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Nature and Heterogeneity of the Antigens of *Neisseria gonorrhoeae* Involved in the Serum Bactericidal Reaction

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Sixty strains of *Neisseria gonorrhoeae* have been classified into four main groups according to their resistance to killing by human complement together with either normal human or immune rabbit antibodies. The rabbit antisera had been raised against 10 of the strains tested. The normal human antibodies had probably been formed against cross-reacting organisms since they could be removed by absorption with *N. pharyngis sicca*, *N. pharyngis flavus*, *N. catarrhalis*, or *Escherichia coli*. Bactericidal antibodies could be absorbed from both normal and immune sera by *N. gonorrhoeae* which had been autoclaved or trypsinized, and by red cells coated with gonococcal lipopolysaccharides. The results suggest that the antigens involved in the bactericidal reaction are lipopolysaccharides of several distinct specificities. Since individual sera always reacted more widely in hemagglutination than in bactericidal tests, it is postulated that surface-blocking antigens may restrict access to the lipopolysaccharides in the intact organisms.

Ninety years after the discovery of gonococci by Neisser, knowledge of its antigenic structure remains rudimentary compared with what is known about streptococci or salmonellae. To a large extent this is due to the difficulties in growing *Neisseria gonorrhoeae* and to the absence of a satisfactory model animal infection or of any test for virulence. Early work on gonococcal antigens was summarized by Wilson and Miles (19). Their rather general conclusion was that there are two main types of *N. gonorrhoeae*, several intermediate types, a number of antigens, and a close antigenic relationship to *N. meningitidis*. More recent developments were the detection of antigens by means of the fluorescent antibody technique (5, 8), the use of hemagglutination techniques (3, 12), the study of types of antibody found (4), and the beginning of a diagnostic precipitin test (2). There have also been some chemical and serological analyses of gonococcal endotoxins (18, 13). Very promising results are appearing from the intensive study now being made of antigens from cell wall and cytoplasmic fractions of gonococci (14) and their reactions with patient's serum (6, 17). For the moment, however, gonococcal antigens remain largely symbols, potentially useful but of uncertain function and doubtful composition.

A more detailed knowledge of gonococcal antigens is essential for the future investigation of immune responses in gonorrhoea. It is now clear (2, 4) that antibodies develop even if immunity does not. A simple, reliable, serological test for early gonococcal infection would be invaluable for the investigation of female patients in whom direct culture is unreliable. A serological classification of *N. gonorrhoeae* would also be useful in epidemiological work.

The killing of *N. gonorrhoeae* by human serum seemed to us to provide a possible test system for detecting gonococcal antigens which could conceivably be of biological significance and could be used as the basis for later chemical investigation.

This paper reports the results of treating 60 strains of *N. gonorrhoeae* with normal human serum antibody or immune rabbit antibody in the presence of human complement. Some preliminary experiments were also made on the nature and specificity of the antigens involved, and the bactericidal results have been compared with those of hemagglutination tests using sheep red cells sensitized with gonococcal extracts.

MATERIALS AND METHODS

Strains of *N. gonorrhoeae* (G1, G3-G20, and G22-G60) were obtained from patients attending the

venereal diseases clinic of St. Mary's Hospital. Urethral or cervical exudates were first cultured on either chocolate-serum-agar or on the medium described by Amies and Garabedian (1) but with the antibiotics omitted. Organisms were identified by Gram staining, oxidase, and fermentation reactions, and were then preserved by lyophilization after the minimal number of subcultures required to ensure purity, usually one to two. A new ampoule was opened for each experiment. Strains G2 and G21, NCTC 8375, and NCTC 8448 were provided by the National Collection of Type Cultures, Colindale, as were *N. meningitidis* NCTC 8339, *N. flavescens* NCTC 8263, *N. pharyngis-sicca* NCTC 4591, *N. pharyngis-flavus* NCTC 4590, and *N. catarrhalis* NCTC 3622. *N. gonorrhoeae* strains 1a, 2c, 42981, 12954, 11413, and 44341 were kindly given to us by A. Reyn of the State Serum Institute, Copenhagen, and have already been described by her (15). *Escherichia coli* WF96 and *Pseudomonas aeruginosa* R 133 were from the culture collection at the Wright-Fleming Institute, St. Mary's Hospital.

Gonococci were incubated at 37 C in specially modified milk churns (16) under a manometrically adjusted atmosphere of 5% CO_2 in air in the presence of excess moisture.

Sera. Samples of normal human serum were obtained from nine adult male donors with no previous history of gonococcal infection and a negative gonococcal complement fixation test. A portion of the serum from eight of these donors was pooled, and serum from the ninth donor (MW) was used as a standard source of complement and normal antibody for the bactericidal test, except where otherwise indicated. All sera were stored at −60 C in 1-ml portions and were thawed once only. Serum MW had a titer of 52 complement H_{50} units/ml, and the pooled serum of 50 complement H_{50} units/ml when assayed by the method of Kabat and Mayer (11).

Rabbit antigonococcal sera were prepared against strains G 1, 2, 3, 4, 7, 22, 37, 38, 50, and 52 by intravenous injection of approximately 10^9 viable gonococci in saline on days 0 and 3. The rabbits were bled on day 17, and the sera were tested and stored at −20 C. Only rabbits whose normal serum showed no gonococcidal activity at a final concentration of 1:100 in the bactericidal assay were chosen for immunization.

Bactericidal test. The overnight growth of gonococci from an ampoule cultured on Amies and Garabedian medium was suspended in broth to a concentration corresponding to 4×10^8/ml. A 0.5-ml amount of this was used to inoculate the biphasic flask culture described by Gerhardt and Hedén (9) modified by leaving out the starch from the broth and reducing the volume. After 3 hr of incubation at 37 C, the liquid phase contained approximately 10^9 gonococci per ml in the early logarithmic stage of growth. These were diluted approximately 1,000 times in Dulbecco's complete balanced salt solution to give a concentration of viable organisms of about 10^6 per ml. This was sufficient to dilute out the anticomplementary effect of broth and made it possible to avoid washing the bacteria which had been found to reduce the viable count significantly. A 0.1-ml amount of a suspension containing approximately 10^5 organisms was added to tubes containing 0.25 ml of fresh or deep-frozen normal human serum (MW) and 0.1 ml of a 1:10 dilution in Dulbecco's solution of rabbit immune serum where appropriate; the volume was made up to 1.0 ml with Dulbecco's solution. The final concentration of complement, therefore, was approximately 13 complement H_{50} units per ml. The tubes were then incubated for 30 min at 37 C in a shaking water bath. Control tubes containing 0.25 ml of inactivated normal human serum with rabbit antibody, where appropriate, were set up in parallel. Samples (0.1 ml) were taken at 0 and 30 min, diluted immediately in Dextrose Starch Broth (Difco) without the starch, and spread on plates of Amies and Garabedian's medium to determine viable counts. Strains were defined as sensitive when the test tube showed a reduction of 90% (i.e., 1 log unit) or more in viable count in 30 min when compared with the control lacking complement. The frequency distribution of percentages of organisms killed in 210 tests of 60 strains is shown in Fig. 1.

All strains were first screened by using normal serum to provide complement and antibody. Resistant strains were retested with the addition to the system of rabbit immune serum.

Other bactericidal tests were carried out by using various normal and absorbed immune sera as sources of antibody. One to two milliliters of a 1:5 dilution of normal human serum or a 1:10 dilution of rabbit antiserum in Dulbecco's balanced salt solution was absorbed twice for 12 hr at 4 C with 10% (v/v) of packed bacteria in sterile tubes [3 by 0.5 inches (7.6 by 1.3 cm)]. All antisera and absorbed sera were checked for significant anticomplementary activity by the use of strain G2, which was sensitive to the bactericidal activity of the standard normal human serum alone. Specificity of absorption was checked by control tubes containing antibody absorbed with heterologous organisms.

Gonococcal extracts. Extracts were prepared from strains G1, G2, G22, G36, and G38 by the phenol-water method (12). The yield of deposit on ultracentrifugation of the water layer was 2 mg/g (wet

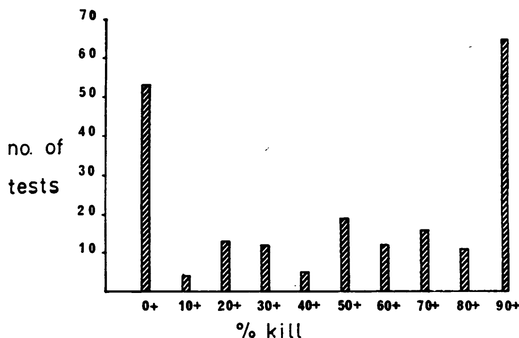

FIG. 1. *Frequency distribution of percentage of organisms killed in 210 bactericidal tests with Neisseria gonorrhoeae, using one normal human serum (MW) and four immune rabbit sera.*

weight) of bacteria. The ultraviolet spectra were similar to those reported by Maeland (12), and there were no absorption peaks at 260 and 280 nm. Preparations from G1 and G36 induced a biphase febrile response and a local Schwartzman reaction in rabbits. Although full chemical analysis has not yet been done, the method of preparation, the absence of detectable protein, the marked endotoxinlike activity (2 μg was pyrogenic), and the ability of alkali-treated material to sensitize red cells suggest that the active fractions concerned were Westphal-type lipopolysaccharides.

To sensitize sheep red cells, the lipopolysaccharide was treated with alkali (7), made up to 12.5 μg/ml in Dulbecco's solution and incubated with an equal volume of 2.5% red cells for 1 hr at 37 C. The cells were washed three times before use.

Hemagglutination was carried out in Perspex trays by using 0.1-ml volumes of twofold dilutions of antibody and 0.1 ml of 0.5% sensitized red cells.

Antisera were absorbed by mixing a 1:10 dilution of antiserum with 20% (v/v) packed sensitized red cells, mixing and keeping for 4 hr or overnight at 4 C. The cells were then removed by centrifugation, and the procedure was repeated four more times.

RESULTS

Serum sensitivity of strains of N. gonorrhoeae. The results of the bactericidal tests on strains of *N. gonorrhoeae* G1-G60 are shown in Fig. 2. There appear to be four main groups, defined by the antibodies necessary for killing by complement to occur.

Group I strains were killed in the presence of antibodies from normal human serum (serum MW).

Group II strains were killed when any of the immune anti-G1, -G2, -G3, -G4, -G37, or -G50 sera were present.

Group III strains were killed in the presence of anti-G37 or anti-G50 but not of anti-G1, -G2, -G3, or -G4 serum.

Group IV was resistant to the antisera just mentioned and also to anti-G7, -G22, -G38, and -G52. This group is of particular interest in that strains G22 and G52 were resistant to their homologous antisera. Strain G7 was also resistant to anti-G7 on our criteria, although there was a 30% kill. G38 is included in the group for the time being, although it was killed when anti-G38 was added.

Detailed examination of Fig. 2 shows several other strains which do not fit exactly into the groups described, e.g., G1 resists anti-G37 and anti-G50. G24 and G25 are slightly sensitive to normal human serum (MW) yet resist anti-G3 and anti-G4 or anti-G37. Exact classification must

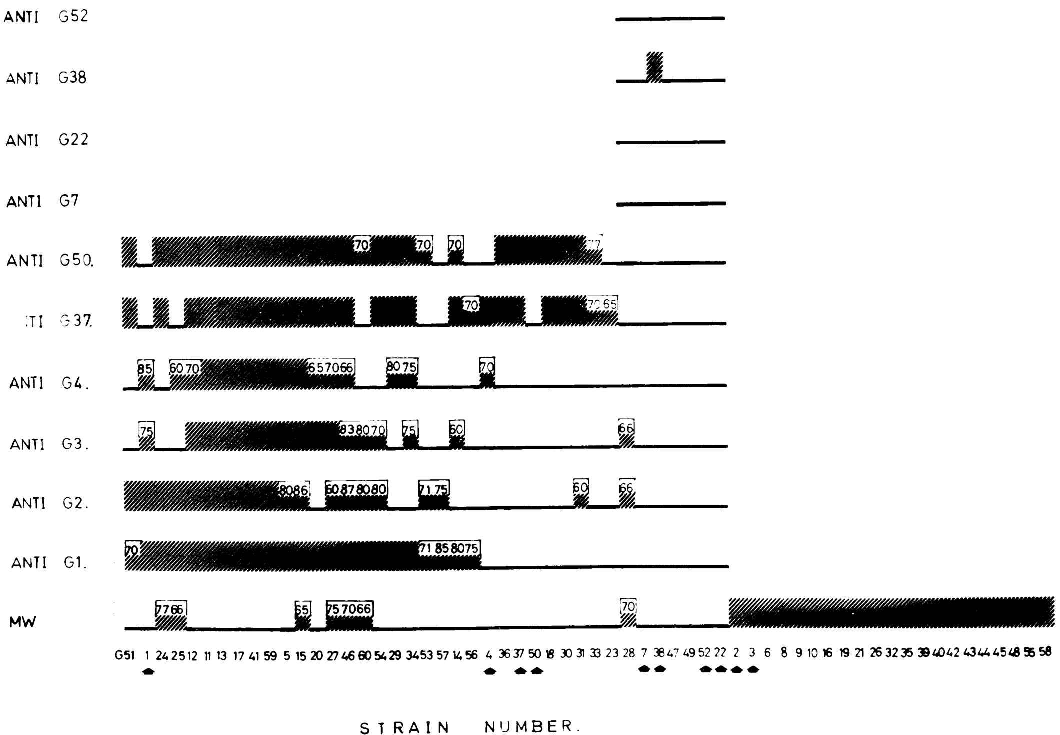

FIG. 2. *Sensitivities of 60 strains of Neisseria gonorrhoeae to 1 normal human serum (MW) and 10 immune rabbit sera. Arrows indicate strains used to prepare antisera. Solid black lines indicate no significant killing. Hatched areas indicate 90% or more killed, except where inset figures indicate between 60 and 89% killed.*

await further analysis. It should be noted that anti-G7, -G22, -G38, and -G52 have not yet been tested against strains in groups I, II, and III. Their ability to kill some of the Danish strains and their hemagglutinating activity showed that they contain specific antibodies.

Two strains, which were not killed by normal human serum, were retested after they had been subcultured two or three times a week for 6 months. They remained resistant.

The serum sensitivities of five Danish strains are shown in Table 1. Strains 2c and 42981 clearly belong in group I, whereas strain 11413 probably belongs in group II. The resistant strains 1a and 44341 would have been placed in group IV were it not for their sensitivities to anti-G1 and anti-G2. There does not appear to be any correlation between the results of the bactericidal tests and the factor formulae previously described (15).

Bactericidal antibodies in normal human serum. One-third of the strains tested were sensitive to normal human serum (serum MW) alone. Although normal human sera vary in activity (Fig. 3), antibodies reacting with some gonococcal antigens must be widely distributed. Absorption of serum MW with heat-killed suspensions of various strains of bacteria (Table 2) suggests that such antigens may be found in some Neisseria species and in *E. coli*. Surprisingly little effect was produced by the strain of *N. meningitidis* tested.

The immune rabbit serum anti-G1 was not affected by absorption with the strains tested, except for *N. gonorrhoeae* strains G1 and G2 and *N. pharyngis-sicca*.

Antigens involved in the bactericidal reaction. The heterogeneity and complexity of gonococcal antigens is shown in Table 3. Strains G1, G29, G34, and G36 were resistant to normal human serum alone but, with the exception of G36, were killed on the addition of anti-G1 serum. Absorption of anti-G1 with the sensitive strains G29 or G34 largely removed all bactericidal activity. Absorption with the resistant strain G36 made the serum ineffective against G29 and less effective against G34 but did not significantly alter the killing of G1. Absorption with the homologous strain G1 removed all bactericidal activity.

The same absorbed sera were tested for their ability to agglutinate sheep red cells coated with G1 lipopolysaccharide (Table 3). Hemagglutinating activity was absorbed not only by strains G1 and G29, as expected, but also by G36. This suggests that, although all four strains have major lipopolysaccharide antigens in common, G1 has an additional antigen involved in killing. This antigen could be another lipopolysaccharide present in such small quantities that it did not reach the red cells in the sensitization procedures, though it was adequate in situ for the bactericidal reaction. Alternatively, it could be of a different chemical nature.

Bactericidal antibodies could be removed from antiserum to G1 by absorption with live G1. The absorbing antigens resisted heat and trypsin (Table 4). Control absorptions with heated samples of strain G36 did not affect bactericidal activity. Antisera to strains G1 and G50, absorbed five times with sheep red cells coated with purified lipopolysaccharide from strains G1 and G36, respectively, showed reduced bactericidal activity (Fig. 4). Specific hemagglutinating activity was almost completely removed. Control sera absorbed with unsensitized sheep red cells were not significantly affected.

The immune sera raised against live subcultured gonococci were also tested for hemagglutinins against sheep red cells coated with lipopolysaccharides from five strains of gonococci (Table 5). Although all the cells were sensitized in the same way, they probably differed in agglutinability, whereas the antisera must also have differed in potency. However, where a serum could be tested against its homologous lipopolysaccharide, the hemagglutination titer was satisfactory.

Even allowing for variations due to these

TABLE 1. *Serum killing of Danish strains of Neisseria gonorrhoeae*

Strain no.	Factor formula	Per cent killed by[a]										
		Normal (MW)	Anti-G1	Anti-G2	Anti-G3	Anti-G4	Anti-G37	Anti-G50	Anti-G7	Anti-G22	Anti-G38	Anti-G52
1a	V	60	92	50	10	0	0	0	0	0	0	0
2c	(V), IX	>99	NT[b]	NT	NT	NT	NT	NT	NT	NT	NT	NT
42981	X, (VI), IX	>99	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT
11413	III, VI	0	>99	>99	75	90	90	96	97	95	95	95
44341	IX, XII	70	>99	>99	0	0	50	50	50	83	15	0

[a] Per cent killed by antisera listed was calculated by comparison with that produced by normal human serum.

[b] Not tested.

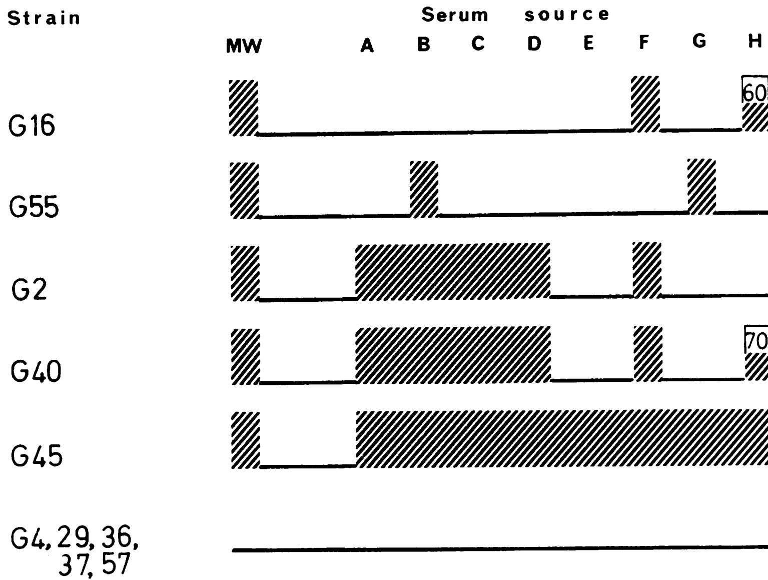

FIG. 3. *Bactericidal antibodies to Neisseria gonorrhoeae in normal human sera. Solid black lines indicate no significant killing. Hatched areas indicate 90% or more killed, except where inset figures indicate 60 or 70% killed.*

TABLE 2. *Absorption of bactericidal antibodies from normal human and immune rabbit serum by Neisseria species, Escherichia coli, and Pseudomonas aeruginosa*

Serum absorbed with	Per cent kill at 30 min of	
	Strain G2, tested with normal human serum (MW)	Strain G1, tested with rabbit anti-G1
N. gonorrhoeae G1	>90	0
N. gonorrhoeae G2	0	0
N. flavescens	>90	>99
N. meningitidis	66	>99
N. catarrhalis	33	>99
N. pharyngis-flavus	0	>99
N. pharyngis-sicca	0	0
E. coli WF96	0	NT[a]
P. aeruginosa R133	>90	>99

[a] Not tested.

factors, it is clear that cross-reactions occurred and it is instructive to compare them with the results of the bactericidal tests.

Strain G1 was readily killed by anti-G1 and anti-G2, doubtfully by anti-G3 and anti-G4, and not at all by anti-G37 and anti-G50. The hemagglutination titers corresponded.

Strain G2 was killed by many normal human

TABLE 3. *Effects on bactericidal and hemagglutination titers of immune sera of absorption with heterologous strains of Neisseria gonorrhoeae*

Antibody	Absorbing strain	Percentage killed at 30 min in strains				Hemagglutination titer aganst G1 lipopolysaccharide
		G1	G29	G34	G36	
Anti-G1		99	98	98	0	640
Anti-G1	G1	0	0	0	ND[a]	<40
Anti-G1	G29	40	0	0	ND	<40
Anti-G1	G34	0	0	33	ND	ND
Anti-G1	G36	92	0	70	ND	40
Normal human serum		0	0	0	0	16

[a] Not done.

TABLE 4. *Nature of the gonococcal antigens subserving the bactericidal reaction*

Anti-G1 serum absorbed with	Killing of G1
G1, live	0
G1, 100 C, 1 hr	0
G1, 121 C, 2 hr	0
G1, 100 C, 0.5 hr, NaCl	0
G1, trypsin	0
G36, 100 C, 1 hr	>90

sera, and its lipopolysaccharide reacted with all the immune sera. Strain G36 was killed by anti-G37 and anti-G50 but not by anti-G4. Its lipopolysaccharide, however, reacted with all three sera. Strains G22 and G38 were not killed by any antisera, although their lipopolysaccharides reacted with several. Whenever tested, therefore, antisera killing a given strain always reacted with the corresponding lipopolysaccharide, but positive hemagglutination was not always paralleled by killing.

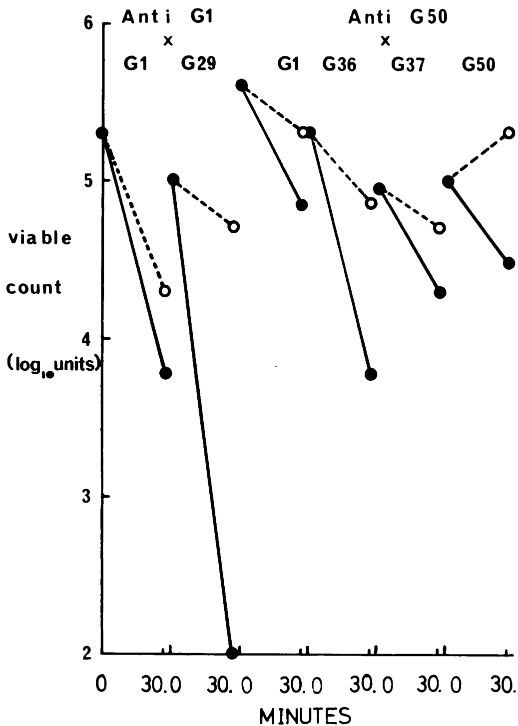

FIG. 4. *Reduction of bactericidal and hemagglutinating activity after absorption of immune sera with lipopolysaccharide-coated red cells. Viable counts with unabsorbed serum (●); viable counts with absorbed serum (○). Anti-G1 absorbed with G1; anti-G50 absorbed with G36. The hemagglutination titer of anti-G1 was 10,000 before absorption and 4 after. The corresponding titers of anti-G50 were 640 and 4.*

DISCUSSION

By means of the bactericidal reactions, 60 strains of *N. gonorrhoeae* can be separated into four main groups. These groups are not homogeneous, and it is evident that additional groups and subdivisions could be defined, given further work on the same lines. As the bactericidal reaction is tedious to perform and, particularly in relation to the gonococcus, difficult to standardize, it would be preferable to detect the antigens involved by some simpler means. In addition, antigens used as taxonomic criteria ought to be clearly defined chemically.

The reactions with normal sera show that cross-reacting antigens occur in other species of *Neisseria* and even in other genera. Moreover, several different antigens are involved since not all normal sera behaved similarly. From the absorption tests, it seems likely that the immune sera were more species-specific.

We have given reasons for believing that the main antigens concerned in serum killing are lipopolysaccharides. However, tests with red cells coated with lipopolysaccharides showed that both normal and immune sera reacted with a far wider range of organisms in the hemagglutination than in the bactericidal reaction. Since the latter is generally taken as the more sensitive test, the most likely explanation is that lipopolysaccharides may be present in gonococci but not necessarily accessible to antibody. This could be due to purely quantitative variations or to the presence of blocking antigens in the intact organisms. The absorption experiments would fit in with this hypothesis, as well as suggesting that there may be several antigenically different lipopolysaccharides per strain. It is also possible, though perhaps less likely, that in some strains the bactericidal reaction is based on other types of

TABLE 5. *Hemagglutination titers of immune antigonococcal sera tested against sheep red cells sensitized with gonococcal lipopolysaccharides*

Sensitizing lipopolysaccharide from strain	Hemagglutinin titers of antisera to strain										Pooled normal human serum
	G1	G2	G3	G4	G7	G22	G37	G38	G50	G52	
G1	1,280	640	40	40	320	80	40	<40	40	640	16
G2	2,560	1,280	1,280	1,280	5,120	2,560	2,560	2,560	640	5,120	16
G22	1,280	<40	640	80	1,280	2,560	80	2,560	1,280	1,280	16
G36	40	40	40	320	80	<40	320	<40	80	<40	8
G38	320	<40	640	160	640	320	160	640	80	640	32

antigen. For example, it would be difficult to explain the sensitivities of strain G36 simply on the presence of blocking antigen. If this blocked anti-G4 which can react with G36 lipopolysaccharide, it ought to have blocked anti-G37 and anti-G50 as well.

If the outcome of bactericidal reactions to a given set of antisera is the result of several categories of antigens, it is unlikely that any other single test would give the same pattern. Further analysis is needed, but, from the diagnostic point of view, it is clear that any serological test for gonorrhoea must not be based on only one or two strains of gonococci but must take into account their antigenic heterogeneity.

It is tempting to compare the antigenic makeup of gonococci with the O and K or Vi systems of enterobacteria. How far such antigens are responsible for virulence is still debated in the enterobacteria (10) and is pure speculation in *N. gonorrhoeae*.

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