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Immunological and Serological Diversity of *Neisseria gonorrhoeae*: Immunotyping of Gonococci by Cross-Protection in Guinea Pig Subcutaneous Chambers

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An in vivo typing system for studying the immunological relationship of gonococcal strains was established. Nine gonococcal strains of proven virulence for guinea pig subcutaneous chambers were selected, and these isolates were used to immunize groups of guinea pigs that were subsequently cross-challenged with graduated numbers of gonococci from these isolates. Resistance to infection was determined by culture of fluid from challenged chambers; results were expressed as the median dose, in colony-forming units, of gonococci required to produce infection in each group of immunized guinea pigs. This information was then used to develop immunotypes of gonococci based on the cross-protection results obtained. Four cross-protecting immunotypes were established from the preliminary nine strains tested.

The recent development of infection models in lower animals (1, 3, 8, 9, 12) has provided investigators with a mechanism for elucidating the role of acquired or induced immunity in gonococcal infections. Although urogenital infection in the chimpanzee most closely resembles human gonorrhea (7), chimpanzees are not readily available for use in large-scale immunological experiments. Because of the ready availability of guinea pigs and the close correlation of previously obtained infection data on these animals with that obtained on chimpanzees (4, 5), we selected the guinea pig subcutaneous chamber model of gonococcal infection to study immunological similarities and diversities of gonococcal strains. This animal model has been used successfully in other studies designed to demonstrate the virulence of gonococcal colony types for subcutaneous chambers (3, 16), to study the development and passive transfer of induced immunity (3, 13), and to obtain preliminary data on the relative strain specificity of gonococcal isolates (3, 15).

The purposes of the present study were to obtain data concerning immunological similarities and differences among a number of gonococcal isolates from various clinical sources and to establish a procedure for the study of gonococcal immunotypes.

MATERIALS AND METHODS

Experimental animals and gonococcal strains. Forty adult male, Hartley strain guinea pigs were received from the Lawrenceville facility of the Center for Disease Control at 1-week intervals for 9 weeks. They were randomly divided into 10 groups of 4 animals each, and 9 groups were used for immunization with different gonococcal strains. One group was not immunized, but was held for use as challenge controls for determining the median infecting dose (MID) in normal animals. Although the study involved approximately 360 guinea pigs, in the series of tests conducted over a 3-month period, no more than 40 animals were challenged at any one time.

Gonococci used in this study were from various sources; strains A, B, C_6, and G were isolated in 1974 from the urethra of different male patients seen at venereal disease clinics in Atlanta, Ga. Strains N9, KF(F62), and 2686 are isolates used in previous studies (1, 10). Strain WX was obtained by J. Kwapinski, Winnipeg, Canada, and WJ1 was isolated at the University of Virginia from the heart valve of a parient with a disseminated gonococcal infection. The nine strains, A, B, C_6, G, N9, KF, 2686, WX, and WJ1 (hereafter referred to as a, b, c, d, e, f, g, h, and i) were streaked onto agar plates (100 by 15 mm) of GC base (GCB) medium enriched with IsoVitaleX, with or without selective vancomycin, colistin, and nystatin inhibitors (Baltimore Biological Laboratories, Cockeysville, Md.) (14). After incubation for 20 h at 36°C in a candle jar, the plates were examined with a Spencer dissecting microscope for bacterial colonies. The type 1 (T1) colonies (10, 11) from each strain were tested for animal virulence by inoculating subcutaneous chambers in guinea pigs (3). Five days later, 0.1 ml of fluid was withdrawn with a needle and syringe from each chamber and was cultured on GCB agar for 20 h at 36°C in a candle jar. The resulting gonococcal growth was suspended in Trypticase soy broth (TSB, 30 g/liter of distilled water with 10% glycerin) and stored in 0.2-ml amounts in glass vials at −70°C for seed cultures.

Animal immunization and tests for cross-protec-

tion. Seed vials of the nine frozen gonococcal strains were thawed, and each strain was streaked onto four agar plates of GCB medium. After incubation as previously described, the T1 cells from each isolate were harvested with a sterile cotton-tipped swab and suspended in sterile phosphate-buffered saline, pH 7.4, with 1.0% gelatin. The optical density (OD) of each antigen suspension was standardized in glass tubes (13 by 100 mm) to 0.4 OD units with a Leitz spectrophotometer at 535 nm. This was determined by culture to contain approximately $10^{8.0}$ colony-forming units (CFU) of gonococci per ml. Living T1 cells from each of the different isolates were used to immunize nine groups of guinea pigs by giving 1.0-ml subcutaneous injections to each animal at four 1-week intervals.

Seven days after the fourth immunization, a coiled stainless-steel chamber (10 by 20 mm) was implanted subcutaneously into each guinea pig by procedures previously described (2). Eight days after implanation, the resistance of each guinea pig to intrachamber infection was tested. Stock T1 cell suspensions of each challenge isolate were prepared in glass tubes (13 by 100 mm) containing 4.5 ml of TSB. The OD of the TSB was set at 1.0 units with the Leitz spectrophotometer at 535 nm and then was adjusted to 0.5 units by adding the challenge strain cells with a cotton-tipped swab. Serial 10-fold dilutions of the stock suspension were prepared in TSB to a $10^{-6.0}$ level. The initial challenge of each guinea pig group consisted of injecting 0.2-ml amounts from the $10^{-6.0}$ dilution into the subcutaneous chamber of each animal. Quantitation of the challenge inoculum was made by culturing 0.1 ml from the $10^{-5.0}$ and $10^{-6.0}$ dilutions on GCB agar. After incubation as previously described, the number of colonies on each plate was counted and the challenge dose of gonococci given each guinea pig was determined in CFU.

The MID of each challenge strain was determined for each group of guinea pigs. When the lowest challenge dose of gonococci produced greater than 50% infection rates, the MID was calculated by interpolation between zero and the dose tested.

Three days after the initial challenge, approximately 0.1 ml of chamber fluid was withdrawn from each animal with a syringe and needle, and 0.05 ml was streaked onto agar plates of GCB. The streaked plates were incubated as before and visually examined the following day. Suspect bacterial growth was confirmed as *Neisseria gonorrhoeae* by the oxidase reaction, by Gram stain, and, when required, by sugar fermentation tests (6). The growth of one or more gonococcal colonies from the streaked chamber fluid was considered evidence that the individual guinea pig had not developed demonstrable immunity. Culture-negative chambers were rechallenged the following day with a 10-fold increase in CFU of gonococci. The duration of the infection in culture-positive chambers was monitored by making cultures at 3-day intervals until two negative test results were obtained.

RESULTS

Results for the guinea pig cross-challenge tests are expressed as the $\log_{10}$ of the MID in CFU of gonococci required to produce a gonococcal infection of 3 days' duration or longer in the subcutaneous chambers of each group of immunized guinea pigs (Table 1). The level of resistance to infection induced by the described course of immunization was significantly different ($P < 0.001$) among gonococcal strains, by the median test. However, due to the wide variation in levels of resistance observed after immunization with the nine strains and with only four animals in each challenge block, further statistical comparison is limited. Guinea pigs immunized and challenged with strain g were only 25 times more resistant than the control animals, and cross-resistance with other challenge strains was not increased. However, post-immunization resistance with strains a and c was approximately 1,000 times that of the controls, and significant cross-resistance with other isolates was observed.

Immunization with strain a increased resistance to infection with strains a, b, f, and h to higher levels than was observed with the controls. Immunization with strain c increased resistance to infection with strains c, b, f, and i, and increased resistance to strain g to as high a level as the homologous g immunization induced. In addition, immunization with strain c induced approximately 25 times greater resistance to strain i infection than did immunization with strain i. In contrast, immunization of guinea pigs with strain d and i increased resistance to infection with the homologous organism to higher levels than were found in the controls but did not increase resistance to the other eight isolates.

Table 2 shows the median number of days

TABLE 1. *Median $\log_{10}$ resistance[a] of immunized and control guinea pigs to cross-challenge with nine gonococcal isolates*

Challenge strains	Gonococcal strains used for immunization of animals[b]									Controls
	a	b	c	d	e	f	g	h	i	
a	3.9	1.1	1.1	1.0	1.0	1.1	1.1	1.0	1.1	1.0
b	6.7	7.0	4.0	1.5	3.0	7.0	1.7	7.0	1.5	1.5
c	2.4	2.4	5.7	2.3	2.4	2.4	2.3	2.3	2.3	2.3
d	2.1	2.1	2.1	7.3	3.3	2.9	2.3	2.2	2.1	2.1
e	1.0	1.0	1.1	1.0	4.3	1.0	1.0	1.3	1.1	1.0
f	6.3	4.3	3.3	1.3	3.3	7.6	1.3	6.3	1.8	1.3
g	2.5	2.4	3.8	2.4	2.7	2.5	3.8	2.5	2.5	2.4
h	4.3	2.0	1.7	1.7	1.7	2.8	1.7	7.3	2.0	1.7
i	2.1	2.1	6.3	2.3	2.1	2.1	2.3	2.5	4.9	2.0

[a] Each table value represents the base 10 logarithm of the median, minimum infecting dose of gonococci in CFU and was calculated from the results obtained by challenging subcutaneous chambers of four guinea pigs in each block with graduated numbers of CFU.

[b] Animals of each group received four 1.0-ml injections containing approximately $10^{8.0}$ CFU of the respective isolate of gonococci. There were 36 guinea pigs immunized with each isolate.

TABLE 2. *Number of days that gonococcal infections persisted in immunized and nonimmunized groups of guinea pigs challenged in subcutaneous chambers with nine different strains of N. gonorrhoeae*

Challenge strain	Median no. of days of infection[a] in immunized and control groups of animals[b]									Controls
	a	b	c	d	e	f	g	h	i	
a	6.5	17.0	9.5	9.5	17.0	9.5	6.5	11.0	8.0	12.5
b	8.0	0.0	9.5	9.5	9.5	0.0	11.0	0.0	11.0	14.0
c	8.0	9.5	5.0	14.0	11.0	11.0	9.5	9.5	11.0	11.0
d	14.0	11.0	23.0	0.0	11.0	8.0	6.5	9.5	12.5	11.0
e	12.0	17.0	9.5	18.5	4.0	20.0	14.0	6.5	17.0	14.0
f	0.0	8.0	8.0	11.0	9.5	2.5	9.5	11.0	9.5	9.5
g	9.5	11.0	6.5	11.0	6.5	9.5	8.0	9.5	9.5	17.0
h	14.0	9.5	14.0	12.5	14.0	8.0	12.5	0.0	11.0	20.0
i	9.5	14.0	0.0	9.5	9.5	9.5	12.5	8.0	6.5	9.5

[a] Persistence of infection was determined by culture at approximately 3-day intervals.

[b] Each table value represents the median number of days that infection persisted with each strain of gonococci and was calculated for each block from the culture results obtained with four guinea pigs.

that gonococcal infections persisted in the immunized and nonimmunized control groups. The medians ranged from 9.5 to 20 days in the nonimmunized groups challenged with the nine gonococcal strains. Immunized groups with various degrees of protection (Table 1) remained infected for a shorter period than the nonimmunized controls that were infected with the respective challenge strains. Protection (MID) corresponded to some degree with the median number of days that gonococcal infections persisted in the animals.

DISCUSSION

The results of this study demonstrated the feasibility of immunotyping gonococci in the guinea pig subcutaneous chamber model. In addition, other important immunological characteristics of the gonococcal strains, including specific protection, cross-protection, and immunogenicity, were ascertained. Guinea pigs immunized with either strains a, c, or h demonstrated increased resistance to infection with three or more heterologous strains; however, guinea pigs challenged with a, c, d, and e showed increased resistance only when immunized with the respective homologous strains. This finding indicates that, in addition to the antigen(s) that these strains shared with other related strains, specific antigens had to be present for homologous protection.

Under similar conditions of immunization, some strains stimulated greater protective immunity than others. Guinea pigs immunized with strain g showed only a slight increase in resistance to challenge with strain g or any of the other eight strains. Moreover, immunization with strain c gave protection to challenge with strain g which was equal to that provided by immunization with strain g and provided better protection to challenge with strain i than did immunization with strain i.

In this study, immunization with strains a, c, d, or e induced 400 times or greater cross-immunity to the nine strains tested. It may be possible to find strains that have the capability of inducing immunity to a larger number of strains so that the required number of immunotypes necessary for developing an effective typing system can be reduced. This in vivo procedure, however, may not be suited to the large-scale screening and typing of clinical isolates of gonococci. Therefore, the results of this study are being correlated with those of experimental serological tests for the immunological typing of gonococcal strains which are now in progress.

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