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Immunological and Serological Diversity of *Neisseria gonorrhoeae*: Gonococcal Serotypes and Their Relationship with Immunotypes

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Strains of gonococcus were shown to be immunologically heterologous. Serum bactericidal activity generally correlated with induced immunity to gonococcal challenge as detected by the guinea pig subcutaneous chamber model. Sera devoid of bactericidal activity reflected the lack of cross-protection in subcutaneous chambers. Factors affecting the bactericidal assay described in this report include (i) source of complement, (ii) concentration of test antigen and complement activity, and (iii) presence of calcium and magnesium ions and bovine serum albumin in diluent. Poor correlation was observed between agglutinating activity of the immune sera and protection.

Knowledge of immunological properties of gonococcal strains is essential both for the understanding of gonococcal immunity and for the development of vaccines. Immunological diversity of nine strains of *Neisseria gonorrhoeae* was demonstrated by cross-protection in guinea pig subcutaneous chambers (4). Since the procedure described for immunotyping is elaborate and is not suitable for large-scale screening or typing of clinical isolates, attempts were made to correlate the results of immunotyping with those obtained by experimental serotyping procedures. Our results are presented in this report.

MATERIALS AND METHODS

Bacterial cultures. The nine strains of *Neisseria gonorrhoeae* used in this study were described previously (4).

Gonococcal immune sera. Antisera for each of the nine gonococcal strains were prepared in Hartley strain guinea pigs weighing approximately 300 g each. The guinea pigs were injected weekly for 11 weeks by the subcutaneous route with type 1 (T1) living cultures containing 10^9 colony-forming units (CFU). Blood was obtained by cardiac puncture with a 25-gauge needle from animals under light anesthesia.

Bactericidal antibody assay. The appropriate gonococcal strains were grown on GC base (GCB) medium supplemented with IsoVitaleX (Baltimore Biological Laboratories, Baltimore, Md.) overnight at 37°C in a candle jar. Bacterial growth was suspended in glass tubes (13 by 100 mm) containing Trypticase soy broth (TSB) (30 g/liter of distilled water). The suspension was adjusted to an optical density of 0.5 units with a Leitz spectrophotometer at 535 nm and was then diluted 1:100 in TSB for use in the bactericidal assay. The diluted suspension contained approximately 10^6 CFU per ml.

The diluent used throughout the assay was phosphate-buffered saline (PBS), pH 7.2, containing 0.006% $MgCl_2 \cdot 6H_2O$, 0.004% $CaCl_2 \cdot 2H_2O$, and 1% bovine serum albumin. Guinea pig complement (Texas Biologicals, Fort Worth, Tex., lot 507) or fresh chimpanzee serum was used. Complement preparations were pretested to determine that the bacteria used were not nonspecifically killed in the complement. Complement was stored at −70°C until used.

All sera to be tested, including reactive and nonreactive controls, were heated for 30 min at 56°C. Serial twofold dilutions were made by using microtiter loops to transfer 0.025 ml of each serum and the controls to microtiter U-bottom plates (Ames, Elkhart, Ind.) containing 0.025 ml of 1:10 dilution of complement. As a serum control, 0.025 ml of the diluent (without complement) was added to 0.025 ml of each heated serum. For complement control, serial twofold dilutions of the complement were made in diluent by using 0.025-ml microtiter loops.

A 0.025-ml portion of the appropriate antigen suspension was added to each serum dilution and the controls, and the mixtures were incubated at room temperature for 90 min.

A micropipetting gun (Clay Adams, Parippany, N.J.) was used to deliver 10 μl of the reaction mixture to plates containing GCB with IsoVitaleX and vancomycin, colistin, and nystatin (7). The plates were air-dried for approximately 20 min at room temperature until the spots of samples were absorbed into the agar. They were then incubated at 37°C in a candle jar for 20 h. The serum dilution that yielded 50% or less gonococcal growth as compared with the antigen control was taken as the titer of the serum.

Serum macroagglutination test. Mouse-virulent T1 colonies of each gonococcal isolate were grown on

GCB plates and incubated for 18 h in a candle jar at 37%C. GCB plates having greater than 95% T1 colony growth were used to prepare suspensions of formalin-fixed gonococcal cells. The bacterial growth was removed with a sterile cotton swab and suspended in PBS, pH 7.4, containing 2.5% formalin. The suspension was adjusted to an optical turbidity of approximately a McFarland no. 5. The cell suspension was incubated at room temperature for at least 3 h before being used in the macroagglutination procedure.

Fourfold serial dilutions of the test and control sera were made in disposable Limbro trays (Flow Laboratories, Rockville, Md.) by using microtiter diluting loops (Cooke Engineering Co., Alexandria, Va.) to transfer 0.025 ml to each well containing 0.075 ml of PBS. In addition to known positive and negative sera, a PBS antigen control was included in each test. The test antigen suspensions were blended in a Vortex mixer for 10 s before 0.05 ml was dropped into the test wells. The autotrays were placed on a platform and rotated at 30 rpm in a 37°C incubator for 15 min. Each tray was then placed on a viewing stand and observed with an overhead light for macroagglutination in the test wells.

RESULTS

Bactericidal activity of immune guinea pig sera. Serum bactericidal titers generally correlated with increased resistance to gonococcal challenge as determined in a previous paper (4) by using the guinea pig subcutaneous chamber model (Table 1). Titers of 32 or above were generally associated with protection; minor exceptions were observed with strains b, f, and h. Strain d reacted with all the nine immune sera, but protection was observed primarily with the homologous organism and, to a much less extent, with challenge strains e and f. Protection in subcutaneous chambers was negligible when the cross-reacting bactericidal titer was 8 or less. Strain f cross-reacted in bactericidal assay with all the immune sera except strain d, but titers of 16 or less were not indicative of protection. Strain g cross-reacted with h antiserum, but no cross-protection was detected between these two strains.

Sera devoid of bactericidal activity except with strain b invariably reflected the lack of cross-protection in subcutaneous chambers among the strains tested. Consistent with immunotyping results (4), the immune sera were most active against the homologous antigen in bactericidal assay, which indicates antigenic heterogeneity of the gonococcal strains.

Factors affecting bactericidal results in the described procedure include (i) source of complement, (ii) concentration of test antigen and complement activity, and (iii) presence of calcium and magnesium ions and bovine serum albumen in diluent.

The effect of complement from two animal species on bactericidal activity is summarized in Table 2. Guinea pig complement generally yielded higher strain-specific titers than chimpanzee complement. Table 3 indicates that the source of complement affected the specificity of the immune sera. More low-grade cross-reactions were observed with chimpanzee comple-

TABLE 2. *Effect of complement from different animal species on bactericidal activity of antisera against homologous strains*

Antigen	Titer[a] with complement from:	
	Chimpanzee	Guinea pig
a	16	64
b	16	64
c	32	16
d	64	128
h	16	128

[a] Titers are expressed as the end-point dilution factor of the serum dilution.

TABLE 1. *Relationship of serum bactericidal activity[a] and immunotyping[b]*

Test antigen (bactericidal activity) or challenge (immunotyping)	Bactericidal activity/protection								
	a	b	c	d	e	f	g	h	i
a	64/2+	0/−	8/−	0/−	0/−	0/−	0/−	4/−	0/−
b	64/4+	16/4+	0/2+	0/−	0/1+	32/4+	0/−	32/4+	0/−
c	0/−	0/−	16/4+	0/−	0/−	0/−	16/−	8/−	8/−
d	16/−	4/−	8/−	128/4+	16/1+	16/1+	16/−	8/−	16/−
e	0/−	0/−	0/−	0/−	64/2+	0/−	0/−	0/−	0)−
f	128/4+	32/2+	32/1+	0/−	16/1+	64/4+	16/−	64/4+	16/−
g	0/−	0/−	4/1+	4/−	0/−	4/−	128/2+	64/−	0/−
h	128/2+	8/−	0/−	0/−	0/−	64/1+	8/−	128/4+	0/−
i	0/−	0/−	16/4+	0/−	0/−	0/−	0/−	4/−	16/3+

[a] Titers are expressed as the end-point dilution factor of the serum dilution.

[b] Protection is graded according to the base 10 logarithm of the median infecting dose in CFU of gonococci as determined in a previous study by using the guinea pig subcutaneous chamber model: 0 to 2.5, negative (−); 2.6 to 3.5, 1+; 3.6 to 4.5, 2+; 4.6 to 5.5, 3+; >5.6 = 4+.

ment than with guinea pig complement. In comparison, guinea pig complement yielded specificity that correlated well with increased resistance in the guinea pig model.

The effect of antigen and complement concentrations on bactericidal activity is presented in Table 4. The optimal concentration of antigen in relation to sensitivity appeared to be 2.5×10^4 to 2.5×10^3 CFU per reaction well. Higher concentrations of antigen decreased sensitivity. Complement concentration did not affect sensitivity except when the dilution was higher than 1:10.

Agglutinating activity of immune sera in relation to protection. Agglutinating activity of the immune sera did not correlate well with protection (Table 5). Strain a agglutinated in a, c, and i immune sera, but protection was observed only with the homologous strain a challenge. Strain b did not agglutinate in a, c, and h antisera, but there was significant cross-protection in guinea pigs immunized with these heterologous strains. Strain c agglutinated in e, h, and i antisera with no detectable cross-protection. Guinea pigs immunized with strains, a, c, e, or h were protected against challenge with strain f with no observable agglutinating activity of strain f in the respective heterologous antisera. Although strain i agglutinated to the same extent in a and c antisera, animals immunized with c were highly protected against i challenge, but had no detectable immunity to strain a. Agglutinating activity of antisera was not indicative of cross-protection.

Poor correlation was observed between agglutinating and bactericidal activities of the immune sera (Table 6). When strain a was used as antigen, a and c antisera were shown to have an agglutinating titer of 256, but bactericidal activity of the a antiserum was eight times higher than that of the c antiserum. Antiserum against i agglutinated strain a at a titer of 64 with no demonstrable bactericidal activity. Antisera a and h had high bactericidal activity against strain b although agglutinating activity was absent. Antiserum a was highly bactericidal to strains f and h with no agglutinating

TABLE 3. *Effect of complement from different animal sources[a] on specificity of immune sera[b]*

Antigen	Antisera																	
	a		b		c		d		e		f		g		h		i	
	CH	GP	CH	GP	CH	GP	CH	GP	CH	GP	CH	GP	CH	GP	CH	GP	CH	GP
a	16	64	0	0	8	0	0	0	8	0	8	0	8	0	8	4	8	0
b	16	64	8	16	0	0	0	0	0	0	16	32	0	0	16	32	4	0
c	0	0	0	0	32	16	8	0	0	0	8	0	16	16	16	8	16	8
d	16	16	0	4	8	8	64	128	16	16	16	16	8	16	8	8	8	16
e	0	0	0	0	0	0	0	0	16	64	4	0	0	0	0	0	0	0
f	32	128	4	32	16	32	8	8	8	16	32	64	16	16	16	64	8	16
g	0	0	0	0	8	4	0	4	0	0	0	4	128	128	8	64	0	0
h	16	128	4	8	4	0	4	0	0	0	8	64	48	—	16	128	0	0
i	8	0	0	0	16	16	0	0	0	0	0	0	8	0	8	4	16	16

[a] CH, Chimpanzee complement; GP, guinea pig complement.
[b] Titers are expressed as the end-point dilution factor of the serum dilution.

TABLE 4. *Effect of antigen and complement concentration on bactericidal activity of guinea pig immune sera*

Dilution of complement (chimpanzee)	Titer[a]				
	2.5×10^{6}[b]	2.5×10^{5}	2.5×10^{4}	2.5×10^{3}	2.5×10^{2}
e serum against e antigen					
1:5	0	16	16	16	NT[c]
1:10	0	16	16	16	NT
1:20	0	8	32	16	NT
1:40	0	0	8	16	NT
1:80	0	0	0	0	NT
c serum against c antigen					
1:5	NT	0	32	64	32
1:10	NT	0	16	32	16
1:20	NT	0	16	32	32
1:40	NT	0	±	±	±

[a] Titers are expressed as the end-point dilution factor of the serum dilution.
[b] CFU used as test antigen.
[c] NT, Not tested.

TABLE 5. *Relationship of serum agglutinating activity and immunotyping*

Test antigen (agglutinating activity) or challenge (immunotyping)	Agglutinating antibody titer[a]/protection[b]								
	a	b	c	d	e	f	g	h	i
a	256/2+	0/−	256/−	0/−	0/−	0/−	0/−	0/−	64/−
b	0/4+	64/4+	0/2+	0/−	0/−	64/4+	0/−	0/4+	0/−
c	0/−	0/−	256/4+	0/−	64/−	0/−	0/−	16/−	64/−
d	0/−	0/−	0/−	256/4+	0/1+	0/1+	0/−	0/−	0/−
e	0/−	0/−	0/−	0/−	256/2+	0/−	0/−	0/−	0/−
f	0/4+	256/2+	0/1+	0/−	0/1+	256/4+	0/−	0/4+	0/−
g	0/−	0/−	0/1+	0/1+	0/1+	0/1+	64/2+	0/−	64/−
h	0/2+	0/−	0/−	0/−	16/−	0/1+	0/−	256/4+	0/−
i	64/−	16/−	64/4+	0/−	0/−	0/−	0/−	16/−	64/3+

[a] Titers are expressed as the end-point dilution factor of the serum dilution.

[b] Protection is graded according to the base 10 logarithm of the median infecting dose in CFU of gonococci as determined in a previous study by using the guinea subcutaneous chamber model: 0 to 2.5, negative; 2.6 to 3.5, 1+; 3.6 to 4.5, 2+; 4.6 to 5.5, 3+; >5.6, 4+.

TABLE 6. *Relationship of bactericidal and agglutinating activities of gonococcal immune sera*

Test antigen	Immune sera (bactericidal/agglutinating)[a]								
	a	b	c	d	e	f	g	h	i
a	64/256	0/0	8/256	0/0	0/0	0/0	0/0	4/0	0/64
b	64/0	16/64	0/0	0/0	0/0	32/64	0/0	32/0	0/0
c	0/0	0/0	16/256	0/0	0/64	0/0	16/0	8/16	8/64
d	16/0	4/0	8/0	128/256	16/0	16/0	16/0	8/0	16/0
e	0/0	0/0	0/0	0/0	64/256	0/0	0/0	0/0	0/0
f	128/0	32/256	32/0	8/0	16/0	64/256	16/0	64/0	16/0
g	0/0	0/0	4/0	4/0	0/0	4/0	128/64	64/0	0/64
h	128/0	8/0	0/0	0/0	0/16	64/0	8/0	128/256	0/0
i	0/64	0/16	16/64	0/0	0/0	0/0	0/0	4/6	16/64

[a] Titers are expressed as the end-point dilution factor of the serum dilution.

activity. When homologous antigens were used, all nine immune sera were demonstrated to have bactericidal and agglutinating activities.

DISCUSSION

Information concerning the heterogeneity and the complexity of gonococcal antigens is essential for defining gonococcal immunity and for exploring the possibility of a gonococcal vaccine. Antigenic heterogeneity of gonococcal strains has been demonstrated in several recent in vitro studies. Glynn and Ward (5) used a bactericidal system to divide 60 strains of gonococci into four distinguishable but overlapping groups. Employing a similar bactericidal system, Tramont and associates (8) demonstrated antigenic differences and similarities among gonococcal strains and cross-reactivity between *N. gonorrhoeae* and *N. meningitidis*. By use of an indirect fluorescent-antibody procedure, O'Reilly and associates (6) identified several broadly reactive strains sharing antigenic properties common to most gonococci but without significant cross-reactions with other species of *Neisseria*. In a previous publication (4), we reported immunological characteristics of nine gonococcal strains as demonstrated in guinea pig subcutaneous chambers (1). Distinct immunotypes were established, and the potential usefulness of immunotyping for epidemiological work and for understanding of gonococcal immunity was discussed.

In the present study, a close correlation between immunotypes and bactericidal activity of immune sera was demonstrated. The profile of cross-protection generally paralleled bactericidal activity of the immune sera. Discrepancies were observed with three strains at low cross-reacting titers between 4 and 16, which may be attributed to the difference in sensitivity of the in vivo immunotyping and the in vitro bactericidal systems, and to the fact that some gonococcal strains were more susceptible to complement-dependent bactericidal activity than others. Immune sera with bactericidal titers of 32 or above were invariably indicative of in vivo cross-protection; however, titer figures should not be overemphasized since they may

be related to the sensitivity of the particular gonococcal strains to the complement used in the assay.

The effects of complement preparations from two different animal species on specificity and titer of the immune sera were interesting. Larger numbers of low grade cross-reactions were observed with chimpanzee complement than with guinea pig complement. In addition, guinea pig complement generally yielded higher bactericidal activity of the immune sera than did chimpanzee complement.

Agglutinating activity did not correlate with protection, nor did it parallel bactericidal activity. The presence of pili on the cells probably was largely responsible for the agglutinating activity, since sera from guinea pigs immunized with T1 cells did not agglutinate T3 cells of the same strain (2). The inability of pili to confer immunity in guinea pigs has been reported by Turner and Novotny (9). The protective role of pili in chimpanzees has not yet been conclusively demonstrated.

With sera from chimpanzees immunized with T1 bacterin, Arko and associates (3) reported that serum bactericidal tests correlated most closely with the resistance of individual chimpanzees challenged in the pharynx and urethra with graduated doses of gonococci 1 month after the last immunization. These findings are consistent with our results obtained with the guinea pig subcutaneous chamber model, and they provide further evidence that bactericidal antibody is probably significant in induced immunity of animals to gonococcal infection.

The immunotyping system previously described (4) yielded valuable epidemiological and immunological information on gonococcal strains. Undoubtedly, more immunotypes will be established as the work continues, but this system is too elaborate for use in the mass screening of clinical isolates. We are using the bactericidal system described in this report for preliminary grouping of isolates from a defined clinical population. Confirmation of a new immunotype is performed in animals.

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