Development and Passive Transfer of Immunity to Gonococcal Infection in Guinea Pigs

R. W. SCALES AND S. J. KRAUS

Center for Disease Control, Atlanta, Georgia 30333

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Gonococcal infections of relatively long duration were produced in guinea pigs with minimal infective doses ranging from 10^1 to 10^2 colony-forming units. After spontaneous eradication and upon rechallenge with $\geq 10^8$ gonococcal colony-forming units, guinea pigs were refractory to infection. Serum from these guinea pigs was bactericidal in vitro and protected virgin guinea pigs from in vivo challenge. The 7S peak, but not the 19S peak, from gel filtered immune serum demonstrated both bactericidal and passive protective properties. In vitro bactericidal activity of whole immune serum and its 7S peak was abolished after heating at 56 C for 30 min. The in vivo protective ability of the 7S peak was not abolished after heating.

Previous studies have indicated that some degree of an immune response, both cellular and humoral, develops in man after natural gonococcal infections (5, 7). However, repeated infection in human males is not uncommon. These perplexing data together with the lack of a susceptible laboratory animal (with the exception of the chimpanzee [3, 10]) have resulted in an inadequate understanding of the immune mechanism(s) involved in gonococcal infection. Recently (1) it was demonstrated that gonococci will grow in chambers subcutaneously implanted in small laboratory animals. The infection of laboratory animals and humans may or may not have similar mechanisms of pathogenesis. However, the small animal model does provide an avenue for studying those immunologic factors responsible for preventing in vivo growth in gonococci.

In this investigation, the first in a series concerned with immunity to gonococcal infection in the guinea pig, we describe immunity to reinfection after spontaneous eradication of the gonococcal infection. This immunity is accompanied by the development of complement-dependent bactericidal antibody. This antibody protects against in vivo challenge with the gonococci.

MATERIALS AND METHODS

Animals. A technique similar to that previously described was used to insert subcutaneous chambers in Hartley strain guinea pigs weighing 750 g each (1). The technique was modified by inserting six chambers per animal; instead of wire coils, the chambers were polyethylene tubes that measured 2.5 cm in length and had a diameter of 1.25 cm. Two weeks were

allowed between chamber insertion and bacterial challenge.

Neisseria gonorrhoeae. Isolate 2868, colony type 2 (6), was initially received from D. Kellogg (Center for Disease Control, Atlanta, Ga.) in 1971 and has been used in several previous reports (4, 7, 15). This gonococcus was grown on GC base media supplemented with Isovitalex® (Baltimore Biological Laboratories) (GCB medium) in a candle extinction jar for 18 h at 36.5 C. Cells were removed from GCB medium with sterile cotton-tipped applicators and suspended in Eagle minimal essential medium (MEM) containing Eagle balanced salt solution. The suspension was adjusted to an optical density of 0.3 at 530 nm. Serial logarithmic dilutions were made in MEM, and the number of gonococci in the suspensions was determined by plating 0.1-ml portions of the higher dilutions on GCB medium and incubating as described above. Colonies were counted on the plate. The number of gonococci in the undiluted sample with an optical density of 0.3 was approximately 10^9 colony-forming units (CFU) per ml.

Initial animal infections. Six guinea pigs were used, and 0.1 ml of inoculum was injected into each of the subcutaneous chambers. The minimal infective dose (MID) in this guinea pig model was determined by placing in each chamber a different number of gonococci: 10^1 , 10^2 , 10^3 , 10^5 , 10^7 , and 10^8 CFU. Every 2 weeks 0.05 ml of fluid was removed from the chambers, streaked on GCB medium, and incubated as before. Gonococci were identified by colony morphology (6), oxidase reaction (1% solution of *N,N*-dimethyl-*p*-phenylenediamine monohydrochloride), and Gram stain. The quantity of growth was estimated and designated 4+ for confluent growth, 3+ for confluent growth in some areas but discrete colonies in other areas, 2+ for discrete colonies >50 CFU, 1+ for <50 CFU, and 0 for no growth. Specimens from the animals were cultured until spontaneous eradication of the infection occurred in all six chambers. Two

consecutive negative cultures were considered proof that there was no longer any infection.

Test of postinfection immunity. The animals were rechallenged, and specimens were cultured in a manner similar to that used in the initial infection except the challenges were initiated at 10^1 CFU and progressed in consecutive log units until reinfection occurred or a maximal challenge dose of 10^8 CFU was administered. If reinfection occurred, the maximal resisted dose was the inoculum just prior to reinfection and according to protocol was one log less than the reinfesting dose.

Collection, titration, and fractionation of sera. After the test of postinfection immunity, 5 ml of blood was obtained by cardiac puncture under general anesthesia with 0.5 ml of fentanyldroperidol. These bleedings were continued weekly until the desired quantity of sera was obtained. Similar bleedings were done on other guinea pigs that had not been infected with *N. gonorrhoeae*. Normal or immune guinea pig serum was fractionated on a column (2.5 by 90 cm; flow rate of 15 ml/h) of Sephadex G-200 equilibrated with 0.1 M tris(hydroxymethyl)aminomethane-hydrochloride, 0.2 M NaCl, 0.02% sodium azide. Sera were made 5% with respect to NaCl for top loading under buffer, and 5-ml fractions were collected. The volume of pooled fractions was reduced to that of the original serum sample by negative-pressure dialysis. All fractions were dialyzed exhaustively against phosphate-buffered saline to remove azide. Before the samples were injected into animals, they were sterilized by passage through a 0.22- μ m membrane filter (Millipore Corp.). Normal or immune sera and fractions were assayed for agglutinins by passive hemagglutination (9). Immunoelectrophoresis was performed according to the method of Scheidegger (14).

In vitro assay of serum bactericidal activity. One- and two-tenths milliliters of test sera or serum fraction was mixed with 0.3 ml of gonococci (isolate 2868, colony type 2) suspended in MEM, and the mixture was incubated at 36.5 C for 1 h. MEM or normal guinea pig serum was substituted for the sera or serum fractions in controls. A sample of the reaction mixture was then removed with a 24-gauge platinum wire inoculating loop that had an internal diameter of 3 mm. This sample was streaked onto GCB medium and incubated, and colonies were enumerated as mentioned before.

Passive transfer of protection. Mixtures of gonococci and test sera or fractions were prepared exactly as in the bactericidal assay. One-half milliliter of each suspension was injected into the subcutaneous chambers of guinea pigs not previously exposed to *N. gonorrhoeae*. These chambers were cultured as before except the initial culture was obtained 3 days after inoculation and a second culture was obtained 10 days after inoculation.

RESULTS

Infection and postinfection immunity. Before the passive transfer experiments were attempted it was necessary to ascertain dose requirements for infection and to establish the

existence of immunity. As shown in Table 1, infections were produced with as few as 10 gonococci. Subsequent experiments demonstrated that the MID for single infections was 10 to 100 CFU/ml (Kraus, unpublished data). This value is the same as the MID observed in multiple simultaneous infections. The duration of the infections showed animal variation and ranged from 45 to 110 days. However, after these infections were allowed to naturally terminate, rechallenge doses were increased tremendously ($\geq 10^7$ CFU), and in some animals infection did not occur with the maximal dose used.

Gel filtration and hemagglutinins. Experiments were performed with whole immune serum or fractions to determine whether protection could be passively transferred. By immunoelectrophoresis, the 19S peak did not show any material characteristic of immunoglobulin G (IgG), nor did the 7S peak show material characteristic of immunoglobulin M (IgM). The hemagglutinin titer of whole serum was represented almost totally by the 7S peak.

Bactericidal activity. Initially as a control for contamination and colony counts, the mixture of gonococci and the appropriate test sample were planted on GC base just prior to animal injection. In doing this, in vitro bactericidal activity was observed. Table 2 shows the results of immune serum, fractions, and the appropriate controls either heated (56 C, 30 min) or unheated. Gonococci suspended in either MEM or normal guinea pig serum grew at all concentrations used. A definite effect at the highest concentration and no growth at the lowest concentration of gonococci were observed with human serum. The 19S peak, whether heated or unheated, did not appear to have bactericidal properties. The 7S peak, however, had a bactericidal effect equivalent to whole immune serum. This effect was abolished after

TABLE 1. Initial infection and test of postinfection immunity

Guinea pig	Initial infection		MRD or MCD rechallenge ^a (CFU)
	MID (CFU)	Infection duration (days)	
A1	10^2	98	10^8 MCD
F2	10	101	10^8 MCD
F3	10^2	61	10^8 MCD
J4	10^2	59	10^8 MCD
J6	10	110	10^8 MRD
R2	10	45	10^8 MCD

^a MRD, Maximal resisted dose; MCD, maximal challenge dose.

heating at 56 C for 30 min.

Passive transfer of protection. Table 3 shows the results of injecting animals with 10^5 to 10^8 CFU/ml ($MID = 10^2$) together with whole immune serum or fractions. The similarity to the in vitro bactericidal experiments is apparent. Animals were infected at all concentrations when the suspending menstruum was MEM or normal guinea pig serum. The 19S peak from immune serum showed a similar pattern. However, when immune serum or the 7S fraction was used, infection did not occur during the period of observation. Interestingly, heating did not affect this ability to deter infection.

DISCUSSION

In this report, we have presented data to show that the immunity developed by guinea pigs infected with *N. gonorrhoeae* can be transferred to normal susceptible guinea pigs by serum or certain serum fractions. Presumably this protection is due to specific antibody. It should be emphasized that this procedure is actually a modified passive transfer because antiserum or

fractions are mixed with the gonococci before injection.

When MIDs were established, it was surprising to observe that guinea pigs could be infected with as few as 10 CFU. The subcutaneous chambers apparently offer optimal conditions for gonococcal growth. Animals once infected remained so for a relatively long time, and this is comparable to the long duration of untreated infection in humans (11).

The initial results of the bactericidal experiments in which serum fractions were used were regarded with some degree of skepticism for two reasons. First, it is often generalized that antibodies to somatic antigens of gram-negative bacteria are principally of the IgM class (9, 12), yet the 19S peak from immune serum did not show any bactericidal properties or in vivo protection. However, it should be emphasized that the immune sera were harvested after many months of infection and consequently at a time when possibly only IgG antibody synthesis was occurring. A definitive answer to the question of IgM activity would require using 19S fractions obtained from early bleedings in which some antibody activity could be demonstrated and in which IgM could be identified as the antibody.

The second reason is that the 7S fraction demonstrated bactericidal activity, and it was assumed that this activity was complement mediated, although no extraneous source of complement was added to the system. This was concluded because the in vitro bactericidal activity was abolished by heating at 56 C for 30 min. When whole fresh immune serum is used, the elimination of bactericidal activity by heating is not difficult to explain. However, in the case of serum fractions, specifically the 7S peak, those complement components necessary for the bactericidal effect must emerge from Sephadex G-200 at an elution volume similar to that for IgG. With the possible exception of C1, which is reported to be excluded from G-200 (13), all other components may possibly elute in the 7S region. It is entirely possible that some C1 may have been included in the 7S peak from the descending shoulder of the 19S peak. In addition, the time (1 to 2 days) involved in gel filtration (room temperature) and volume reduction apparently was not qualitatively injurious to the function or the complement pathway. Although complement titration was not performed, a decrease in titer may have occurred but apparently not to a level insufficient for bactericidal activity.

As C3 is heat stable, it is possible that the

TABLE 2. *In vitro* bactericidal effects of serum and fractions at different concentration of gonococci^a

Suspending medium	Final <i>N. gonorrhoeae</i> concn		
	10 ⁸ CFU/ml	10 ⁷ CFU/ml	10 ⁵ CFU/ml
MEM	4+	3+	2+
Normal guinea pig	4+	3+	2+
Immune guinea pig	3+	1+	0
Immune 19S	4+	3+	2+
Immune 19S (heated)	4+	3+	2+
Immune 7S	3+	1+	0
Immune 7S (heated)	4+	3+	2+

^a 4+, Confluent growth; 3+, confluent growth in some areas but discrete colonies in other; 2+, discrete colonies >50 CFU; 1+, <50 CFU; 0, no growth.

TABLE 3. *Effect of serum and fractions on in vivo growth of gonococci*

Inoculum diluent	<i>N. gonorrhoeae</i> in inocula		
	10 ⁸ CFU/ml	10 ⁷ CFU/ml	10 ⁵ CFU/ml
MEM	4+/3+ ^a	3+/3+	4+/3+
Normal guinea pig	4+/4+	4+/3+	4+/3+
Immune guinea pig	1+/0	0/0	0/0
Immune 19S	3+/3+	4+/3+	4+/3+
Immune 19S (heated) ...	4+/3+	4+/3+	4+/3+
Immune 7S	0/0	0/0	0/0
Immune 7S (heated)	0/0	0/0	0/0

^a Growth at 3rd day to the growth at 10th day.

alternate pathway for complement fixation could have been activated at the C3 step via mechanism analogous to C3 proactivator.

We feel that the data demonstrate clearly that immunity to reinfection develops in the course of an experimental gonococcal infection. At least part of the immunity to gonococcal infection in the guinea pig may be antibody mediated and contained in the 7S peak of gel filtered immune serum. On the basis of the above gel filtration studies and without the appropriate absorption studies it is not possible to state whether IgG₁, IgG₂, or IgA is mediating the observed effects in this system. However, previous data that show an inability of IgG₁ to fix complement (2) does suggest that guinea pig IgG₂ is the antibody involved.

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