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Neisseria gonorrhoeae Acquire a New Principal Outer-Membrane Protein When Transformed to Resistance to Serum Bactericidal Activity

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Resistance to the complement-dependent bactericidal activity of normal human serum is found in nearly all *Neisseria gonorrhoeae* strains causing disseminated gonococcal infection. Transformation of serum-sensitive gonococcal strain NRL 7189 to serum resistance using deoxyribonucleic acid from three separate disseminated-infection gonococci was accompanied by simultaneous structural and antigenic changes in the principal outer-membrane protein (POMP) of the transformants. In each of 10 separate transformations, there was a reduction in subunit molecular weight of the POMP from that in the recipient (39,000) to that in the deoxyribonucleic acid donors (36,500). Also, in each instance the POMP antigenic type, as measured by enzyme-linked immunosorbent assay, converted from that of the recipient to an antigenic type common to each DGI donor strain. This conversion of POMP antigen was reflected in part by changes in the surface fluorescence of the transformed gonococci to the microimmunofluorescence pattern of the donor strains. These results suggested that serum resistance of gonococci is related to the possession of a POMP of characteristic subunit molecular weight and antigenicity.

Neisseria gonorrhoeae strains resistant to the complement-dependent bactericidal activity of normal human serum were first observed by Spink and Keefer (19) in 1937 and subsequently further characterized by Schoolnik et al. (17). To further characterize this property, we transformed serum-sensitive gonococci to serum resistance using deoxyribonucleic acid (DNA) from serum-resistant organisms, then evaluated the associated characteristics of the transformants. The transformation to serum resistance required two steps. The first transformation produced gonococci with intermediate resistance to bactericidal activity. Repeat transformation of these intermediate transformants yielded second-step transformants that were equally as resistant to killing by normal human serum as the DNA donor. The outer-membrane protein compositions of both the first- and second-step transformants were compared with those of the DNA donor and recipient strains. These changes in outer-membrane proteins (4, 7, 9) were further correlated with alterations in antigenicity of outer-membrane proteins (T. M. Buchanan, in press; T. M. Buchanan and J. F. Hildebrandt, submitted for publication) and surface immunofluorescence (21).

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MATERIALS AND METHODS

Bacteriological techniques and transformation procedure. Three strains of *N. gonorrhoeae*, NRL 1384, 2031, and 6305, were isolated from patients with disseminated gonococcal infection (DGI). The DNA from these strains was prepared as described previously (5) and used to transform a single recipient strain, NRL 7189, using 10 μg of DNA per ml, as described previously (14, 18). Serum-resistant transformants were selected by testing transformed cell suspensions in the serum bactericidal system (below; 12, 17). Four first-step transformants (NRL 7220, 7652, 7657, and 8032) with intermediate resistance to serum bactericidal activity were again transformed with DNA from the donor strains to yield 10 second-step transformants (NRL 7221, 7653-7657, 8075-8077, 8080) that were completely resistant to killing by normal human serum (Table 1).

All isolates were identified as *N. gonorrhoeae* by oxidase reaction, Gram stain, and sugar fermentation reactions (2). Gonococcal strains were preserved at −70°C in a medium consisting of 5% (wt/vol) bovine serum albumin and 5% (wt/vol) monosodium glutamate (6).

In preparation for extraction of outer-membrane antigens, organisms were first subcultured twice on GC agar base medium (Difco Laboratories, Detroit,

TABLE 1. *Transformation sequences by strain*

Sequence	DNA donor strains	Recipient strains	Intermediate transformants	Full transformants
I	6305	7189	7220	7221
II	6305	7189	7220	7655
III	6305	7189	7220	7657
IV	6305	7189	7220	7653
V	6305	7189	7652	7656
VI	1384	7189	7657	7654
VII	2031	7189	8032	8075
VIII	2031	7189	8032	8076
IX	2031	7189	8032	8077
X	2031	7189	8032	8080

Mich.) containing 1% defined supplement (23) and were then inoculated onto 15-cm-diameter plates of the same medium composition for mass production. After incubation for 18 to 22 h at 36°C in an atmosphere with 5% CO_2, organisms were harvested into 0.1 M NaCl and processed as described below for analysis of their outer-membrane proteins.

Transformation and selection for increased resistance to serum killing were performed as follows. Recipient organisms were exposed to DNA from donor strains at 37°C in a previously described broth (15). Samples were removed at 1- to 2-h intervals for up to 22 h and assayed for transformants to serum resistance to determine the time required for phenotypic expression of this trait. After we learned that transformation to serum resistance was not detected until after 10 h of incubation, and that it reached a plateau at 11 to 12 h, all subsequent transformation incubations were continued for 12 h. Aliquots of the transformation suspension were diluted to 5×10^3 colony-forming units per ml, and 50 μl of this suspension was added 50 μl of heat-inactivated, pooled human serum (diluted 1:20 in medium 199) together with 50 μl of a 1:2 dilution of a complement source consisting of serum from a patient with agammaglobulinemia. The viable count was determined for each experiment by including wells that contained 50 μl of bacterial suspension and 100 μl of medium 199. The contents of each well were plated, incubated, and enumerated. An equivalent portion of the cell suspension not treated with DNA was run in parallel as a control along with the serum and complement controls. In addition, a parallel transformation experiment, with another equivalent portion of the cell suspension, was performed using DNA from the serum-sensitive strain NRL 6777. This transformation mixture served as a control for possible nonspecific effects of DNA on the expression of serum resistance by recipient cell suspensions. Control and transformation mixtures were assayed at least in triplicate. The number of colonies from each well was enumerated after 24 h of incubation. Alternatively, serum-resistant transformants were selected by a procedure based upon the bactericidal assay of McCutchan et al. (12). After incubation for 12 h, the cells that had been exposed to DNA, and the control cell suspensions, were diluted in medium 199 to 2×10^4 to 10^5 colony-forming units per ml. Twofold serial dilutions of each cell suspension were prepared, and 50-μl amounts of each dilution were placed in microtiter trays. Fresh normal human serum (50 μl of a 1:4 dilution in medium 199) from an individual with no history of gonorrhea was added to each well. Determinations were done in triplicate. Incubation and plating was identical to the previous selection procedure. Serum-resistant transformants were isolated from a point in the twofold serial dilution of the cell suspension at which no growth was present on the controls but ≥5 colonies were present from the transformed mixture. Both methods used for the selection of serum-resistant transformants gave equivalent results.

Serum bactericidal assay. Gonococci were measured for their resistance to complement-dependent killing by normal human sera by the method of Schoolnik et al. (17). The bactericidal test was performed in a microtiter system using disposable U-well trays and 50-ml diluters (Cooke Lab Products Div., Alexandria, Va.). Serum from 10 people with no history of gonorrhea was pooled, filter sterilized, and heat inactivated before use. The source of complement was a person with x-linked agammaglobulinemia whose serum lacked bactericidal activity for *N. gonorrhoeae*.

The reactive mixture contained 50 μl of bacteria suspension (600 colony-forming units harvested in log phase), 50 μl of serum in different dilutions, and 50 μl of complement in medium 199 (activity, 50% hemolytic complement units per ml). The incubation time at 37°C in a shaking incubator was 60 min before 1 drop of the suspension from each well was seeded onto prewarmed plates. Growth medium was GC base, supplemented with 1% Kellogg supplement (23). Plates were incubated for 24 h at 37°C in a 5% CO_2 atmosphere. The colonies were counted, and the reciprocal of the serum dilutions producing 75% reduction in colony-forming units compared with the controls was defined as the bactericidal titer.

ELISA for POMP antigen. Gonococcal principal outer-membrane protein (POMP) antigen was purified as previously described (Buchanan and Hildebrandt, submitted for publication). Briefly, the 130,000 × *g* outer-membrane pellet material (10) was chromatographed over a Sepharose 6B column (1.5 by 90 cm) containing 1.5% sodium deoxycholate and 5 mM glycine buffer (pH 9.0). The POMP eluted at the void volume free of contaminating lipopolysaccharide and other proteins. This protein in 2-μg/ml concentrations was used to coat polystyrene tubes for an enzyme-linked immunosorbent assay (ELISA) (Buchanan, in press, Buchanan and Hildebrandt, submitted for publication). The enzyme conjugate was horseradish peroxidase linked to goat anti-rabbit immunoglobulin (Cappell Laboratories, Cochranville, Pa.), and the chromagen was *o*-phenylenediamine. Serotyping of gonococci based specifically on POMP antigen was performed by using gonococci to inhibit the binding of antibody to purified POMP antigen coated to tubes. Organisms containing POMP of the same antigenic type as the POMP coated to tubes inhibited the ELISA test color development, whereas organisms with heterologous POMP antigens did not (Buchanan and Hildebrandt, submitted for publication). For this study, strains were tested against only one POMP serotype. This POMP antigen was obtained from strain 7122, a strain with the characteristics associated with an ability to cause DGI (11, 17, 24). This POMP antigenic type was chosen because each of the three

DGI DNA donor strains inhibited antibody binding to strain 7122 POMP antigen in the ELISA test, but did not inhibit antibody binding to any of five other purified POMP antigenic types (Buchanan and Hildebrandt, submitted for publication).

Extraction of POMP. Bacteria were suspended in 0.15 M NaCl (4°C) and centrifuged for 10 min at 12,000 × *g*. The bacterial pellet was suspended in a buffer containing 0.2 M lithium acetate and 0.01 M ethylenediaminetetraacetic acid adjusted to pH 5.9 in a proportion of 1:10 (weight of organisms per volume of buffer) and then shaken vigorously at 45°C for 2 h in the presence of glass beads (6 mm OD). The fluid was then centrifuged for 10 minutes at 12,000 × *g* and the supernatant was retained and centrifuged for 40 min at 30,000 × *g*. This supernatant was then spun at 130,000 × *g* for 1 h. The pellet obtained from this procedure consisted of a crude outer-membrane preparation (10). This pellet was suspended in 1 ml of buffer [0.01 M tris(hydroxymethyl)aminomethane (pH 7.6), 0.15 M sodium chloride, 0.02% sodium azide] and preserved at −70°C until used.

Polyacrylamide gel electrophoresis. Sodium dodecyl sulfate–10% polyacrylamide slab gel electrophoresis (13, 20) was performed for comparative analysis of the partially purified gonococcal POMP preparations. A 50-μl volume the POMP material was mixed with an equal volume of 2% sodium dodecyl sulfate and 2% 2-mercaptoethanol (pH 7) and heated in boiling water for 5 min. After cooling, 50 μl of bromophenol blue in 80% (wt/vol) sucrose was added, and a 50-μl sample of the mixture was applied to the gel.

The gel (15 by 11 cm) was subjected to electrophoresis at 90 mA for 6 h and then fixed in 5% trichloroacetic acid overnight. The fixed gel was then stained for 2 h in 0.25% Coomassie brilliant blue R (Sigma Chemical Co., St. Louis, Mo.) dissolved in 50% methanol–10% acetic acid. Destaining was performed in 10% acetic acid–20% methanol containing Dowex 1 × 8 anion exchange and Dowex 50 W-X8 cation exchange resins (BioRad Laboratories, Richmond, Calif.).

Micro-IF immunotyping. Microimmunofluorescence (micro-IF), immunotyping was performed as described by Wang et al. (21). Briefly, Formalinized whole gonococci were used as test antigen and as immunogen for the preparation of antisera in mice. By using pen points, dots of homologous and heterologous strain antigens (each mixed with normal yolk sac fluid) were placed on the microscope slide so that a single bacteriological loop of serum could cover a group of antigens. By using a template, nine groups of an identical set of antigens were placed on a single slide so that serial dilutions of an antiserum could be tested on the same slide. An indirect fluorescent antibody technique was utilized. The highest dilution of serum resulting in a definite fluorescence of the rim of the bacterial cell was considered the serum titer for that particular strain. The titers obtained for immune serum to heterologous strains were expressed as the percentage of the titer obtained for that serum produced to the homologous strains. Antigenic similarities were based upon cross-reaction patterns. By extensive cross-testing, strains have been classified into types A, B, and C, and further to A_1, A_2, A_3, B_1, B_2, B_3, C_1, or C_2.

RESULTS AND DISCUSSION

It was of interest that transformation to serum resistance required two separate steps, and required 10 to 12 h for each step. This suggests that two separate genetic events may be required to produce strains that possess serum resistance equal to strains isolated from patients with the DGI syndrome. The transformation of first-step transformants to full serum resistance occurred at a frequency of approximately 0.2%. The prolonged time required to produce serum-resistant transformants contrasts with transformation of most previously reported gonococcal genes, which required 2 to 4 h for phenotypic expression (14, 18). Some traits, for example resistance to penicillin or loss of a growth requirement for uracil, have required up to 6 h for full phenotypic expression (14). Inoue et al. (8) have presented evidence to show that the one-hit theory of immune hemolysis could be extended to the immune bactericidal reaction. This could explain the unusually long time required for phenotypic expression. If the entire outer layer of the gonococcus must be replaced to eliminate even a single site of bactericidal action before we could detect any increase in resistance to serum, several generations would be required. Under the growth conditions used, about 10 generations would take place during incubation before serum-resistant gonococci could be detected.

The gonococcal serum resistance selected by passage in serum and reported by McCutchan et al. (12) was unstable and probably did not represent DNA-mediated transformation or other genetic alteration at two loci. Instead, it may have represented a phenotypic change stimulated by growth conditions, which reverted upon transfer to other media. The fully serum-resistant transformants in this study have retained their strain 7122 POMP antigenic type, serum resistance, and micro-IF characteristics despite multiple passages on artificial solid agar media.

As shown in Fig. 1 and 2, the transformation of serum-sensitive gonococci to serum resistance (Tables 1 to 3) was accompanied by simultaneous change in one or more proteins of the 130,000 × *g* pellet material. As shown previously by Johnston and Gotschlich (9), and confirmed by Buchanan and Arko (4) and by Heckels (7), at least one of these proteins, one with a subunit molecular weight in the rage of 36,000 to 39,000 was an outer-membrane protein. This protein has been termed POMP. A consistently observed change in each of the 10 separate transformations analyzed in these studies was a reduction in subunit molecular weight by approximately 2,500 in the POMP for each of the fully

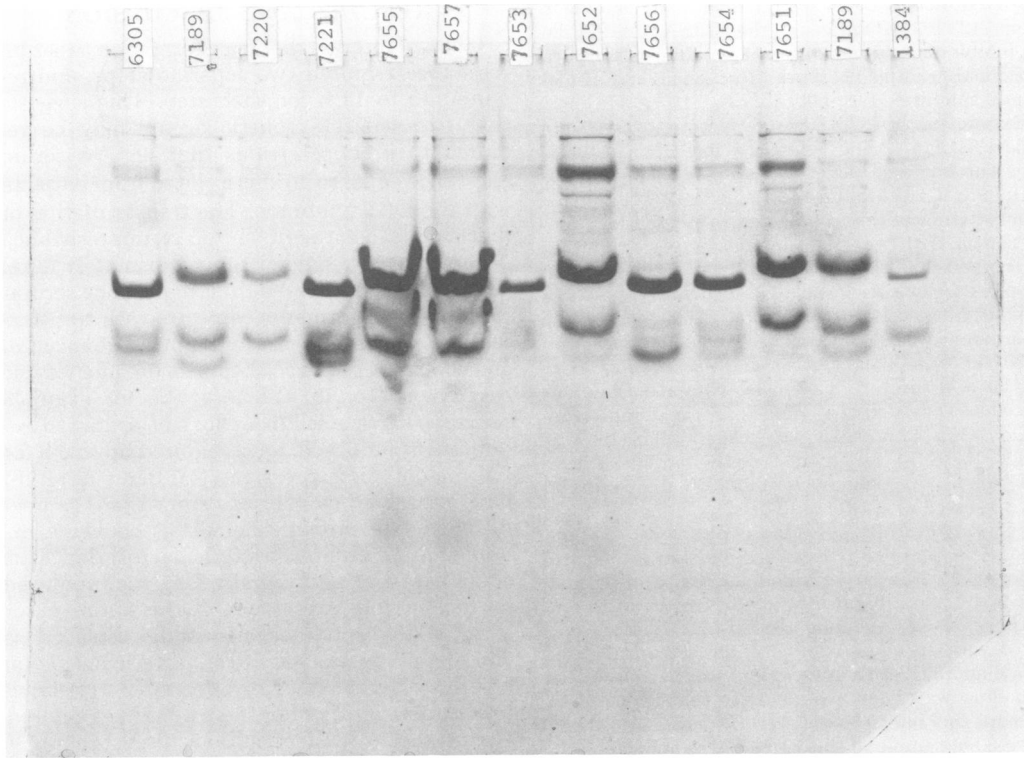

FIG. 1. *Sodium dodecyl sulfate–10% polyacrylamide slab gel of partially purified outer-membrane preparations (130,000 × g pellet material) or gonococci. Sequence of transformation: DNA donor-recipient-intermediate transformant-full transformant. Sequence (i) 6305-7189-7220-7221; (ii) 6305-7189-7220-7655; (iii) 6305-7189-7220-7657; (iv) 6305-7189-7220-7653; (v) 6305-7189-7652-7656; (vi) 1384-7189-7651-7654.*

resistant transformants, as compared to gonococci of intermediate or extreme serum sensitivity (Fig. 1 and 2, Tables 1 to 3). Furthermore, all fully serum-resistant strains (donors and fully serum-resistant transformants) contained POMPs of nearly identical subunit molecular weights (Fig. 1 and 2). This POMP subunit molecular weight was approximately 36,500, and this particular POMP may be in part responsible for the resistance of these strains to complement-dependent killing by normal human serum. It has been demonstrated that lipopolysac-

TABLE 2. *Biological and immunological properties of N. gonorrhoeae strains listed in Fig. 1*

SDS-PAGE[a] well no.	Strain	Bactericidal titer	Micro-IF serotype	ELISA (strain 7122 POMP)
1	6305	≤2	A_2	+
2	7189	256	B_2	−
3	7220	32	B_2	−
4	7221	≤2	A_2	+
5	7655	≤2	A_2	+
6	7657	≤2	A_2	+
7	7653	≤2	A_2	+
8	7652	16	B_2	−
9	7656	≤2	A_2	+
10	7654	≤2	A_2	+
11	7651	16	B_2	−
12	7189	256	B_2	−
13	1384	≤2	A_3	+

[a] SDS-PAGE, Sodium dodecyl sulfate–10% polyacrylamide slab gel electrophoresis.

TABLE 3. *Biological and immunological properties of N. gonorrhoeae strains listed in Fig. 2*

SDS-PAGE[a] well no.	Strain	Bactericidal titer	Micro-IF serotype	ELISA (strain 7122 POMP)
1	2031	≤2	A_3	+
2	7189	256	B_2	−
3	8032	≥64	B_2	−
4	8080	≤2	B_2/A	+
5	8075	≤2	B_2/A	+
6	8076	≤2	B_2/A	+
7	8077	≤2	B_2/A	+
8	2031	≤2	A_3	+
9	7189	256	B_2	−
10	8032	≥64	B_2	−

[a] SDS-PAGE, Sodium dodecyl sulfate–10% polyacrylamide slab gel electrophoresis.

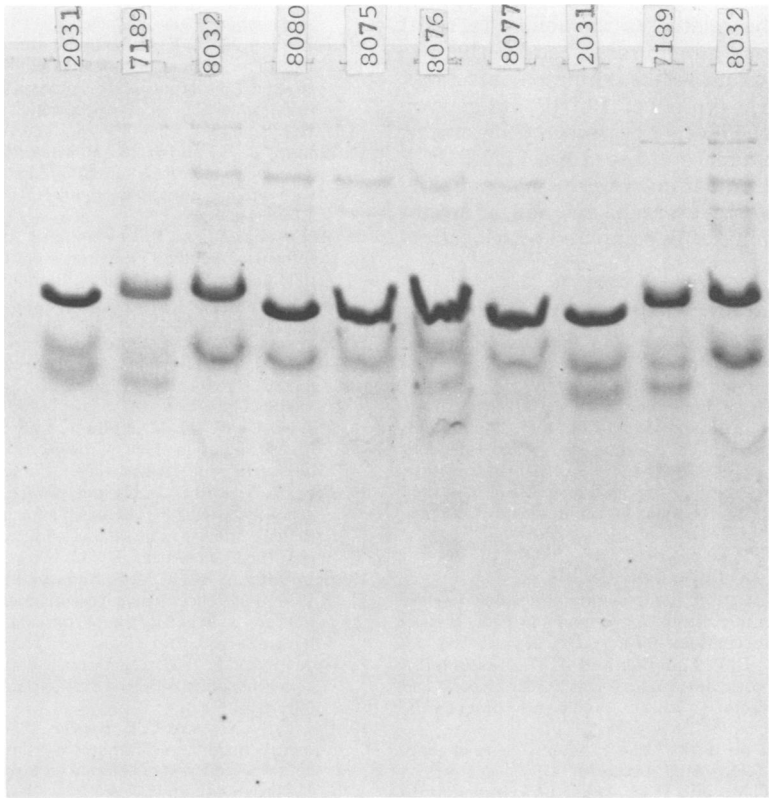

FIG. 2. *Sodium dodecyl sulfate-10% polyacrylamide slab gel of partially purified outer-membrane preparations (130,000 × g pellet material) of gonococci. Sequence of transformation: DNA donor-recipient-intermediate transformant-full transformant. Sequence (i) 2031-7189-8032-8080; (ii) 2031-7189-8032-8075; (iii) 2031-7189-8032-8076; (iv) 2031-7189-8032-8077.*

charide antigens react with bactericidal antibodies to result in killing of gonococci (16, 20). One might hypothesize, therefore, that this protein in some way masks lipopolysaccharide surface antigens to prevent their recognition by bactericidal antibodies in human serum (17).

Strains of *N. gonorrhoeae* that cause DGI are characteristically serum resistant (17), and Wang et al. have determined that the great majority of strains fall into similar immuno-subtypes A_2-A_3 in the micro-IF test (21). For example, the strains used as DNA donors for these studies (NRL 1384, 2301, and 6305) were all DGI strains, and all were classified as members of subtypes A_2-A_3 (serogroup A) by the micro-IF. Of 37 Seattle strains obtained from patients with the DGI syndrome, 35 were subtypes A_2-A_3 (21).

As shown in Tables 1 to 3, intermeidate transformants retained the same micro-IF serogroup as the recipient (subtype B_2, serogroup B), whereas transformants to full serum resistance acquired complete (9/10) or partial (1/10) immunofluorescence characteristics of immunotype A coincident with a change in the subunit molecular weight of their POMP to approximately 36,500. Interestingly, all 10 of the fully serum-resistant transformants also acquired strain 7122 POMP antigen on their surfaces, the same POMP antigen type expressed on the surface of each of the donors (Tables 2 and 3).

It is likely that the shift to immunotype A was due at least in part to a change in POMP antigen, since this was the only consistent protein change observed (Fig. 1 and 2) and since Apicella (1) has determined that lipopolysaccharide serotypes vary extensively within DGI strains (unpublished collaborative data). This suggests that the POMP is at least one of the antigens involved in surface immunofluorescence of gonococci as measured in the micro-IF system and confirms Heckels' (7) suggestions that this protein is involved in surface immunofluorescence. The combination of both shared antigenicity and common subunit molecular weight of the POMP for each of the donor and fully serum-resistant transformants suggests that this

POMP may be related to an ability to resist killing by normal human serum, and perhaps to the capacity to cause disseminated infections. Further ELISA typing of POMP antigens in many strains of gonococci is needed to determine the actual prevalence of the strain 7122 POMP antigenic type in serum-resistant as compared with serum-sensitive organisms, and in strains causing disseminated as compared with localized infections.

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