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Peter A. Rice, Dennis L. Kasper

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IgG immunoconcentrated against outer membrane protein (OMP) derived from ser^r gonococci showed 40-fold increased blocking activity over normal IgG (wt/wt) and lacked antibody activity directed against gonococcal lipopolysaccharide by ELISA. Using direct immunoabsorption of IgG with purified gonococcal OMP; ser^r-OMP was found sixfold more effective than ser^s-OMP in neutralizing the blocking of immune serum killing of ser^r gonococci, and 10-fold more effective in systems that used excess blocking IgG, NHS, and ser^s gonococci. Blocking IgG preabsorbed with whole ser^r gonococci lost 75% […]

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INTRODUCTION

Resistance to killing by normal human serum (NHS)[1] and complement is exhibited by most strains of *Neisseria gonorrhoeae* that cause disseminated infection, (DGI) (1). Although gonococcal lipopolysaccharides are the major target of bactericidal antibodies in human sera (2, 3), unique outer membrane protein antigens have been identified in certain gonococcal strains that resist killing by human serum (4); therefore susceptibility of individual gonococci to human serum may represent a varied and complex interaction between several surface antigens of the gonococcus and the host. Strains that resist killing by normal human serum (ser^r strains), however, often are killed by convalescent human DGI serum (1, 5) and by normal rabbit serum (6) in complement-dependent reactions, indicating that these ser^r strains indeed are susceptible to lysis by bactericidal antibody and complement. Complement-dependent killing of human ser^r gonococci by normal rabbit serum, however, can be blocked by normal human IgG (6). This may indicate that naturally occuring antibodies bind to the surface of gonococci and interfere with the lytic attack by bactericidal antibody and complement. The studies reported here were designed to investigate the blocking action of normal human IgG in human gonococcal bactericidal systems in order to characterize serum resistance of gonococci. We also examined the relative effect of blocking upon the killing of human serum-resistant

[1] *Abbreviations used in this paper:* BSA, bovine serum albumin; C', complement; DGI, disseminated infection; KDO, 3-deoxy-*manno* octulosonic acid; LISA, enzyme-linked immunosorbent assay; LPS, lipopolysaccharide; NHS, normal human serum; OMP, outer membrane protein; PAGE, polyacrylamide gel electrophoresis; SDS, sodium dodecyl sulfate; ser^r, serum-resistant; ser^s, serum-sensitive.

ANTIGEN PREPARATION

N. gonorrhoeae (whole cells)

EDTA buffer (pH 7.4)
shear
ultracentrifugation

Outer membranes
NaD buffer (pH 9.0)
gel filtration
dialysis (pH 9.0)

Outer membrane proteins(OMP)   LPS

dialysis (pH 9.0)
10% EtOH

dialysis (pH 9.0)   glycine buffer (pH 9.0)

OMP   OMP
(lyophilized)   (solution)

ANTIBODY PREPARATION

Immunoabsorption   Normal human serum   Immunoabsorption
(solid phase, ser'OMP)   (soluble)

NH₂C₂H₄NH₂+CH₃COOH   TRIS buffer (pH 7.5)
buffer (pH 7.0)   gel filtration
Ion exch. chrom.   dialysis (0.15 M NaCl)
dialysis (0.15 M NaCl)
                                              IgM

IgG

Na Acetate (pH 4.3)
pepsin digestion
TRIS buffer (pH 7.4)
gel filtration
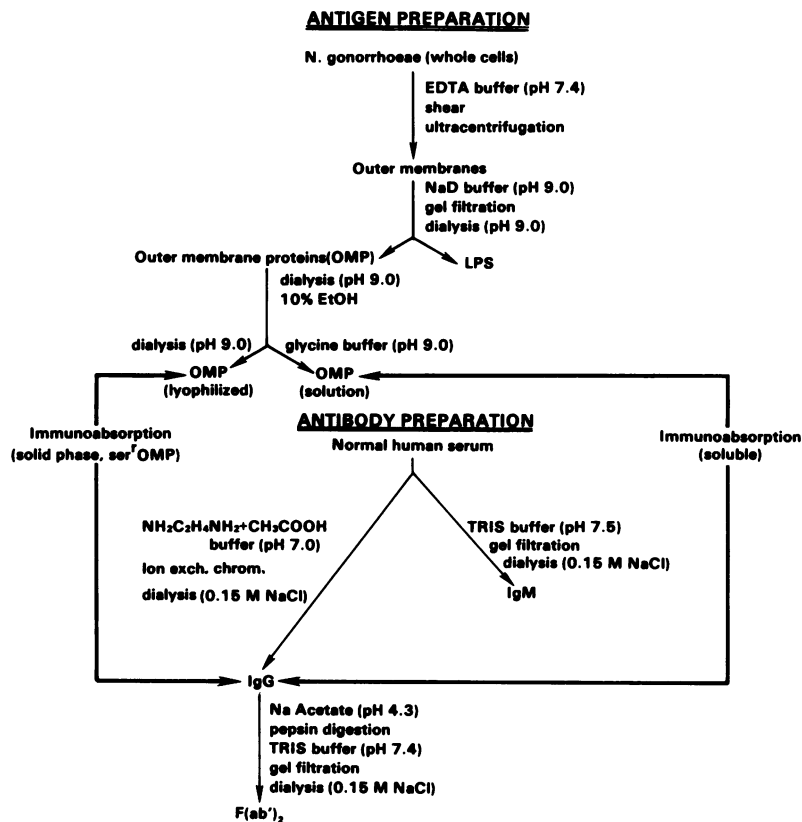dialysis (0.15 M NaCl)

F(ab')₂

FIGURE 1   Flow diagram representing schematically the preparation of gonococcal outer membrane antigens, human immunoglobulins and their derivatives, and immunoabsorption of immunoglobulins (antibodies) with outer membrane proteins.

and serum-sensitive strains and the corresponding antigens from these strains that were the binding sites of blocking antibody.

METHODS

Strains and serums

Isolates of *N. gonorrhoeae* were identified according to standard methods (7) and were stored frozen at −70°C in trypticase soy broth: glycerol (4:1, vol/vol). We used two strains: one was a serum-sensitive (ser^s) strain that was killed by the serum of each of 10 volunteers in dilutions of >1:100 when supplemented with an exogenous source of complement from a patient with hypogammaglobulinemia; the other was a serum-resistant (ser^r) strain isolated from the blood of a patient with DGI that resisted killing by the same 10 fresh normal human serum at a 2:3 dilution.

Normal human serum was the source of killing activity in experiments that used the sensitive strain, and convalescent serum from the DGI patient infected with the ser^r strain was the source of bactericidal activity directed against the otherwise resistant strain.

The methods of antigen and antibody preparation to be described are represented schematically in the flow diagram (Fig. 1).

Media and growth conditions for preparation of antigen

Transparent, nonpiliated phenotypes of gonococci were grown in mass culture in petri dishes containing a clear media of trypticase and thiotone peptones (BBL, Div. Becton, Dickinson & Co., Cockeysville, MD), plus salts and soluble starch, prepared according to the methods of James and Swanson (8). A solution of a supplement (1%, vol/vol) identical to Isovitalex (BBL) was added to the autoclaved media after it had cooled. One loopful of thawed organisms was transferred to each of 20 plates that were then grown for 16 h in a candle extinction jar at 37°C. Organisms from each plate were used to heavily inoculate 10 additional plates using cotton-tipped applicators. The 200 plates were grown for 16 h and the organisms were harvested with rubber spatulas, washed three times with 0.15 M NaCl, and checked for contamination. For radiolabeling experiments, organisms were grown in a liquid media similar to the solid media described, lacking only the agar and containing [^{3}H] sodium acetate, according to methods we have described (3).

Preparation of outer membrane complex

Pelleted organisms were suspended at room temperature in a buffer containing 0.05 M Na₂PO₄, 0.15 M NaCl, and

0.01 M EDTA adjusted to pH 7.4 (EDTA buffer). The suspension of organisms was 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 Blender (Waring Products Div. Dynamics Corp. of America, New Hartford, CT).

Whole organisms were separated from the suspension by centrifugation at 12,000 g (4°C) for 20 min. Centrifugation of the supernatant at 80,000 g for 2 h (4°C) pelleted the outer membrane complex. Both centrifugations were repeated, and the final pellet was suspended in water and lyophilized. We have shown previously that such preparations yield outer membranes judged pure by their uniform density, ρ^0 = 1.27 g/cm^3 and absence of d lactate dehydrogenase (LDH) activity (3).

Separation and characterization of outer membrane antigens

Outer membranes were dissolved in a buffer containing 0.05 M glycine, 0.001 M EDTA, and 1.5% sodium deoxycholate adjusted to pH 9.0 with NaOH (NaD buffer), which has been shown to disaggregate gonococcal endotoxin (3). Outer membrane suspensions were solubilized in this buffer by alkalinization to pH 11.0 briefly; the clear suspensions were then promptly chromatographed on a 2.6 × 35-cm column of Sephadex G-100 (Pharmacia Fine Chemicals, Uppsala, Sweden) equilibrated in the NaD buffer, and 4-ml fractions were collected.

Fractions that showed absorption of ultraviolet light at 280 nm were pooled and dialyzed exhaustively against dilute NaOH (pH 9.0) at 4°C to free the preparation from NaD. Further purification was accomplished by the addition of ethanol (10%, vol/vol) to displace deoxycholate bound to protein; this was followed by terminal dialysis against 0.025 M glycine in 0.15 M NaCl adjusted to pH 9.0 with NaOH (glycine buffer). Protein antigens were concentrated, using pressure filtration (Amicon Corp., Scientific Sys. Div., Lexington, MA), and these solutions were used as stocks for subsequent experiments. Protein concentration was measured with Folin phenol reagent, using bovine serum albumin (BSA) as a standard (9). Peptide patterns of outer membranes and proteins extracted from outer membranes were analyzed by sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis (PAGE) (10, 11). 12.5% acrylamide and 0.34% N,N'-methylene bisacrylamide gel was prepared in 0.37 M Tris buffer and 0.1% SDS (pH 8.8), poured in a glass mold 11.0 cm × 13.0 cm × 0.15 mm, and allowed to harden overnight. A comb with 0.8-cm teeth for slots was inserted into an overlying stacker gel that contained 3.9% acrylamide and 0.10% bisacrylamide in 0.14 M Tris buffer and 0.1% SDS (pH 6.8). Samples for electrophoresis were boiled (100°C) for 10 min in a solution of 4.5% SDS in 0.19 M Tris–HCl, and 15% glycerol (pH 6.8). 25 μl of this solution (20–40 μg protein) was applied to the gel slots. 25 μl of a similar solution containing 0.25% bromophenol blue was applied to an empty slot as a tracker dye. After electrophoresis at 150 volts (30–60 mA) for 3.5 h, the slab gel was immersed overnight in a staining solution of 0.05% Coomassie-Brilliant Blue and 0.05% cupric sulfate dissolved in ethanol-acetic acid-water (2:1:5, vol/vol/vol). The gel was immersed subsequently in a decolorizing solution of ethanol-acetic acid water (1:1:8, vol/vol/vol) until the gel background was clear. Retardation factors (R_f) of bands were calculated from known protein standards (Bio-Rad Laboratories, Richmond, CA) run simultaneously. Molecular weights of proteins were estimated using phosphorylase B (94,000), BSA (68,000), ovalbumin (45,000), carbonic anhydrase (30,000), and soybean trypsin inhibitor (21,000) as markers on the same gel.

To assess the relative solubility of outer membrane proteins in glycine buffer, protein antigens were ultracentrifuged at 50,000 g for 30 min and the supernatants were checked for decrease in protein concentrations (9) before their use in blocking antibody-binding experiments.

Lipopolysaccharide (LPS) was collected from later fractions (250–350 ml) of the Sephadex G-100 column (3), recovered, and purified from NaD similarly to outer membrane protein. The first dialysis, however, was carried out using mol wt 8,000 cutoff tubing (Spectrapor, Fisher Scientific Co., Pittsburgh, PA) to retain low molecular weight disaggregated LPS. A concentration of 3-deoxy-manno octulosonic acid (KDO), a constituent sugar of gonococcal LPS (5), was determined for outer membrane antigens by the thiobarbituric acid reaction, modified to compensate for the presence of excess polysaccharide (12, 13). 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.

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 outer membrane antigens. The reactions were provoked 21 h later by the intravenous injection of 0.5 ml of the corresponding samples. Hemorrhage 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 (14).

Immunoglobulin separation

IgM was separated by gel filtration (15) from serum of a volunteer that had been shown to kill the ser^s strain. 9 ml of the serum was applied to a 1.6 × 85-cm column containing Bio Gel A-5M (Bio-Rad Laboratories) equilibrated in 0.1 M Tris and 0.15 M NaCl buffer (pH 7.5) at 4°C. Fractions were assayed for immunoglobulin content by rate nephelometry (Beckman Instruments, Inc., Fullerton, CA) (16). The effluent containing IgM was pooled, concentrated, and dialyzed against 0.5 M saline. Final adjustment of volume was performed (∼4 ml) to approximate the concentrations of IgM in whole serum and sterilized by filtration. IgG, IgA, and IgM content of the concentrate were measured by rate nephelometry (Beckman Instruments, Inc.) with antisera directed against the three immunoglobulins (16).

IgG was separated by anion exchange chromatography (17) from the same serum after it was determined that the whole serum blocked killing of the ser^s strain by the convalescent serum obtained from the DGI patient (see Blocking assay). QAE-Sephadex A-50 gel was equilibrated in a 0.048-M ethylene diamine, 0.073 M acetic acid buffer (pH 7.0), then 5 ml of the serum was mixed with an equal amount of buffer and applied to a 13 × 2.6-cm column of the gel mixture. Fractions were assayed for immunoglobulin content by rate nephelometry using antisera directed against IgG (Beckman Instruments, Inc.) (16). The effluent containing IgG was pooled, concentrated, and dialyzed against 0.15 M saline. The volume (∼3 ml) was adjusted to approximate the concentration of IgG in whole serum and sterilized by filtration.

Solid phase immunoadsorption

2 g of cyanogen bromide-activated Sepharose 4B gel (Pharmacia Fine Chemicals) were swollen and washed with 0.001 M HCl. Lyophilized outer membrane protein (∼10

mg) was suspended in 10 ml of 0.1 M $NaHCO_3$ buffer containing 0.5 M NaCl (pH 8.3) and gently mixed with the swollen gel mixture for 2 h at room temperature. After washing the gel with the $NaHCO_3$ buffer, remaining active groups were blocked by reacting the gel with 1.0 M ethanolamine (pH 9.0) for 2 h at room temperature. The gel was then washed with 0.1 M acetate buffer in 1.0 M NaCl (pH 4.0), followed by 0.1 M borate buffer in 1.0 M NaCl, (pH 8.0). The gel was placed into a 0.9 × 15-cm column, equilibrated with 0.2 M borate buffer in 0.15 M NaCl, pH 9.0, (borate buffer), and washed free of protein (18). Mock elution was performed by passing 20 ml of 2.0 M potassium iodide in borate buffer (KI buffer) through protein columns (19). Fractions were collected and monitored for protein antigen leakage (9–11), but none was detected.

1 ml of purified IgG (450 mg/100 ml) was dialyzed against borate buffer and applied to the column. Fractions were collected until IgG was no longer detectable by rate nephelometry (16). An additional 20-ml of buffer was collected and handled exactly as the subsequently eluted IgG, as a buffer control. 20 ml of KI buffer were applied to the column to elute the immunoadsorbed IgG. 20 ml of eluant were collected, dialyzed against 0.15 M saline, concentrated to 1 ml, and filter sterilized before its use in subsequent blocking experiments. IgG concentration was measured by rate nephelometry (16). IgG that had been immunoconcentrated against ser^r outer membrane protein was assessed for relative purity by performing rebinding experiments. IgG was directly absorbed with an excess of ser^r outer membrane protein (8 mg protein/mg IgG) in PBS (pH 7.4) and incubated for 24 h at 4°C. Immune complexes were centrifuged (8,000 g), and the remaining IgG in the supernatant was measured (16).

Preparation of F(ab')₂

Purified IgG prepared from normal human serum with blocking activity was dialyzed against 0.1 M sodium acetate, (pH 4.3), and then incubated with pepsin (Sigma Chemical Co.) at an enzyme/substrate ratio of 1:100 for 7 h at 37°C; the reaction was stopped by increasing the pH to 9.0 with a few drops of 2.0 M Tris–HCl in 1.0 M NaOH, (20). The digested IgG preparation was dialyzed overnight against 0.05 M Tris–HCl in 0.15 M NaCl, (pH 7.4). F(ab')₂ fragments were separated from the other constituents of the digest by gel filtration over a 2.8 × 85-cm column of Sephacryl S-200 Superfine (Pharmacia Fine Chemicals), equilibrated at 4°C with 0.05 M Tris–HCl-0.15 M NaCl (pH 7.4) containing 0.002% Hibitane (Stuart Pharmaceuticals, Wilmington, DE) as a preservative. The separation resolved the digest into three major peaks (21). The second peak (mol wt 90,000) was rechromatographed over Sephacryl S-200, Superfine, and a single peak recovered. This was dialyzed against 0.1 M phosphate buffer (pH 7.0) and further purified from small amounts of residual IgG by passage over protein A-Sepharose CL-4B (Pharmacia Fine Chemicals) equilibrated in the same buffer. Following dialysis against 0.15 M NaCl, F(ab')₂ was concentrated until the light chain concentration measured by anti-λ activity (Calbiochem-Behring, Corp., American Hoechst Corp., San Diego, CA) in agar gel diffusion experiments was equivalent to that of the original IgG. Likewise, Fc activity in the two preparations was assessed using anti-Fc antiserum (Calbiochem-Behring Corp.) and F(ab')₂ preparations were found to contain ≤25% the Fc activity of original IgG. These preparations were then sterilized by filtration.

Immunoglobulin preparations and their derivatives were stored in 0.15 M phosphate and 1.0 M NaCl (pH 7.4) to minimize the formation of aggregates (22), and dialyzed against 0.15 M saline before their use.

ELISA

Direct binding activity of the IgG preparations for outer membrane antigens was determined by ELISA (23). In experiments that used outer membrane proteins, 12 × 75-mm polystyrene tubes (Falcon Labware, Div. of Becton Dickinson & Co., Oxnard, CA) were coated at 37°C for 3 h with $NaCO_3$ buffer (pH 9.6) solution of outer membrane protein (5.0 μg/ml) derived from the two strains (24). The 5.0-μg/ml outer membrane protein coat concentration was predetermined to give optimal readings (23).

Experiments were performed in triplicate using 1.0-ml vol. After the coating the tubes were washed with 0.05% Tween-20 in phosphate-buffered saline (pH 7.4) and then reacted with normal IgG diluted in PBS-0.05%-Tween-20 for 1 h at 37°C. Antihuman IgG alkaline phosphatase conjugate (γ-chain specific 1:1,000) (Sigma Chemical Co.) was added after another wash and allowed to react for 1 h at 37°C. After the final wash, the substrate, disodium-p-nitrophenyl phosphate, in 0.09% diethanolamine buffer (pH 9.8) was added, and the reaction was allowed to proceed for 1 h at room temperature, and 0.2 ml of 3.0 N NaOH was added to halt the reaction; readings were performed immediately at 405 nm on a spectrophotometer (Gilford 250, Gilford Instrument Laboratories Inc., Oberlin, OH). Controls included tubes coated with outer membrane protein, and reacted with conjugate (antigen control), mock coated wells reacted with IgG and conjugate (antisera control), and mock-coated wells reacted with conjugate alone (conjugate control). Optical density of control readings were ≤0.1. Readings ≥0.2 were considered positive. Experiments that used soluble electrodialyzed triethylamine LPS salts (25), as target antigens, were performed using a barbital-acetate coating buffer (pH 4.7) (26), and optimal coating concentrations of the ser^r LPS salt at 7.5 μg/ml (27).

Bactericidal assay

The bactericidal assay used in this study was a modification of the described procedure (28). Reaction mixtures were performed in 12 × 75-mm capped tubes. Test mixtures contained 0.05 ml of complement, 0.075 ml of dilution of test serum or immunoglobulin fraction, and 0.025 ml of gonococci.

REAGENTS

Complement. Freshly drawn serum of an individual with acquired hypogammaglobulinemia containing IgG <20 mg/100 ml, IgA < 3.8 mg/100 ml, and IgM < 4.9 mg/100 ml, was allowed to clot for 15 min. The serum was separated by centrifugation at 3,000 g for 10 min at 24°C, and then immediately stored at −70°C. Total hemolytic complement activity of the serum was 125 C'H₅₀ U/ml (N, 111±15).

Serum. DGI patient serum and a normal serum, which had been stored at −70°C, were used in a bactericidal reactions with the ser^r and ser^s strains, respectively. Purified human serum immunoglobulin fractions were similarly examined.

Gonococci. Nonpiliated transparent colony types iden-

tified on clear agar (8) were grown in a proteose peptone liquid broth (28) to mid-log phase concentration of ~6 × 10⁸ organisms/ml and diluted 10^{-4} in broth. 0.025 ml of the diluted bacterial suspension were inoculated into each reaction mixture. Comparisons of chamber counts of ser^s and ser^r gonococci to numbers of colony-forming units revealed that >95% of the bacteria present were viable.

Method. The reaction mixtures were incubated at 37°C with continuous shaking in a water bath (New Brunswick Scientific Co., Inc., Edison, NJ). Viable colony counts were performed at 0 and 30 min by plating duplicate 0.025-ml samples of each reaction mixture. Killing was accorded to those reactions where > a 50% reduction in colony-forming units was observed after the 30-min incubation period, compared with the numbers of organisms present in the same tube after the immediate plating (t$_0$). Active complement controls and heat-inactivated (56°C for 30 min) serum controls of immunoglobulin controls were included in each experiment and maintained ≥100% viability during the 30-min incubation period.

Blocking assay

Reagents. The following reagents were tested for their ability to block killing of the ser^r DGI strain by homologous convalescent DGI serum: (*a*) the 10-volunteer serums, tested individually, (*b*) IgG derived from normal human serum, (*c*) normal IgG with specific anti-ser^r outer membrane protein activity prepared by immunoadsorption, and (*d*) F(ab')$_2$ fragments prepared from normal IgG with blocking activity. Also tested was the ability of IgG from normal human serum to block killing of the ser^s strain by normal human serum.

Method. Heat-inactivated (56°, 30 min) normal serum, IgG preparations, or F(ab')$_2$ fragments were diluted serially in 0.05 ml with 0.15 M saline and incubated for 15 min with diluted ser^r organisms (0.025 ml) at 37°C. Thereafter, 0.025 ml of a dilution of convalescent DGI serum that had produced ~ a 1-log$_{10}$ kill in the bactericidal assay, was added to the reaction mixture together with 0.05 ml of complement. Controls included the DGI serum plus complement to ensure an adequate killing system and other controls routinely used in the bactericidal assays. To ensure that inhibition was specific immune inhibition and not anticomplementary activity, normal serum, IgG, and F(ab')$_2$ concentrations shown to inhibit killing of ser^r gonococci were added to a similarly designed complement-dependent bactericidal test that used a strain of nontypable *Haemophilus influenzae* and human serum that had been titered to produce a 1-log$_{10}$ kill.

We also used IgG derived from normal serum to attempt blocking of the killing reaction of diluted normal serum plus complement against the ser^s strain. IgG used in these experiments were purified from the same normal serum used as the source of killing antibody.

Blocking inhibition assay

Reagents. Varying concentrations of outer membrane protein and outer membrane-derived lipopolysaccharides from the two strains were used to attempt dose-related inhibition of blocking activity directed against each of the two strains.

Method. Outer membrane antigen preparations were serially diluted in 0.05 ml of glycine buffer and incubated (37°C, 15 min) with concentrations of IgG that were shown capable of complete blocking in the bactericidal assays. Thereafter, these IgG-antigen mixtures were used as the

source of blocking activity, and they were incubated with organisms as described in the blocking assays. Dilutions of killing serum and complement were added to complete the reaction mixtures. Because regenerated killing was the end point in these assays, antigen controls that contained the highest concentrations of antigen used in the assays added to complement and blocker IgG were included in each experiment to ensure adequate survival of organisms in the absence of killing antibody. Absorption of IgG blocking activity also was attempted using ser^r and ser^s gonococci at IgG/organism ratios that blocked immune lysis of the ser^r strain. Absorption conditions duplicated those used to fix IgG to gonococci in the blocking assays. Unabsorbed IgG was recovered in the supernatants of centrifuged absorption mixtures and used in blocking assays.

RESULTS

Preparation and characterization of outer membrane antigens. Ultraviolet light absorption at 280 nm of fractions of outer membrane prepared from the ser^s and ser^r strain after passage through the Sephadex G-100 column equilibrated in the NaD buffer revealed single major protein peaks at the void volume (Fig. 2). Nucleic acid contribution to the protein peak was estimated at <1.5% ($R_{280/260}$ > 1.25). Protein antigens freed from NaD and suspended in glycine buffer were judged to be relatively dispersed by their ability to remain in solution after ultracentrifugation.

Chemical analysis of protein preparations derived from the ser^s and ser^r outer membranes revealed protein concentrations of 92 and 89%, respectively. SDS-PAGE analysis performed on the dialyzed single protein peaks from outer membranes of each of the strains recovered from Sephadex G-100 columns showed that major peptide bands present in outer membrane preparations were preserved throughout the purification procedure, although there was some loss of a few minor bands (Fig. 3). The largest outer membrane protein bands resolved at molecular size 36,500 for ser^r outer membrane and its derived outer membrane protein antigen, and slightly lower for ser^s outer membrane antigens. Other major membrane protein bands varying in size from 27,000 to 32,000 were seen in the profiles of antigens from both strains. Outer membrane proteins showed no detectable KDO or Shwartzman activity when 750 µg of each antigen was tested, indicating <0.9 and 0.8% LPS contamination of ser^s and ser^r proteins, respectively, by chemical assay, and <0.5 and 1.6% contamination by biologic assay.

LPS antigens derived from ser^s and ser^r outer membranes from later fractions of the Sephadex column showed no protein detectable (<1.0%, wt/wt) in either preparation. KDO concentrations determined for ser^s and ser^r LPS were 6.5 and 8%, respectively, and the sensitizing doses (ID$_{50}$) of LPS in the dermal Shwartzman reactions were 4 and 12 µg.

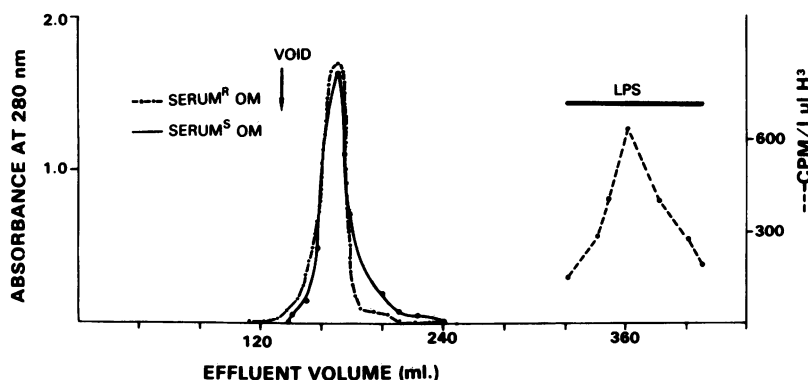*Bactericidal assays.* The bactericidal titer of the

FIGURE 2 Elution profile of ser^s and ser^r gonococcal outer membranes on a 2.6 × 85-cm column of Sephadex G-100 equilibrated in 0.5 M glycine, 0.001 M EDTA, 1.5% sodium deoxycholate (NaD), pH 9.0 buffer. Membranes were brought up in the buffer, solubilized by increasing the pH to 11.0, neutralized to pH 9.0, applied to the column, and eluted with NaD buffer, (ultraviolet light 280 nm absorbance ser^s———and ser^r- · - · -membranes). Radioactivity/1 μl [^{3}H] for gonococci grown in the presence of ^{3}H sodium acetate, (-----). ^{3}H labeling of first peak not shown, 2nd peak confirmed LPS by presence of KDO. LPS peak the same for both antigens.

normal serum was 1:384 against the ser^s strain. No bactericidal activity against the ser^r strain by normal serum was noted at the lowest dilution tested (2:3). Acute serum from the DGI patient did not kill the ser^r strain. Convalescent DGI serum had a titer of 1:12. 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.

The results of bactericidal assays using twofold serial dilutions of purified IgM and normal serum (whole IgM) are shown in Fig. 4. No contaminating IgG (<1.5 mg/100 ml) or IgA (<3.8 mg/100 ml) was detected in the IgM preparation containing 100 mg IgM/100 ml. These experiments demonstrated that a fivefold
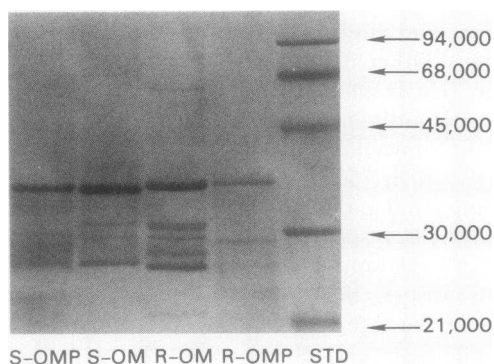

S-OMP S-OM R-OM R-OMP STD

FIGURE 3 SDS-PAGE of the outer membrane complex extracted from serum-sensitive (S-OM) and serum-resistant (R-OM) gonococci. First peaks (Fig. 2, Sephadex G-100 elutions) of ser^s (S-OMP) and ser^r (R-OMP) outer membrane are shown on either side of OM.

greater concentration was required for killing of the ser^s strain by whole serum IgM, compared with purified IgM in the complement-dependent reaction and suggested that blocking activity might be present in whole normal serum, which interferred with IgM-mediated killing of the ser^s strain. Purified IgM plus complement, however, did not kill the ser^r strain.

Blocking assays. 7 of the 10 volunteer serums completely blocked killing of the ser^r strain by immune serum and complement. Two serums partially blocked and one had no effect. IgG prepared from a blocking volunteer serum was examined for its ability to block killing of gonococci by bactericidal antibody and complement. No contaminating IgM (<4.9 mg/100 ml) or IgA (<3.8 mg/100 ml) was detected in IgG preparation containing 900 mg IgG/100 ml. These purified IgG fractions (plus complement) used in concentrations equaling those present in normal sera were shown incapable of killing either strain in the bactericidal assay.

The results of the experiments demonstrating the blocking effects of purified IgG upon bactericidal activity are demonstrated in Fig. 5. These experiments used the preincubation of serial dilutions of the IgG preparation with ser^r organisms before the addition of serum from the patient with DGI, and complement. Also shown are the concentrations of IgG that inhibited or blocked killing of the ser^s strain by the normal human serum. The ratios represent an excess of normal IgG necessary to inhibit or block killing of the strains by each of the killing sera. A comparison of the IgG excess at the extrapolated 50% inhibition points revealed a 16-fold lower concentration of IgG needed to block killing of the ser^r strain by immune serum, compared with the concentration needed to block kill-

ing of the sensitive strain by normal serum. Serial dilutions of normal serum used as a source of blocking antibody produced results similar to the purified IgG upon comparing the IgG concentrations in each of the two reagents needed to block killing of the ser^r strain by DGI serum. Blocking activity of F(ab')$_2$ preparation with light chain (γ) concentration equal to that of IgG was equivalent to IgG. No inhibition was demonstrated in bactericidal assays that used nontypable *Haemophilus influenzae* and human serum when IgG or F(ab')$_2$ concentrations shown to inhibit killing of ser^r gonococci were preincubated with nontypable *Haemophilus influenzae*, thereby ensuring the presence of bacteriolytic complement in the experiments with *N. gonorrhoeae*.

Blocking inhibition. To examine the role of gonococcal antigens in the recognition and binding of IgG-blocking antibodies, we used both outer membrane proteins and lipopolysaccharides as absorbants for blocking IgG; however, we reasoned that outer membrane proteins might be the primary target of the blocking antibodies because lipopolysaccharide antigens have been shown to be the major target of gonococcal bactericidal antibodies (2, 3). In preliminary experiments, we measured IgG antibody concentrations directed against both ser^s and ser^r outer membrane proteins in normal serum and purified normal IgG, by ELISA. The titer against ser^r OMP was 1:500, fivefold greater than the 1:100 titer against ser^s outer membrane protein. We also tested blocking activity of purified IgG that had been absorbed to and subsequently eluted from ser^r outer membrane protein that had been affixed to the immunoabsorbant column. The adsorbed IgG preparation with a measured IgG concentration of 11 mg/100 ml showed a 40-fold in-
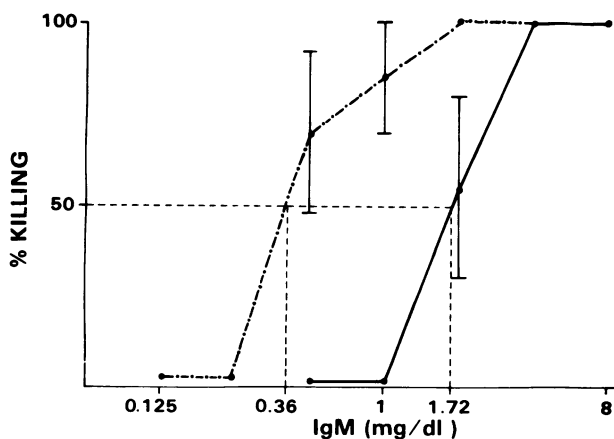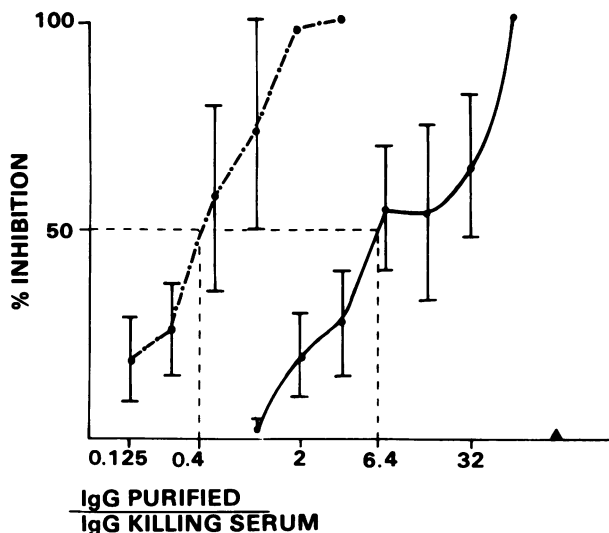

FIGURE 5 Ratio of concentration of purified IgG to IgG in whole killing sera: experiments used increasing concentrations of purified IgG to inhibit killing of the otherwise ser^r strain by convalescent serum from the DGI patient (–•–•–), and increasing concentrations of purified IgG to inhibit killing of the ser^s strain by normal human serum (———). Purified IgG heated to 65°C (▲); n = 2±range.

creased blocking ability per milligram of IgG compared with unabsorbed IgG in assays using the ser^r DGI strain and convalescent bactericidal antibody. Buffer controls recovered from the immunoabsorption columns showed no blocking activity. Relative purity of adsorbed IgG was assessed by direct reabsorption of IgG with ser^r outer membrane protein. 8% of immunoadsorbed IgG rebound to outer membrane protein.

Direct absorption experiments with outer membrane protein antigens and blocking IgG were performed to examine a dose-response effect of antigen absorption and to compare the relative blocking efficacy of these two different outer membrane antigen preparations. LPS antigens also were tested in these assays.

Fig. 6 illustrates experiments where increasing concentrations of proteins prepared from the ser^r and ser^s strains were used to absorb blocking dilutions of IgG before preincubation with organisms. A comparison of concentrations of the two antigens revealed that a fivefold lower concentration of ser^r protein was needed to inhibit the blocking action of IgG, compared with the ser^s protein. A similar experiment performed with normal serum and the ser^s strain showed that the ser^r (heterologous) protein inhibited blocking IgG at a 10-fold lower concentration than ser^s protein (Fig. 7). Protein antigen concentrations used in both experiments displayed no toxicity to the organism when used

FIGURE 4 IgM concentration that killed ser^s *N. gonorrhoeae* with added complement from the hypogammaglobulinemic source; purified IgM (–•–•–) vs. whole serum IgM (———); n = 2±range.
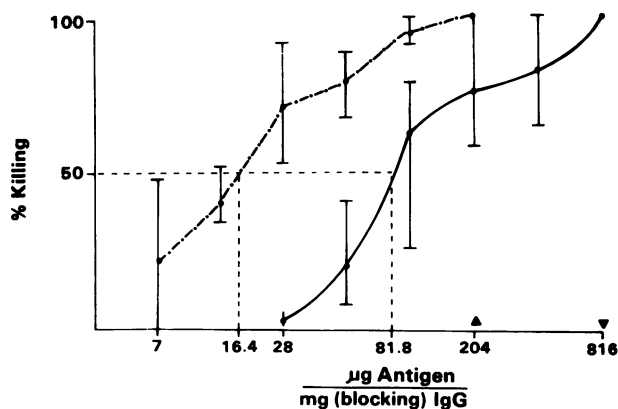
FIGURE 6 Concentrations of antigens per milligram of blocking IgG used to inhibit the blocking effect and regenerate the DGI serum killing system directed against the ser^r strain: increasing concentrations of ser^r outer membrane protein used to absorb blocking IgG before blocking assay was performed (– • – • –) and the same experiments with ser^s outer membrane protein (——), ser^r LPS (▲), and ser^s LPS (▼); n = 4±range.

without antibodies. When using absorption conditions simulating those in the blocking assays, IgG absorbed against whole ser^r gonococci underwent a fourfold reduction in blocking titer compared with control IgG when retested in experiments using the ser^r strain and immune serum. No change in titer was observed in IgG similarly absorbed against ser^s gonococci and tested against the ser^r strain and immune serum.

To assess the possible role of low level LPS contamination of the outer membrane protein, we attempted inhibition of blocking activity with LPS from the two strains. Concentrations of LPS higher than those of outer membrane proteins shown to inhibit 75% of the blocking action of IgG (Figs. 6, 7) demonstrated no inhibiting effect. In addition, anti-LPS activity was not detected by ELISA in the purified IgG preparation with increased blocking activity that had been immunoadsorbed against ser^r outer membrane protein.

DISCUSSION

Serum bactericidal antibodies are associated with protection of the human host against invasive infection with *Neisseria meningitidis* (29, 30) and *Haemophilus influenzae* (31). Currently the role of complement-dependent serum bactericidal activity in the host defense against the three major categories of gonococcal infection; uncomplicated, locally invasive (i.e., pelvic inflammatory disease in women), and bacteremic infection is incompletely defined. Although serum bactericidal activity directed against gonococci that cause uncomplicated local infection does not appear to pro-

tect against this disease, its absence in women with pelvic inflammatory disease infected with gonococci otherwise sensitive to normal human serum may indicate susceptibility to locally invasive strains in these women (28). Moreover, resistance to the lytic action of normal human sera of most strains that cause bacteremic infection may prove to be an important factor that enables these strains to invade, disseminate, and produce distinct manifestations of the disseminated syndrome (32).

The complex interaction between the surface antigens of gonococci and the host may reflect both the inflammatory potential and the sensitivity to serum of *Neisseria gonorrhoeae*. Although lipopolysaccharides have been shown to be the major targets of human bactericidal antibody and may prove to be important antigens in the pathophysiology of specific inflammatory states (2, 3, 5), one or two outer membrane protein serotypes (33), characterized on SDS-PAGE by a similarly sized principal outer membrane protein (protein 1) (4), comprise the majority of disseminated strains that resist killing by normal human serum (33). A second major class of proteins (protein 2) present in gonococcal outer membranes are associated with a number of phenotypic features of gonococci including colonial opacity (34), leukocyte association (35), resistance to low molecular weight antibiotics (36), adhe-
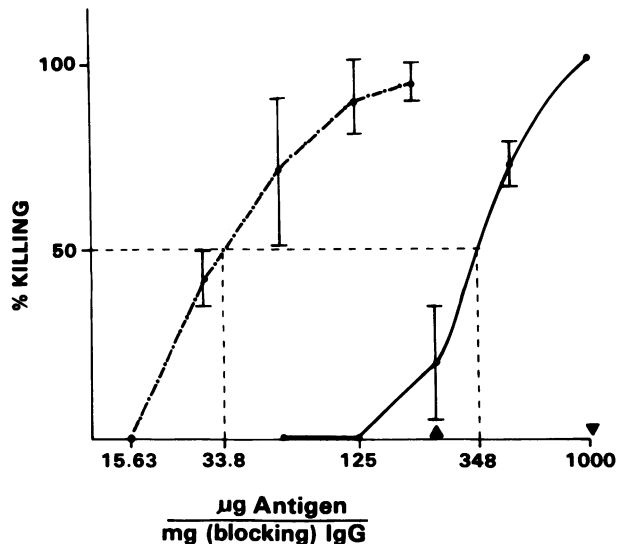

FIGURE 7 Concentrations of antigens per milligram of blocking IgG used to inhibit the blocking effect and regenerate the normal human serum killing system directed against the ser^r strain: increasing concentrations of ser^r outer membrane protein used to absorb blocking IgG before blocking assay was performed (– • – • –) and the same experiments with ser^s outer membrane protein (——), ser^r LPS (▲), and ser^s LPS (▼); n = 2±range.

sion to human buccal epithelial cells (36), and serum-resistance (in at least one strain) (37). These proteins are heat-modifiable (38, 39) and can be distinguished from a third outer membrane protein (protein 3), which although not modifiable by heat, can be modified by chemical reduction from a protein that migrates on SDS-PAGE with an apparent molecular weight of 30,000 to one of higher molecular weight (38, 40). In our studies we prepared antigens derived from gonococcal outer membranes, containing these and other proteins to investigate the role of these antigens, separate from lipopolysaccharides, in serum resistance of gonococci. Although protein 1 is only partially soluble in sodium deoxycholate at pH < 8.5 (38, 41, 42) we used brief alkalinization of outer membranes to pH 11.0 in sodium deoxycholate and EDTA followed by chromatographic separation of proteins from LPS and subsequent suspension of proteins at pH 9.0 in glycine buffer to permit relative solubilization.

That differences in cell surface structure of ser^s and ser^r strains may be important in explaining serum resistance is reflected perhaps by the different human immunoglobulin classes responsible for the killing of gonococci. "Natural" bactericidal activity against serum-sensitive gonococci has been shown here and by others to be mediated by IgM (43); however, the killing of strains resistant to normal human serum is accomplished by IgG isolated from convalescent DGI sera, which is directed also against lipopolysaccharide antigens (3). These isolated immune IgG fractions often are capable of killing heterologous DGI strains but not ser^s strains (unpublished observations), suggesting that cross reactive antigens shared by DGI strains may include common lipopolysaccharide determinants in addition to outer membrane proteins. However, in these studies, we have shown that normal IgG may interfere with natural as well as immune serum killing of both ser^s and ser^r gonococci, respectively. This blocking activity occurred via the F(ab')$_2$ portion of the IgG molecule and was dependent upon light chain concentration, indicating, therefore, that blocking antibodies recognize specific antigenic targets (33). Although seemingly paradoxical that a single immunoglobulin class may both kill (immune IgG) and block killing (normal IgG) of gonococci, perhaps IgG subclass specificity for different antigenic targets (44, 45) with a variable ability to activate complement (46), dictates whether killing or blocking will supervene. The added observation that bactericidal antibody development in patients convalescing from DGI is often nonexistent or meager (32) despite high titered antibody rises measured by indirect immunofluorescence (47), suggests that blocking antibodies also may result from specific antigenic challenge. IgG also has been shown

capable of inhibiting the killing of other gram-negative bacteria (48–50) in addition to *Neisseria gonorrhoeae* (6). Convalescent sera from patients infected with *Neisseria meningitidis* may show diminished bactericidal activity against the infecting strain of the meningococcus and also may inhibit killing of meningococci by sera from normal humans (51). Furthermore, sera taken from patients early in the course of meningococcal disease may have lytic activity against the infecting strain that is unmasked only when the sera have been freed of IgA (51–52). Similar inhibition also has been demonstrated in the sera of patients chronically infected with common enteric gram-negative bacilli and in the IgA fractions of sera taken from patients with chronic brucellosis (53).

Although we have shown that killing of gonococci can be blocked by human IgG antibodies, greater blocking efficacy of IgG against ser^r vs. ser^s strains and possibly higher titers of natural IgG against ser^r proteins measured by direct binding assays suggest that quantitative differences in blocking antibodies attaching to ser^r gonococci may contribute to impaired access of lytic antibody and complement to lipopolysaccharide targets due to steric hindrance. Antigenic differences in the outer membrane proteins of strains also may be important in the recognition and avidity of natural blocking antibodies. Neutralization of standardized blocking concentrations of IgG by lower concentrations of ser^r vs. ser^s outer membrane protein antigens for sensitive as well as resistant gonococci indicates, however, that the antigens are cross-reactive. Perhaps the location of proteins in the outer membrane and therefore the accessibility of these antigens to blocking antibodies may combine with intrinsic antigenicity as additional important factors. Surface labeling of whole gonococci with ^{125}I using the lactoperoxidase method labels proteins 1 and 2 (38, 54), although the efficiency of protein 2-labeling using this method may vary (24), suggesting limited association of this protein with the outer surface membrane (38). Protein 3 has been shown to be positioned near protein 1 in the outer membrane by demonstrating cross-linking of these two proteins when vesicles of outer membrane are treated with protein cross-linking reagents (39). The specific targets of blocking antibodies are yet unknown, however, the exposure of the protein antigen receptors at the surface of the gonococcus may be an important factor in recognition of gonococci by these antibodies.

ACKNOWLEDGMENTS

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