. of Becton, Dickinson & Co., Cockeysville, Md.) (11). The 40-ml aliquots were grown to a concentration of 10^8 organisms/ml with continuous stirring in 250-ml nephelometry flasks (Bellco Glass, Inc., Vineland, N.J.) (4–6 h), and the total volume (40 ml) was then inoculated into approximately 1,600 ml of the modified broth in a 2-liter Erlenmeyer flask. After overnight incubation with 5% CO_2 in air bubbled through the flasks, the organisms were centrifuged at 8,000 *g* (4°C); all preparations were washed three times with 0.15 M NaCl, except in one experiment where the organisms were only washed once. All cultures were checked for contamination at the end of the growth cycle. For some experiments intrinsic radioactive labeling of organisms was accomplished by growing them in the presence of 5 mCi of [^{3}H]sodium acetate and 1 mCi of [^{3}H]leucine (New England Nuclear, Boston, Mass.) per liter of broth.

Purification of the outer membrane complex

Pelleted organisms were suspended at room temperature in a buffer containing 0.05 M Na_2PO_4, 0.15 M NaCl, and 0.01 M EDTA adjusted to pH 7.4 (EDTA buffer). The suspension of organisms was incubated at 60°C for 30 min, subjected to mild shearing by passage through a 25-gauge hypodermic needle attached to a 50-ml syringe (manual pressure), and mixed for 10 s in a Waring Blendor. In one experiment the organisms were incubated for 30 min at room temperature rather than 60°C, but were otherwise treated similarly. The membranes isolated from this experiment were assayed for *d*-LDH activity; therefore heating was undesirable.

Whole organisms were separated from the suspension by centrifugation at 12,000 *g* (4°C) for 20 min. Centrifugation of the supernate at 80,000 *g* for 2 h (4°C) pelleted the outer membrane complex. Both centrifugations were repeated, and the final clear, gel-like pellet was suspended in water and lyophilized. Density measurements of outer membrane material were performed to assess purity. Pellets of outer membrane were suspended in 1.2 ml of a 25% (wt/wt) sucrose solution in the EDTA buffer to a concentration of 5 mg outer membrane material per milliliter, and applied to a discontinuous gradient prepared by layering sequentially 1.2-ml samples of sucrose solutions ranging in concentration from 65 to 25% (wt/wt) in nitrocellulose centrifuge tubes. Samples were centrifuged in a SW-41 rotor (Beckman Instruments, Inc., Fullerton, Calif.) for 18 h at 280,000 *g* to isopycnic conditions. Tube contents were fractionated by puncturing the bottom of the tubes and collecting drops; absorption of uv light at 280 nm was measured for the fractions, and the corresponding sucrose densities were measured on a refractometer (Bausch & Lomb, Inc., Rochester, N.Y.).

Fractionation of the outer membrane

Lyophilized outer membranes were dissolved in 5 ml of an endotoxin-disaggregating buffer containing 0.05 M glycine, 0.001 M EDTA, and 1.5% sodium deoxycholate (12) adjusted to pH 9.0 with NaOH (NaD buffer). This materal was then chromatographed on a 1.6 × 85 cm column of Sephadex G-100 (Pharmacia Fine Chemicals, Uppsala, Sweden) equilibrated in the same buffer, and 2-ml fractions were collected. Absorption of uv light at 280 and 260 nm was measured for the fractions (0–190 ml). The void volume of the column was determined with blue dextran (Pharmacia Fine Chemicals). When required, radioactive counts were measured (Beckman Instruments, Inc., LS-230) in 20-μl samples taken from each fraction and suspended in 10 ml of a scintillation solution consisting of 60% toluene and 40% ethylene glycol monomethyl ether and containing 4 g/liter of Omnifluor (New England Nuclear).

The void volume material (fraction 60–100 ml, Sephadex G-100 column) obtained after elution was pooled and recovered by precipitation with 4 vol of absolute alcohol. This was washed twice with 80% alcohol to rid the antigen of residual NaD buffer and lyophilized. The remaining smaller molecular size material (fraction 100–140 ml, Sephadex G-100 column) was recovered from the column and treated similarly. After precipitation of this second fraction with 80% ethanol, the precipitate was re-extracted with absolute alcohol to remove the alcohol-soluble loosely bound lipid. For studies of reaggregation of lipopolysaccharide, the alcohol-insoluble material was suspended in Tris-HCl buffer (pH 7.6) and applied to a 40 × 0.9-cm column of Sepharose 4B (Pharmacia Fine Chemicals). The void volume of this column was also determined with blue dextran.

Polyacrylamide-gel electrophoresis (PAGE)

Membranes and extracts of membranes were analyzed by sodium dodecyl sulfate (SDS)-PAGE (8). A 2% SDS, 9.8% acrylamide, 0.25% N,N′-methylene bisacrylamide gel was used. 6 × 60-mm gels were prepared in glass tubes. 50-μl samples (50–100μg protein) to be electrophoresed were treated at 100°C for 2 min in a solution of 2% (wt/vol) SDS, 3 mM sodium phosphate, 0.006% bromphenol blue, and 13.4% glycerol. Samples were electrophoresed at 4 mA/gel for 3 h at room temperature. Staining and de-staining procedures have been described earlier (12). Retardation factors (R_f) of bands were calculated from known protein standards run simultaneously.

d-LDH assay

An enzymatic assay for *d*-LDH was performed on outer membrane preparations (5), and specific activity of *d*-LDH was expressed as micromoles of substrate converted per minute per milligram of protein in the preparation.

Chemical analysis

Protein concentration was measured for the two fractions with the Folin phenol reagent (13), using bovine serum albumin as a standard, and 2-keto-3-deoxyoctonate (KDO) concentrations were measured by the thiobarbituric acid reaction (14), modified to compensate for the presence of excess polysaccharide (15). KDO standards (Sigma Chemical Co., St. Louis, Mo.) were run simultaneously, and maximum absorption was determined by scanning at several wavelengths between 500 and 600 nm.

Analysis of carbohydrate composition by gas-liquid chromatography (GLC) was performed by preparing trimethylsilyl derivatives of the second fraction after absolute alcohol extraction (16). Briefly, 1 mg of this dried material was suspended in 2 ml of a 0.5 M methanolic-HCl solution and heated at 80°C overnight. The methanol-HCl solution was extracted three times (22°C) with equal volumes of hexane. The methanol-HCl phase was neutralized to pH 7.0 with NH_4OH and dried on a rotary evaporator (40°C). 2 ml of methanol were added to the dried sample, and the specimen was redried with N_2 (N_2 evaporator, Organomation Associates, Inc., Northborough, Mass.). 0.2 ml of a solvent mixture composed of trimethylchlorosilane, hexamethyldisilazane, and pyridine (1:4:10, vol/vol/vol) was added to the dried sample. The mixture was kept refrigerated (4°C) overnight, then centrifuged at 120 *g* for 10 min. The supernate was recovered and concentrated with the N_2 evaporator; samples were studied on a gas-liquid chromatograph (Packard Instrument Co., Inc., Downers Grove, Ill., model 421). The temperature program was set at 140°C for 3 min, followed by increments of 20°C per minute for 2 min with maintenance at 180°C for an additional 5 min. Flow rate of the sample was 30 ml/min. A 6-ft U-shaped glass tube packed with 3% SE-30 on 80/100 Gas Chrom Q (Supelco, Inc., Bellefonte, Pa.) was employed, and sugars were detected with a flame ionization detector. Cochromatography with known sugar standards previously characterized by mass spectroscopy was performed to identify sugars in the specimen.

Tests for biologic activity

The limulus lysate assay was performed on the two pools and the whole outer membrane by Dr. William R. McCabe (Boston University) (17). *Salmonella typhi* 0901 endotoxin and the NaD buffer were run simultaneously as controls.

Skin sites for testing of the dermal Shwartzman reaction were prepared in groups of five 2- to 3-mo-old New Zealand white rabbits (1.0–1.5 kg) by the intradermal injection of twofold serial dilutions of 0.25-ml samples of the two pools, whole outer membrane, and *S. typhi* 0901 endotoxin. The reactions were provoked 21 h later by the intravenous injection of 0.5 ml of the corresponding samples. Hemmorhage or necrosis of the skin within 6 h after the provocative dose was given was recorded as a positive reaction, and 50% endpoints were calculated (18).

Separation of immunoglobulins

The patient's IgG was separated from whole serum, taken on day 23, by ion-exchange chromatography employing QAE–Sephadex A-50 (19) (Pharmacia Fine Chemicals). The gel was first equilibrated in a 0.048 M ethylene diamine, 0.073 M acetic acid buffer (pH 7.0), then 0.5 ml of serum was mixed with an equal volume of buffer and applied to a 6 × 0.5 cm column of the gel mixture and the effluent pooled. The pool was concentrated to 0.5 ml employing an ultrafiltration cell (PM-30 membrane, Amicon Corp., Lexington, Mass). This concentrate was dialyzed against phosphate-buffered saline, pH 7.2, to a final volume of 0.5 ml and filter sterilized through a 0.22-μm millipore membrane. IgM was separated from the patient's whole serum by gel chromatography on BioGel A-5M (Bio-rad Laboratories, Richmond, Calif.) on a 2.6 × 90-cm column equilibrated in 0.1 M Tris and 0.15 M NaCl (pH 7.5) at 4°C (20). Fractions were assayed for immunoglobulin content using goat antihuman IgG, IgA, and IgM (Cappel Laboratories, Inc., Downingtown, Pa.). The IgM fractions were concentrated to 0.5 ml with an ultrafiltration cell and filter

sterilized. Immunoglobulin concentrations in each pool were determined by radial immunodiffusion (21).

Bactericidal assay

The bactericidal assay employed in this study was a modification of the procedure previously described (11). Reaction mixtures were set up in 12 × 75-mm glass tubes with gauze plugs. Test mixtures contained 0.05 ml of complement, 0.05 ml of dilution of test serum or immunoglobulin pool, and 0.05 ml of broth containing organisms. The rabbit and patient sera were tested against the homologous immunizing (infecting) strain.

Complement. Freshly drawn human blood of an individual deficient in natural antibody to the case isolate was allowed to clot for 15 min. The serum was separated by centrifugation at 3,000 *g* for 10 min at 24°C, and the separated serum was immediately stored at −70°C.

Serum. The pre- and postimmunization rabbit sera and the four patient serum specimens, which had been stored at −70°C, were tested with use of serial dilutions (sterile water). All rabbit or patient sera were tested simultaneously in each run. The separated patient immunoglobulin pools were similarly examined.

Gonococci. Organisms were grown in a broth media (11) to a mid-log phase concentration of 3×10^8 organisms per ml and diluted with this media to obtain an inoculum with a concentration of 3×10^4 organisms per ml. 0.05 ml of this bacterial suspension was inoculated into each reaction mixture.

The reaction mixtures were incubated aerobically at 37°C with continuous shaking in a water bath (Aquaferm, New Brunswick Scientific Co., Inc., New Brunswick, N.J.). Viable Colony counts were performed at 0 and 30 min by plating duplicate 0.025-ml samples of each reaction mixture, and the bactericidal titer of the sera was taken as that dilution of serum that killed at least 50% of organisms when standardized against controls (11). An active complement control and a heat-inactivated (56°C for 30 min) serum control were also tested in each experiment.

Bactericidal inhibition assay

Sera. We have examined the capability of gonococcal cell wall antigens to inhibit the bactericidal capacity of the serum of the rabbit immunized intravenously with live organisms and of the patient with disseminated infection.

Antigens tested for inhibitory capacity. Outer membranes and the two fractions were purified from both the strain used to immunize the rabbit and the strain isolated from the patient with disseminated disease. Antigens were only tested against the homologous serum except when they were examined for anticomplementary activity.

Inhibition methodology. Whole outer membrane and the two fractions were diluted serially in 0.025 ml of water and incubated for 60 min at 37°C with 0.025 ml of the dilution of each serum (rabbit, postimmunization, patient, 37 days after onset of clinical symptoms) that had produced approximately a 1 $\log_{10}$ kill in the bactericidal assay. Mixtures of serum and antigen were thereafter used as a source of antibody in the assay. Additional controls included: (*a*) the test serum plus complement without antigen to ensure adequate killing by the test serum, and (*b*) a companion to each serum-antigen mixture that contained only antigen at the specified concentration plus complement to ensure that the antigens were not by themselves toxic to the organism. Percent bactericidal inhibition was expressed according to the formula:

$$\%\ \text{inhibition} = 1 - \frac{(\%\ \text{killing, antigen system})^*}{(\%\ \text{killing, control system})\ddagger}$$

$$^*1 - \frac{\left(\frac{\overline{\text{CFU}}_{t30}}{\overline{\text{CFU}}_{t0}}\right)_{\text{antigen+serum+C}'}}{\left(\frac{\overline{\text{CFU}}_{t30}}{\text{CFU}_{t0}}\right)_{\text{antigen+C}'}}$$

$$\ddagger 1 - \frac{\left(\frac{\overline{\text{CFU}}_{t30}}{\overline{\text{CFU}}_{t0}}\right)_{\text{serum+C}'}}{\left(\frac{\overline{\text{CFU}}_{t30}}{\overline{\text{CFU}}_{t0}}\right)_{\text{C}'} \cdot \left(\frac{\overline{\text{CFU}}_{t30}}{\overline{\text{CFU}}_{t0}}\right)_{\text{serum}}}.$$

The formula includes standardization for the antigen control and also corrects for the survival of organisms in diluted serum alone.

To ensure that inhibition was specific immune inhibition and not anticomplementary activity, concentrations of LPS, which had inhibited in the homologous patient's sera, were added to two other rabbit serum bactericidal systems, employing another gonococcal strain and a strain of *Hemophilus influenzae* each with their respective immune serums containing bactericidal antibody. In addition, the antigen was added to a complement-dependent group B streptococcal opsonophagocytic system (22) with immune serum.

$C'H_{50}$ levels on test mixtures simulating actual test conditions (23) were performed to check for the presence of adequate complement levels in antigen- and immune complex-containing test mixtures.

Double diffusion in agar

Double diffusion in agar was done by the method of Ouchterlony.

RESULTS

Purification and fractionation of the outer membrane complex. Analysis of fractions obtained by isopycnic centrifugation of the outer membrane complex, derived from the strain isolated from the patient with DGI, by absorption of uv light at 280 nm revealed a single band of turbidity at a gradient density of $\rho^0 = 1.27$ g/cm^3 (Fig. 1). *d*-LDH assay of the unheated outer membrane preparation showed specific activity of less than 0.1 μmol of substrate converted per minute per milligram of protein. Gas-liquid chromatographic analysis showed no muramic acid. SDS-PAGE of an outer membrane preparation extracted from once- and thrice-washed cells of the DGI strain are shown in Fig. 2*A* and *B*. Major bands are seen at molecular sizes of 34,500 and 25,000 in each. No loss of major or minor protein bands was found by triple washing the cells.

UV light absorption at 280 nm of fractions of outer membrane after passage through the Sephadex G-100 column equilibrated in the NaD buffer revealed a single major protein peak at the void volume (Fig. 3). Nucleic acid contribution to the protein peak was estimated at less than 1.5% ($R_{280/260} > 1.25$). Two peaks

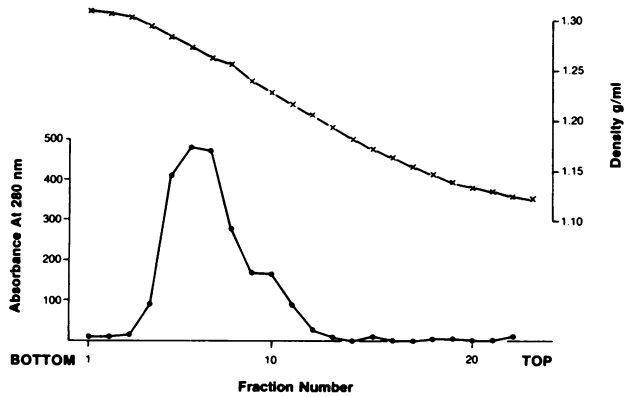

FIGURE 1 Density of outer membrane, DGI strain. Outer membrane preparations were layered on a discontinuous sucrose gradient 62–25% (wt/wt) and ultracentrifuged (280,000 *g* for 18 h) to isopycnic conditions.

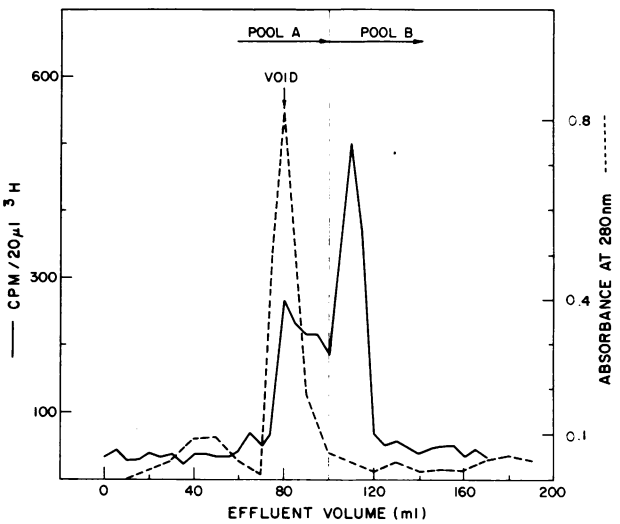

FIGURE 3 Elution profile of DGI strain outer membranes on Sephadex G-100 (Pharmacia) equilibrated in 0.5 M glycine, 0.001 M EDTA, 1.5% sodium deoxycholate (NaD), pH 9.0 buffer. 80,000 *g* pellet of outer membranes was suspended in the NaD buffer, applied to the column and eluted with NaD buffer. UV light (280 nm) absorbance (– – –); radioactivity/20 μl ^{3}H (——). VOID represents void volume measured with blue dextran.

of radioactivity were demonstrated: a minor one corresponded with the protein peak, and the second major peak eluted with the smaller molecular size material (Fig. 3). Two pools, designated pool A and pool B, were created from the eluted fractions, each encompassing one of the radioactive peaks as depicted in Fig. 3.

Suspension of dried pool B material in Tris-HCl resulted in its reaggregation as shown by elution on Sepharose 4B. The pool B front was eluted from this column at the void volume (exclusion size $> 4 \times 10^6$);

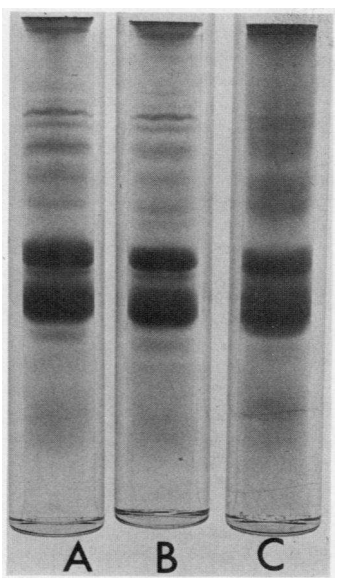

FIGURE 2 Sodium dodecylsulfate–polyacrylamide-gel electrophoresis (SDS-PAGE) of the outer membrane complex extracted from once-washed (*A*) and thrice-washed (*B*) cells and pool A (*C*) (Fig. 3: Sephadex G-100 elution) from the DGI strain.

further purification from small molecular sized contaminants (e.g., traces of NaD buffer constituents) was also accomplished by this procedure.

Chemical and biologic analysis of the fractions. Chemical analysis of the two pools (Table I) revealed protein concentrations (wt/wt) of 63.8% in pool A and 4.0% in pool B. SDS-PAGE of the pool A material (Fig. 2 *C*) showed that both major protein bands found in the intact outer membrane were present and unchanged in pool A. There was, however, some loss of a few minor large molecular weight bands found in the intact outer membrane. No detectable 2-keto-3-deoxyoctonic acid was found in pool A, while pool B contained 6.1% (wt/wt). Absorption maxima were encountered at 549 nm for both pool B and the KDO standard. Sialic acid, which also gives maximum absorption at this wavelength in the thiobarbituric acid

TABLE I
Chemical Analysis of Outer Membrane Antigens (%, wt/wt) by Colorimetric Testing, DGI Strain

	Pool A	Pool B
Protein*	63.8	4.0
2-Keto-3-deoxyoctonate‡	ND§ (<0.1)	6.1

* Reaction with Folin phenol reagent (13).
‡ Thiobarbituric acid reaction (14), modified to compensate for the presence of excess polysaccharide (15).
§ Not detectable.

TABLE II

Carbohydrate Composition (Relative) of Pool B Antigen (%,wt/wt) by Gas-Liquid Chromatography, DGI Strain*

Carbohydrate	%
Glucose	61.8
Galactose	20.5
Heptose	7.8
Glucosamine	~1–5‡

* Antigen washed free of loosely bound lipid with absolute alcohol.

‡ Glucosamine peak overridden by β-glucose; hence the exact amount was indeterminate.

assay (14), was absent in analysis by gas-liquid chromatography (GLC) (16). Relative carbohydrate composition (Table II), determined by GLC for hydrolyzed pool B material, after the removal of alcohol-soluble loosely bound lipid (28%, wt/wt) showed 7.8% heptose, 61.8% glucose, 20.5% galactose, and 1–5% glucosamine (wt/wt), a pattern similar to endotoxins from rough enteric gram-negative bacilli (24).

Gelation of limilus lysate was accomplished by intact outer membrane and each of its constituents, protein (pool A) and LPS (pool B), at similar concentrations (Table III). *S. typhi* 0901 endotoxin was approximately two- to tenfold more potent in this assay than outer membrane or its fractions

Determination of the sensitizing dose that yielded positive dermal Shwartzman reactions in each group of rabbits given outer membrane or its fractions revealed that whole outer membrane was approximately one-third as potent as the control *S. typhi* 0901 endotoxin (Table IV); the LPS fraction, however, was 12.5 times more potent than the *S. typhi* control. The protein fraction did not react in the test at the highest concentration employed (500 μg), indicating that it was essentially void of endotoxic activity.

Bactericidal assays and immunoglobulin separation. No bactericidal antibody was demonstrated in the rabbit serum before immunization. After immunization the bactericidal antibody titer was 1:40. Bactericidal assays performed with the patient's sera obtained on 4 separate days yielded titers of 1:48 on day 18 after the onset of symptoms, 1:384 on days 23 and 37, and 1:96 on day 61 (Table V). Duplicates of reaction mixtures varied by 10–15%, and titers obtained on successive determinations performed on different days did not vary by more than a single twofold dilution.

Concentration of immunoglobulins and bactericidal activity in the patient's whole serum and in each of the two serum pools are shown in Table VI. Separation of the IgG into a relatively pure fraction containing 193 mg/100 ml was accomplished with IgA and IgM concentration in this pool at less than 5 and 4 mg/100 ml, respectively. The IgM pool contained 15.5 mg/100 ml of IgM and no detectable IgA or IgG. Bactericidal activity was demonstrated in the purified IgG immunoglobulin pool, but not in the purified IgM pool. Considering that the whole serum had a titer of 1:384, the IgG pool was at least 64 times more active than the IgM pool in the killing of gonococci.

Inhibition of bactericidal activity. The results of bactericidal inhibition assays performed using twofold serial dilutions of the three separate homologous cell wall antigens mixed with the rabbit's and patient's sera are shown in Fig. 4 ($n = 2$ experiments) and Fig. 5 (n = 3 experiments), ±range. Endotoxin

TABLE III

Limulus Lysate Testing, Outer Membrane Antigens, DGI Strain

Antigen	Concentration*
	ng/ml
Outer membrane	0.07
Protein fraction (pool A)	0.08
Lipopolysaccharide fraction (pool B)	0.16
S. typhi 0901 endotoxin	0.015
NaD buffer	85

* Lowest concentration giving a positive test.

TABLE IV

Sensitizing Doses of Outer Membrane Antigens Employed in the Dermal Shwartzman Reactions, DGI Strain

Antigen	LD_{50}* (sensitizing dose)
	μg
Outer membrane	139
Protein fraction (pool A)	>500
Lipopolysaccharide fraction (pool B)	4
S. typhi 0901 endotoxin	50

* 50% endpoint estimation (18).

TABLE V

Gonococcal Bactericidal Antibody Titers, Patient with Disseminated Infection*

Day after onset of symptoms	Reciprocal titer
19	48
23	384
37	384
61	96

* ≥ 50% killing of the infecting strain (11).

TABLE VI

Immunoglobulin Concentrations (mg/100 ml) and Corresponding Gonococcal Bactericidal Activity (% Killing), Whole Serum (23 Days after Onset of Symptoms) and Serum Pools, Patient with Disseminated Infection

	IgG	IgA	IgM	% Killing
Whole serum diluted 1:6	248	44	13.3	100
IgG pool	193	<5	<4	77
IgM pool	<5	<5	15.5	0

and whole outer membrane inhibited bactericidal activity in these assays. Duplicate culturing of 25-μl aliquots of reaction mixtures at the onset and after 30 min varied by 10–15%. In separate experiments, we have also shown that a complement source containing as few as 15 total hemolytic complement units per milliliter is sufficient to drive the bactericidal reaction. The complement source used in these experiments routinely had activity greater than 100 $C'H_{50}$ units per ml, and this activity was not decreased by the addition of antigens or immune complexes in the concentrations used to demonstrate inhibition (Table VII). Furthermore, gonococcal LPS employed in concentrations used to demonstrate inhibition in the homologous assays did not inhibit complement-dependent killing in the heterologous assays. Of interest, pool A (protein) concentrations of 400 μg in the rabbit system and 250 μg or more in the human system per reaction mixture (0.15 ml) were found to be toxic to organisms and did not permit growth in the antigen controls, even at time zero. A similar but less pronounced effect, also unexplained, was observed in those whole outer membrane reaction mixtures employing concentrations of antigen of 100 μg or more in the rabbit assays.

Double diffusion in agar. Pool A and once- and thrice-washed outer membranes gave a line of identity when tested against rabbit antisera prepared with the

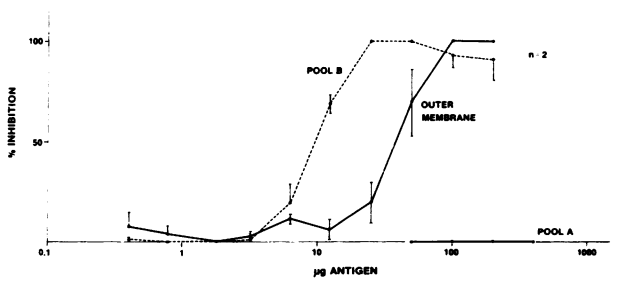

FIGURE 4 Inhibition of bactericidal activity in rabbit antiserum prepared with live intravenous inoculation of the PID strain by the three antigens derived from the homologous strain; n = 2 experiments, ±range.

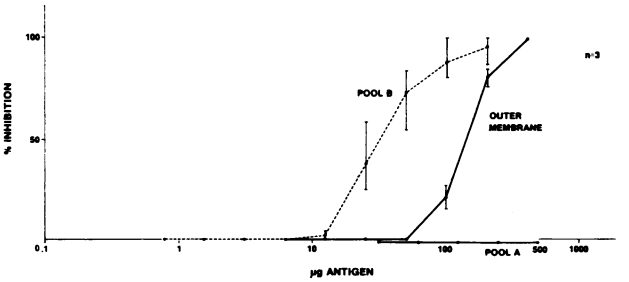

FIGURE 5 Inhibition of bactericidal activity in the DGI patient's serum taken 37 days after onset of clinical symptoms by the three antigens derived from the infecting strain; n = 3 experiments, ±range.

DGI strain, indicating that both the antigenic integrity of the thrice-washed outer membranes and the pool A material has been preserved.

DISCUSSION

Recent investigations have shown renewed interest in the antigenic structure of *N. gonorrhoeae* and in the existence of antibodies directed toward specific outer membrane protein and lipopolysaccharide antigens (8, 25).

Using gentle techniques to separate outer membranes from whole organisms, we have isolated material of a single density that corresponds to purified outer membrane (4, 5). The outer membranes isolated by the techniques described contain negligible quantities of *d*-LDH and no muramic acid, demonstrating that the preparation is not contaminated with significant quantities of either cytoplasmic membrane or peptidoglycan.

We have chemically and biologically characterized endotoxic material from the outer membrane of a strain of *N. gonorrhoeae*. This component has been separated from the protein moiety of outer membrane by column

TABLE VII

$C'H_{50}$ Units, Antigen and Immune Complex Mixtures Employed in Gonococcal Bactericidal Inhibition Experiments, DGI Patient and Infecting Strain

Mixture	v/v/v	$C'H_{50}$
		U/ml
C′, 50 μg endotoxin fraction (pool B), H_2O	1/1/1	129
C′, 50 μg endotoxin fraction (pool B), serum* (1:32)	1/1/1	113
C′, 200 μg OM,‡ H_2O	1/1/1	129
C′, 200 μg OM,‡ serum* (1:32)	1/1/1	129
C′, H_2O	1/2	129

* Serum, 23 days after onset of symptoms.
‡ Outer membrane.

chromatography after its disaggregation into small of monomeric units in NaD buffer (12), and its has also been shown to reaggregate when removed from the buffer.

Chemical characterization of this endotoxin has revealed minimal contamination with protein and relatively high concentrations of KDO and heptose and a glucose-galactose ratio of 3:1. This carboxydrate composition is similar to rough enteric gram-negative bacterial endotoxins that are composed of a low molecular weight core oligosaccharide attached through KDO to the lipid A residue. Heptose is also part of the core oligosaccharide, and measurement of its concentration and that of KDO enables the biochemical classification of these endotoxins as rough or smooth (24). These two sugars have also been detected in the LPS of different strains of gonococci, but in widely variable concentrations (4, 26–28). Measurement of KDO and heptose concentrations in the LPS preparations of a single strain extracted either as T_1 or T_4 type has revealed concentrations of KDO and heptose less than 1.0% in the T_1 endotoxin compared with 7.3 and 3.8%, respectively, in the T_4 endotoxin (28). Based on these findings and those of differing glucose-galactose ratios in these endotoxins, it has been suggested that T_1 organisms contain smooth and T_4 rough endotoxins, by analogy with *Salmonella* mutants (24). However, the concentrations of KDO and heptose seen in our endotoxin preparation of T_3 and T_4 organisms and the serologic activity mounted by the patient suggest that the rough endotoxin might be a major immunologic determinant despite infection with T_1 organisms.

Although in our experiments whole outer membrane and its protein and LPS constituents gelled the limulus lysate, the dermal Shwartzman reaction clearly discriminated between these three fractions, demonstrating the potent endotoxic activity of gonococcal LPS. The results of testing for limulus lysate gelatin are consistent with the high degree of nonspecificity of reactions frequently observed with this highly sensitive assay (29); however, the results of the dermal Shwartzman reactions suggest that separation of endotoxin from the protein was accomplished. Other endotoxin preparations of gonococci also have biologic activity in the dermal Shwartzman reaction (30).

Protection of rabbits from the generalized Schwartzman reaction elicited by endotoxin (31) can be accomplished by both IgG and IgM prepared against several heat-killed gram-negative enteric organisms. Our patient developed IgG bactericidal antibody in response to his infection. Although the specific classes of immunoglobulin responsible for gonococcal bactericidal activity have not been reported, immunization of rabbits with living cholera vibrios yields vibriocidal antibodies that are also predominantly 7S (32). In contrast, administration of killed cholera vaccine results primarily in a 19S antibody response. Therefore, the class of bactericidal or protective antibodies elicited by gram-negative infection or immunization may be heterogeneous, it may vary amongst species, and it may be directed against different antigenic determinants (33).

In the search for important cell wall antigens, we considered it important to define these antigens according to their capacity to induce natural immunity. For example, *Vibrio cholerae* endotoxin prepared by aqueous ether extraction of whole organisms (34) inhibits vibriocidal antibody activity. This antigen has been presumed, therefore, to be important in cholera immunity, though more purified LPS preparations, probably with less protein contamination, has been less effective in this reaction (33). Strains of *N. gonorrhoeae* have been shown to share multiple antigenic determinants with *Neisseria meningitidis* (6). Because of these antigenic similarities and the established role that bactericidal antibody to *N. meningitidis* plays in protecting against meningococcal disease (35), we chose to identify serologically important gonococcal cell wall antigens by their capacity to inhibit acquired bactericidal antibody activity in immune sera. A step-wise reduction of gonococcal bactericidal antibody titers has been demonstrated in rabbit antiserum prepared against viable *N. meningitidis* (administered intravenously) when the antiserum was first absorbed with surface protein from the immunizing meningococcal strains followed by a second absorption with its LPS (6). Inhibition of gonococcal bactericidal activity in rabbit antiserum by the LPS of the immunizing gonococcal strain prepared by phenol water extraction of whole organisms has also been demonstrated (6). Absorption of bactericidal antibodies by erythrocytes coated with gonococcal LPS (7) further supports the role of LPS as a major determinant for bactericidal activity and also suggests that hemagglutinating antibodies that develop in patients with gonorrhea may also have bactericidal activity. Our experiments have shown that major outer membrane proteins remain both chemically and antigenically intact after extraction. Although we cannot exclude the possibility that minor protein determinants, important in bactericidal activity, have been altered by extraction, another recent investigation has also shown no inhibition of homologous serum bactericidal activity by outer membrane proteins extracted from the infecting strain of a patient with gonococcal urethritis. However, these investigators have also shown that these proteins are capable of inhibiting bactericidal activity in heterologous immune serum (36).

Several studies have shown that strains of *N. gonorrhoeae* vary in their susceptibility to serum bac-

tericidal activity (6, 37–39). It is controversial whether variation in colonial morphology (T type) influences this susceptibility (6, 39). Although no specific protective role of gonococcal bactericidal antibody has yet been identified, our own studies have shown that women who are either long-term asymptomatic carriers (>1 mo) or who have clinically severe pelvic inflammatory disease develop bactericidal antibodies against their infecting strains (11). On the other hand, patients with DGI do not predictably develop bactericidal antibodies in response to their infection. In studies performed 40 yr ago, examination of whole defibrinated blood from patients with gonococcal arthritis did not uniformly reveal bactericidal activity (40). Recently, two reports have indicated low rates (two of six) and (two of nine) (37, 38) of measurable bactericidal antibodies against homologous infecting strains in patients with DGI. However, some patients with DGI for example, our own reported here, the 4 patients (of 15 studied) cited above (37, 38), 1 of whom was deficient in the eighth component of complement (41), and a number of patients in the report of 40 yr ago (40) had bactericidal activity in their sera. The apparent inconstancy of bactericidal antibody development in patients with disseminated infection (37, 38, 40) and the report that strains isolated from patients with disseminated infection are resistant to the bactericidal action of normal human serum (37) suggest that heterogeneity of antigenic structure in strains causing disseminated infection is the rule. That this heterogeneity and the corresponding sensitivity to serum may be related to the structure and immunogenicity of the gonococcal endotoxin, as it is with endotoxins of gram-negative enteric organisms (42, 43), is suggested by the development of bactericidal antibodies in only some patients with disseminated infection.

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