

# Immunological and Serological Diversity of *Neisseria gonorrhoeae*: Identification of New Immunotypes and Highly Protective Strains

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Gonococci, irrespective of serotype or immunotype, varied significantly in their capacity to induce immunity in animal models, and in vitro serological relatedness did not always insure in vivo cross-protection. By using a serum bactericidal assay followed by in vivo cross-protection studies, new immunotypic strains which were highly protective were identified from cultures isolated in different geographical areas and from patients with various clinical manifestations. Beta-lactamase production and gonococcal immunotype did not appear as related characteristics in that certain penicillin-sensitive strains were highly effective in immunizing animals against infection with beta-lactamase producers. The findings of this study emphasize the importance of using appropriate biological tests and strains for the investigation of gonococcal immunity and vaccine development. Immunization with a combination of a few major gonococcal immunotypic immunogens may provide substantial protection against the majority of penicillin-sensitive and beta-lactamase-producing gonococci. Investigation of isolated immunotypic immunogens is in progress.

We previously reported the immunological and serological characteristics of nine gonococcal strains by in vivo cross-protection in guinea pig subcutaneous chambers (3) and by in vitro antibody activities in immune sera (23). With nine strains of gonococci from various clinical sources and geographical locations, four distinct immunotypes were identified. Immunization with a strain of one immunotype induced specific protection against challenge with strains of the same immunotype but afforded little or no protection for immunotypically unrelated strains. Agglutinating activity in immune sera or chamber fluids, primarily due to pilus antibody, did not correlate with protection, but complement-dependent bactericidal activity usually did reflect immunity.

These findings indicate that: (i) immunotyping of strains from various clinical manifestations and geographical regions would identify new immunotypes and highly protective strains with the capacity to induce immunity to a broad spectrum of related challenge strains. (ii) Since the in vivo cross-protection model is technically elaborate and time consuming, the bactericidal assay with prototype antisera may be used to select cultures as candidates of new immunotypes for in vivo study. The present report shows that these projections have been substantiated by the isolation and identification of such strains

from a large number of gonococcal isolates. The immunological and serological attributes of these strains are discussed in relation to gonococcal immunity and vaccine development.

## MATERIALS AND METHODS

**Bacteria.** More than 300 strains of *Neisseria gonorrhoeae* were randomly selected from our culture collection accumulated over the last 2 years. They were originally sent to our laboratory for various tests and included cultures isolated in different locales of the United States, Europe, and the Far East from cases of disseminated gonococcal infection, uncomplicated gonorrhea, pelvic inflammatory disease, and asymptomatic carriers. Some strains were beta-lactamase producers (17). The nine prototype gonococcal strains a, b, c, d, e, f, g, h, and i were from our previous studies (3, 23).

The gonococci were grown on GC base medium enriched with IsoVitaléX (Baltimore Biological Laboratory, Cockeysville, Md.) overnight at 37°C in 5% CO<sub>2</sub>. Only piliated (P+) cell types were used for this study.

**Preliminary serological screening.** The nine typing sera from guinea pigs immunized with living cells of gonococcal strains a, b, c, d, e, f, g, h, and i, respectively, were from our previous study (23). Preliminary screening of the gonococcal cultures was performed with the nine typing sera by the complement-dependent bactericidal antibody assay as previously described (23), with one modification. The diluent for the present test was modified by the addition of 1%

polyvinylpyrrolidone (Sigma, St. Louis, Mo.) and 0.1 M MgCl<sub>2</sub> to reduce nonspecific cell lysis during the 90-min incubation period in microtiter wells. Strains which showed low degrees of activity or failed to react with the typing sera were selected for further study as potential new immunotypes.

The selected strains were used as antigens to prepare antisera in Hartley strain guinea pigs weighing approximately 300 g each. Colonies of piliated cells were suspended in phosphate-buffered saline, pH 7.4, with 1% gelatin and were standardized optically with a Leitz spectrophotometer at 535 nm to contain approximately 10<sup>8</sup> colony-forming units of gonococci per ml. The guinea pigs were injected weekly for up to 10 weeks by the subcutaneous route with 1 ml of the freshly prepared antigens. Beginning 1 week after the third injection, blood was obtained periodically by cardiac puncture with a 25-gauge needle from the animals under light CO<sub>2</sub> anesthesia.

For determining serological relatedness, a differential of fourfold or greater in bactericidal titer among the strains tested simultaneously in one assay was considered as serologically different. Included in each assay for cross-reference was a control consisting of a reference serum and a reference strain of gonococcus as antigen.

**Determination of immunoprotective properties of selected strains.** The gonococcal strains which were selected by the bactericidal assay as candidates for new immunotypes were tested for their ability to induce protection by the guinea pig subcutaneous chamber model (2) and the complement-enhanced immunity model in mice (5). Piliated cells from each of the selected strains were harvested into phosphate-buffered saline, and the suspension was optically adjusted to contain approximately 10<sup>8</sup> colony-forming units of gonococci per ml. Aliquots of 10% neutral formaldehyde were then added to the cell suspension to a final concentration of 0.1% formaldehyde. The suspension was held at 4°C for 72 h before use for immunization.

Groups of young adult Hartley strain guinea pigs were immunized subcutaneously with formaldehyde-treated cells in 1.0-ml amounts at four 1-week intervals. Four days after the last injection, a coiled stainless-steel chamber was implanted subcutaneously into each animal (2). One week after implantation, the resistance of the animals to intrachamber infection was assayed by challenging the chambers with gonococci as previously described (3). Three days after each challenge, a specimen of approximately 0.1 ml of chamber fluid was aspirated with a syringe and needle from each chamber, and 0.05 ml was streaked onto GC base agar. The plates were incubated at 36°C in 5% CO<sub>2</sub> for 24 to 48 h. Growth of gonococci was confirmed by the oxidase reaction, Gram stain, and, when necessary, sugar utilization tests (6). The growth of one or more gonococcal colonies from the chamber fluid was considered evidence of infection.

The 50% infectious dose of each challenge strain was determined for each group of guinea pigs by interpolation between the challenge dilutions giving greater than and less than 50% infection rates and was expressed as the log<sub>10</sub> of the colony-forming units of gonococci required to produce an infection of 3 days

or longer in duration in the subcutaneous chambers of each group of immunized and control guinea pigs.

Because of its comparative simplicity and inexpensiveness, the recently developed complement-enhanced immunity model in mice (5) was used simultaneously with the guinea pig subcutaneous chamber model for obtaining information on the protective properties of the selected gonococcal strains. By injecting exogenous complement into subcutaneous chambers, it is possible to determine the effects of complement-mediated immunity in an animal which is normally complement deficient (5). Adult female mice of the Institute of Cancer Research strain were immunized by intraperitoneal injection of formaldehyde-treated cells in 0.3-ml amounts at three 1-week intervals. Stainless-steel chambers were implanted subcutaneously into the animals within 1 week after the last immunization. One week after chamber implantation, a 0.1-ml aliquot of guinea pig complement (50% hemolytic complement units > 200) was injected into each chamber approximately 1 h before challenge with 0.1 ml of the appropriate gonococcal cell suspension, as previously described (5). Immunity as indicated by resistance to intrachamber infection was measured by the procedures previously described for the guinea pig subcutaneous chamber model.

Results on protection from the two animal models were analyzed statistically by the chi-square test (19).

## RESULTS

**Screening cultures for new immunotypes.** Immune sera to the nine gonococcal strains which were prepared in guinea pigs in a previous study (23) were used to screen cultures for new immunotypes by the bactericidal assay. Representative results are presented in Table 1. From more than 300 strains, five cultures, namely, N65, 196, 335, V1, and V2, were selected for further investigation because of the low levels or lack of cross-activities, which indicated antigenic differences between these cultures and the nine strains previously studied (3, 23).

Immune sera to the five selected strains were raised in guinea pigs, and their complement-dependent bactericidal antibody activities against homologous and heterologous prototype strains are summarized in Table 2. Strain differ-

TABLE 1. Representative results of screening gonococcal strains for new immunotypes by bactericidal assay

Strain no.	Bactericidal titer in typing sera:								
	a	b	c	d	e	f	g	h	i
N65	<2	<2	<2	<2	<2	<2	<2	<2	<2
187	16	<2	16	<2	16	256	64	<2	<2
196	<2	<2	<2	<2	<2	<2	<2	<2	<2
335	<2	<2	<2	<2	<2	<2	<2	<2	<2
339	512	<2	<2	<2	16	64	64	16	<2
V1	4	<2	<2	<2	<2	<2	<2	<2	<2
V2	<2	<2	4	<2	<2	<2	<2	<2	<2

ences in antigenicity were evident. Strains V1 and 196 were highly antigenic, inducing serum bactericidal antibody titers of 256 to the homologous antigens in animals after three weekly injections. In comparison, strains N65 and 335 were poor antigens. The bactericidal antibody titers in the animals immunized with either N65 or 335 did not exceed 16 throughout the experiment, which lasted for 11 weeks with 10 weekly injections of antigens and bleeding of animals 6 to 7 days after the 3rd, 7th, and 10th injections.

Antisera to strains V1 and V2 showed the broadest range of cross-activities with 14 test strains (Table 2). Although the V2 antiserum gave a titer of only 16 with homologous V2 cells, this appears to be due to the greater resistance of V2 cells to the *in vitro* effects of complement and bactericidal antibody, since strains N65, b, c, d, f, and g all gave titers of 16 or greater with the same antiserum.

Strains N65, 196, and V1 were isolated in

TABLE 2. *In vitro* antigenic relationship of new immunotypic candidate strains with various gonococcal strains

Strain no.	Bactericidal activities in typing sera:				
	N65	196	V1	335	V2
N65	16	<2	<2	<2	16
196	<2	256	<2	<2	8
V1	16	16	256	16	<2
335	16	<2	64	16	NT <sup>a</sup>
a	<2	<2	32	NT	<2
b	<2	<2	32	NT	64
c	16	<2	<2	NT	16
d	16	<2	<2	NT	64
e	<2	<2	16	NT	<2
f	<2	16	16	NT	16
g	<2	<2	<2	NT	16
h	<2	<2	<2	NT	<2
i	<2	<2	<2	NT	8
V2	8	<2	8	NT	16

<sup>a</sup> NT, Not tested.

Atlanta, Ga., and their antigenic relationship with other strains from Atlanta and with strains from the Philippines (17) was tested. Of the 41 randomly selected Atlanta isolates, strain N65 antiserum reacted with 19 strains, and sera against strain 196 and V1 reacted with 32 and 15 strains, respectively. Table 3 summarizes the cross-activities of N65 and V1 antisera with a group of cultures from the Philippines, including penicillin-sensitive and beta-lactamase-producing strains. Approximately 89% of the beta-lactamase producers and 64% of the penicillin-sensitive strains reacted with strain N65 and strain V1 immune sera. These results indicate the existence of a broad antigenic relationship and possibly cross-protecting properties among the strains isolated in the two geographical areas.

**Immunoprotective properties of selected strains.** The immunoprotective properties of strains N65, 196, V1, and V2 selected as candidates for new immunotypes by bactericidal screening were studied in animals by utilizing the subcutaneous chamber model in guinea pigs and the recently developed complement-enhanced immunity model in mice (5). These strains varied greatly in their ability to induce immune protection (Table 4). Immunization of guinea pigs with strain V1 induced >10<sup>5</sup>-fold more protection against homologous challenge compared with nonimmunized control animals. In contrast, similar immunization with strain

TABLE 3. *Broad antigenic relationship of two immunotypic candidate strains*

Strains (no. tested)	Bactericidal activity of immunotypic antisera (% strains tested)		
	V1	N65	V1 and N65
Penicillin sensitive (89)	36	8	20
Penicillinase producing (27)	52	22	15

TABLE 4. *Comparison of protective activities of strains selected as candidates for new immunotypes*

Subcutaneous chamber	Immunogen and challenge strain	Immunized		Nonimmunized		Significance of difference between immunized and nonimmunized animals	
		No. of tests	ID <sub>50</sub> <sup>a</sup>	No. of tests	ID <sub>50</sub>	χ <sup>2</sup> value	P
Guinea pig	N65	11	2.6	12	1.5	0.41	<0.70
	V1	45	>7.0	48	<1.7	>21.3	<0.001
Mouse	N65	16	6.3	12	<4.0	6.07	<0.02
	V1	22	7.0	12	<4.0	10.53	<0.01
	196	12	<4.0	12	<4.0	0.88	<0.50
	V2	57	>5.9	12	<2.9	>21.3	<0.001

<sup>a</sup> Infectious dose, 50% (ID<sub>50</sub>), was determined by interpolation between the challenge dilutions giving greater than and less than 50% infection rates and was expressed as the log<sub>10</sub> of the colony-forming units of gonococci in the challenge dose.

N65 induced only 10 times better protection than in the controls.

Comparable results were observed in mice and guinea pigs except that strain N65 appeared to be more protective in mice, with an increase of >100-fold in resistance in immune mice. Nonetheless, it was still significantly less protective than strain V1. Similarly, strain 196 was a poor vaccine in mice, whereas strains V1 and V2 were highly effective in inducing immunity in this species.

The immune specificity and spectrum of cross-protection induced by strains V1 and V2 in mice are summarized in Table 5. Strains V1 and V2 represented two distinct immunotypes with little cross-protection to each other. In addition, each strain offered a different spectrum of cross-protection to challenge with heterologous strains, and they were immunotypically quite different from the previously established immunotypes (3). Strain V1 protected against challenge with strains a and b as indicated in Table 5 but not vice versa (our unpublished data).

The spectrum of cross-protection induced by strain V1 against challenge with isolates from the Philippines is given in Table 6. The cultures that were reactive with strain V1 immune serum in the bactericidal assay as summarized in Table 3 were randomly selected to challenge the mice

TABLE 5. Immune specificity and spectrum of cross-protection of two distinct immunotypic strains

Challenge strain	Protection (ID <sub>50</sub> ) <sup>a</sup> induced by strain:			
	V1	V2	Homologous	Nonimmunized controls
V1	6.9 <sup>b</sup>	<2.9		<2.9
196	6.0	NT <sup>c</sup>	<4.0	<4.0
389	6.0	NT	NT	<3.5
a	>6.3	4.1	NT	<3.3
b	>6.8	NT	NT	<3.0
V2	<2.9	>5.9		<4.0
1930	3.7	>6.0	>6.0	<4.0
369	NT	5.7	>6.0	<4.0
c	<3.3	4.3	<3.9	<3.3
d	3.8	4.3	>6.9	<3.3
g	3.5	4.0	NT	3.0
N65	<3.5	<3.9	6.3	<4.0
67T	4.0	<4.0	<4.0	<4.0
e	<3.5	<3.5	NT	3.0

<sup>a</sup> Infectious dose, 50% (ID<sub>50</sub>), determined by interpolation between the challenge dilutions giving less than and greater than 50% infection rates with five or more test mice per challenge dilution and expressed as the log<sub>10</sub> of the colony-forming units of gonococci in the challenge doses.

<sup>b</sup> A differential of 10-fold or greater in ID<sub>50</sub> values between immunized and control groups was considered significant for immunogrouping of strains.

<sup>c</sup> NT, Not tested.

TABLE 6. Cross-immunity induced by immunotypic strain V1 to isolates from the Philippines

Challenge culture	Protected strains <sup>a</sup> / strains tested (%)
Penicillin susceptible	10/13 (77)
Beta-lactamase producers	12/12 (100)

<sup>a</sup> Fourfold or greater resistance in immunized mice as compared with nonimmunized controls.

which had been immunized with strain V1. Increased resistance to infection was demonstrated in all the animals challenged with the beta-lactamase-producing strains and in 77% of the animals challenged with the penicillin-sensitive strains.

## DISCUSSION

Gonococci are serologically and immunologically polymorphic (1, 3, 8-11, 14-16, 20, 21, 23, 24), and regional prevalence of certain serotypes has been reported (11, 21, 24). Although the mechanisms involved in human immunity to gonococcal infection are poorly understood and the disease does not seem to confer immunity against reinfection, the quest for vaccines against gonorrhoea has recently received much attention because of the rampant epidemic of the disease and the emergence of beta-lactamase-producing *N. gonorrhoeae* (13, 17) in many parts of the world. If immunity to gonorrhoea is type specific, serological and immunological diversity of the organism would be a major consideration in vaccine development and control through immunoprophylaxis.

Several findings of the present study may be pertinent to the understanding of gonococcal immunity and vaccine development. First, in addition to being heterogeneous in serotypes and immunotypes, gonococci varied greatly in their capacity to induce immunity in animals. Strains V1 and V2 were highly protective, inducing >10<sup>3</sup>- to 10<sup>5</sup>-fold resistance in immune animals, but strains 196 and N65 were poor vaccines. In fact, strain V1 induced better protection against challenge with strain 196 than immunization with strain 196 itself. Should this observation be relevant in human infection, the ability of the infecting strain to induce protection would be an important factor in determining disease-induced immunity.

Second, in vitro bactericidal activities (16, 23) and microimmunofluorescence typing (14, 21) generally correlated with the in vivo immunotyping (3) of gonococcal strains, but our results showed that serological cross-activities of certain strains did not ensure their in vivo protective capacity. Strain 196 induced reasonably high bactericidal titers in guinea pigs, but it was not protective in the in vivo challenge test. Serolog-

ically "senior" strains by microimmunofluorescence (21; S. P. Wang, personal communication) such as e, g, and h were less protective and induced cross-protection for fewer strains than did their "junior" strains such as a and c (3). Strains c and i appeared to be identical by microimmunofluorescence, but in the in vivo cross-protection test strain c was significantly more protective than strain i. In fact, immunization with strain i induced only weak protection against homologous challenge (3). These discrepancies require that a distinction be made between in vitro serotyping and in vivo immunotyping by cross-protection, and the latter is necessary for immunological characterization of gonococcal strains.

Third, the apparently extensive serological and immunological diversity of gonococci may not be as complex a problem as it seems in the development of vaccines. There exist strains with a high capacity to induce immunity to a broad spectrum of heterologous strains. Such a strain, V1, isolated in Atlanta, induced immunity to infection with immunotypically related strains isolated in the Philippines. It is possible that a combination of a few major immunotypic immunogens may offer substantial protection against a majority of the strains causing disease in humans.

Finally, the ability to produce beta-lactamase is not related to the immunotypic immunogen. Strain V1, which is sensitive to penicillin, protected against both beta-lactamase producers and penicillin-sensitive isolates. The finding that strain V1 protected against a higher percentage of beta-lactamase producers than penicillin-sensitive strains from the same geographic population may be related to the greater sensitivity of beta-lactamase producers to complement. It was observed that significantly more beta-lactamase producers were sensitive to complement activity than penicillin-sensitive strains (24). Recent studies in animals (4) and in humans (7, 18) have linked susceptibility of gonococcal strains to complement to virulence or invasiveness. In guinea pig chambers T1 cells of strain c were >1,000-fold more virulent than T3 cells of the same strain in an environment with high complement activity, but both cell types were about equal in virulence when the complement activity was low or absent (4). In studies of human infections, gonococci causing disseminated disease were more resistant to serum complement and were highly sensitive to penicillin (12, 22). As a corollary, beta-lactamase producers with a high sensitivity to complement should have less propensity to cause systemic infection, and if complement-mediated immunity could be in-

duced at the site of infection, beta-lactamase producers should be more susceptible to immunophylaxis.

The findings of this study emphasize the importance of using the appropriate strains for immunological studies and vaccine development. It is apparent that there are qualitative and quantitative differences in immunotypic immunogens of gonococcal strains. Investigation of properties of isolated immunotypic immunogens is in progress.

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