NATURAL AND IMMUNE BACTERICIDINS FOR THE *GONOCOCCUS*

By Y. B. ABDOOSH (*Cairo*)

From the Bacteriological Department, Lister Institute, London

The work detailed in the present communication was undertaken with a view to amplifying the scanty information we possess on the capacity of the normal sera of various mammals, including man, to destroy gonococci *in vitro* and on the change that takes place in the serum of man in the course of, and after recovery from, gonococcal infection.

Torrey (1908) studied the bactericidins for gonococci present in normal and immune rabbit and guinea-pig sera. He found that the susceptibility of gonococci to these antibodies varied greatly and that there was a considerable rise in the bactericidin titre during the process of active immunization.

Martin (1911), comparing the bactericidins active against the gonococcus and the meningococcus in various sera, came to the conclusion that the sera of cats and rabbits contained gonococcal bactericidins, whereas the sera of man and guinea-pigs did not.

Jötten (1921) separated gonococcal strains into four groups by agglutination and complement fixation tests and found that members of two of his groups were more resistant to natural bactericidins than were members of the other two groups.

Mackie *et al.* (1932) reported that the sera of sheep, horses and rats gave a "uniformly positive but relatively weak reaction" with this organism in the bactericidal test.

There is no adequate mention, however, in the literature reviewed of the bactericidal content of sera of patients suffering from gonorrhoea.

Technique

Three freshly isolated strains of gonococci referred to as H_1, H_3 and H_4 were used. They were cultivated on serum-agar slopes (horse serum 8 per cent., *p*H 7·4) for 20 hours, the tubes being sealed with paraffin wax.

Emulsions in serum broth (inactivated horse serum 8 per cent., *p*H 7·4) were made from the three strains. Equal volumes of these emulsions were mixed together, and the multivalent mixture was diluted suitably to match a standard opacity tube (about 150×10^6 gonococci per c.c.).

From this standard emulsion further dilutions of 1 : 50, 1 : 100, 1 : 200, 1 : 400 and 1 : 800 were made in serum broth, with the adequate precautions

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necessary for making bacterial dilutions for viable count, a fresh pipette being used for each dilution.

For each serum to be examined a series of small sterile tubes was used.

In each one of these tubes 0·4 c.c. of one of the various bacterial dilutions and 0·4 c.c. of the serum to be examined were mixed. The mixtures were incubated at 37°C. for 5 hours. A capillary pipette delivering 50 drops to 1·0 c.c. was used to transfer one drop from each tube to a plate containing 0·8 c.c. inactivated horse serum and 12 c.c. of agar, melted and cooled to 45°C. The plates were mixed and incubated at 37°C., counts being usually made after 48 hours. Preliminary tests showed that equal quantities of gonococcal emulsions spread on blood-agar plates and poured in serum-agar plates gave almost identical counts. The possibility of a noticeable reduction in count due to the temperature of the molten agar was thus excluded. In most cases further subcultures were made on blood-agar tubes from the various dilutions after an overnight incubation to test the action of the serum after the longer period of incubation.

The number of organisms added to each tube was established by plating from a control tube containing the highest bacterial dilution (1/800) before the commencement of incubation and again after it. An increase in numbers in the proportion of 7 to 8 was usually established for these two counts. In rare cases a deterioration in the count in the broth control tube was noticed by the end of the 5 hours' incubation. This spontaneous dying out of the organism was usually traced to a broth of *p*H more acid than 7·4 and sometimes to a specimen of serum of low nutritive value. Such tests were discarded as unsuitable.

Natural bactericidins

The sera used in this investigation were drawn from specimens of clotted blood and were used either within a few hours or after being kept overnight in the cold room.

After each experiment the number of organisms killed by 0·4 c.c. of the serum under test was calculated, taking as an end-point the most concentrated bacterial suspension that was sterilized by the serum.

Table I gives the results of a typical experiment. In the last column is recorded the average number of organisms killed in a series of experiments, the number of these being given in the preceding column.

Consideration of this table shows that the sera of the horse, ox, sheep, pig, goat, cat, monkey, rabbit and rat possess natural bactericidins, some of them of very high order, that the serum of the guinea-pig has a low titre, while the sera of man and the mouse exert no bactericidal effect at all when examined by the technique here described.

Mouse serum

Ritz (1911) showed that fresh mouse serum failed to haemolyse sensitized red blood corpuscles. He traced this to a deficiency of its complement in

end-piece, mid-piece alone being present. Addition of end-piece prepared from guinea-pig complement was necessary to complete the haemolytic system.

This fact suggested that a negative bactericidal action obtained in an *in vitro* test with fresh untreated serum of mice may not suffice to exclude the possibility of the presence of an active bactericidin in the mouse serum. An *in vivo* bacteriolysis test was, therefore, carried out, three mice and three small rats being used. A thin emulsion of gonococci was made in serum broth, and 0·5 c.c. of this emulsion was injected intraperitoneally into each mouse and each rat. After $\frac{1}{2}$, $1\frac{1}{2}$ and 3 hours one mouse and one rat were killed and the peritoneal fluid examined for gonococci by hanging-drop and stained preparations.

It was seen that although lysis of the injected gonococci was somewhat delayed, it took place in the peritoneal cavity of the mouse, just as it did in that of the rat, an animal with normal anti-gonococcal bactericidins demonstrable *in vitro*. A similar lack of lysis *in vitro* but the occurrence of it *in vivo* has been observed in the case of the mouse when tested with certain strains of *B. typhosus*.

It would seem probable that either end-piece as such, or some constituent capable of replacing it, is anchored to the tissue cells and shares in the *in vivo* defensive reaction of the mouse, which may thus be regarded as possessed of active bactericidins for the gonococcus.

Table I. *A comparison of the bactericidal power of a variety of mammalian sera*

Sera	No. of colonies growing after plating 0·02 c.c. of serum mixture containing equal quantities of undiluted serum and gonococcal emulsion: Standard (undiluted)	Dilutions of gonococcal emulsion: 1 : 20	1 : 50	1 : 100	1 : 200	1 : 400	1 : 800	No. of sera examined	Average no. of organisms killed by 0·4 c.c. serum expressed in terms of 10^3
Man	∞	∞	∞	∞	∞	∞	300	17	0
Mouse	∞	∞	∞	∞	∞	300	260	30	0
Guinea-pig	∞	∞	∞	300	5	0	0	20	13
Sheep	∞	50	0	0	0	0	0	6	74
Goat	300	50	0	0	0	0	0	4	190
Ox	300	0	0	0	0	0	0	4	220
Pig	300	0	0	0	0	0	0	5	220
Horse	50	0	0	0	0	0	0	5	640
Rabbit	50	0	0	0	0	0	0	6	1700
Rat	50	0	0	0	0	0	0	6	4000
Cat	25	0	0	0	0	0	0	2	5000
Monkey	3	0	0	0	0	0	0	5	6000

The symbol ∞ indicates that the plate presented over 400 colonies.

Guinea-pig serum

Two observations were made in connexion with this serum, firstly, the variability of its individual level, some guinea-pigs showing bactericidin titre ten times higher than others, and secondly, the relative sluggishness of its

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action, the maximum effect being obtained after 8–10 hours' incubation rather than after 5 hours as with other sera.

Human serum

Dilute human serum, as will be illustrated later, can successfully activate an inactivated immune rabbit serum in a bactericidal system, when the gonococcus is employed as the test organism. Moreover, a specimen of human serum which shows no bactericidal action against the gonococcus can exert a powerful action on *B. typhosus*.

These two facts show that the incapacity of human serum to kill the gonococcus is due to absence of the corresponding antibody and not to a deficiency in "bacteriolytic" complement.

It should be mentioned that a small minority of human sera effected a slight reduction in count in the higher dilutions (1 : 800). For example, one serum gave 140 colonies, whereas the serum broth control of the same dilution had 420 colonies. This may be due to the presence of a very low bactericidin titre, much lower than that of any other mammal.

A possible explanation of this low titre is the presence in the individual of a clinical or subclinical infection of the respiratory tract with *M. catarrhalis* or some other similar organism. Oliver (1929) and Price (1931), investigating the value of the complement—fixation test for gonorrhoea, encountered instances of "cross-fixation" in some human sera of cases suffering from respiratory affection but free from gonococcal infection. Mascall (1931) attributed a similar rôle to infection with *N. flava* No. 1.

During this investigation a considerable rise in the bactericidal titre in the serum of one person was definitely associated with successive injections of anticatarrhal vaccine. From the normal human level of almost failing to show any reduction in count in the highest dilution (1 : 800), the titre rose gradually after every injection, and 4 days after the 5th inoculation, 0·4 c.c. of the serum killed as many as 10×10^3 gonococci.

Immune bactericidins

A. *Bactericidins resulting from inoculation*

Sera of rabbits immunized by giving four graduated doses of the gonococcal strains H_1, H_3 and H_4 at 3 days' interval, were collected aseptically and stored in the cold room without the addition of antiseptics.

Inactivated immune serum was sufficiently diluted with saline for its use with gonococcal emulsions of customary strength. It was then reactivated by the addition of dilute fresh human serum and tested for bactericidal power against its homologous and the heterologous strains. Guinea-pig serum (diluted 1 : 4) was later substituted for human serum with the same satisfactory results. To 0·4 c.c. of diluted immune serum were added 0·4 c.c. of dilute complement and 0·2 c.c. of the various dilutions of gonococcal emulsion; the mixture was

incubated for 5 hours and then plated out. Suitable broth and complement controls showed that the doses of the various strains used were very similar. Table II gives the result of a typical experiment of this kind.

Table II. *Bactericidal effect of immune rabbit serum on homologous and heterologous strains*

Immune serum	Test strain	No. of colonies growing after plating 0·02 c.c. of serum mixture containing diluted serum and gonococcal emulsion				
		Standard (undiluted)	Dilutions of gonococcal emulsion			
			1 : 20	1 : 40	1: 80	1 : 160
H_1	H_1	∞	20	0	0	0
H_1	H_3	∞	∞	∞	∞	∞
H_1	H_4	∞	150	100	20	0
H_3	H_1	∞	∞	∞	∞	300
H_3	H_3	∞	100	0	0	0
H_3	H_4	∞	∞	∞	∞	300
H_4	H_1	∞	∞	∞	200	0
H_4	H_3	∞	∞	∞	∞	350
H_4	H_4	∞	0	0	0	0

The symbol ∞ indicates that the plate presented over 400 colonies.

Table II demonstrates the serological heterogeneity of these gonococcal strains; this is evidenced by the fact that each serum reacts best with its homologous strain, H_3 differing notably from H_1 and H_4. These two strains show a definite relationship to one another in the bactericidin test as they do also in the agglutination test.

B. *Bactericidins resulting from infection*

For the examination of bactericidins resulting in man from infection with *Micrococcus gonorrhoeae* the technique already described in connexion with the investigation of natural bactericidins was adopted. Sera from patients suffering from gonococcal urethritis at different stages of the illness were obtained through the kind offices of Col. L. W. Harrison.

Some of these sera were examined against mixed emulsions of the three strains; others were examined against H_3 as the most salt-stable of the three. In view of the heterogeneity of the gonococcal antigens as evidenced by experiments with immune rabbit sera, already described, it would perhaps have been more satisfactory to have tested each individual against the strain he was actually harbouring. Table III gives a summary of these bactericidal tests.

Table III. *Bactericidal content of serum in relation to duration of illness*

Duration of illness	No. of sera examined	No. of sera preventing growth of gonococcal emulsion diluted					
		Standard (undiluted)	1/50	1/100	1/200	1/400	1/800
1–15 days	18	0	0	0	0	0	1
15–60 days	10	0	0	0	0	3	4
2–5 months	24	0	9	4	4	0	2

Table III shows that of the eighteen sera taken during the first 15 days after infection, only one was capable of preventing growth and then only in the highest dilution. The remaining seventeen sera permitted growth in the 1/800 dilution tubes, though some of them showed a considerable reduction in the counts obtained; thus, thirty colonies grew in one case as compared with 550 colonies on the serum-broth control plate. This implies a very low bactericidin titre, and for all practical purposes the results indicate that sera of patients during the first 15 days of illness are devoid of any appreciable amount of gonococcal bactericidins.

Among the ten sera taken between the 15th and 60th days, four could inhibit growth in the 1/800 dilution and three could do so in the 1/400 dilution, while among the twenty-four sera whose donors were at the 2nd to the 5th month of illness, as many as nine were capable of sterilizing the 1/50 dilution. Yet even at this late period in the disease there were five sera still unable to inhibit growth even in the highest 1/800 dilution. It is possible that, as we have already suggested, the strains infecting these cases may have been so heterologous to those used in the experimental emulsions that the antibodies in the serum remained unobserved.

Discussion

This investigation reveals the interesting phenomenon that laboratory animals which are naturally immune and experimentally refractory to gonococcal infection are distinguished from the naturally susceptible human beings by the possession in their sera of bactericidins for gonococci.

Although the effect of such bactericidins is regarded now as being of less importance than it was once supposed to be, these bodies certainly operate with certain organisms and under certain conditions. The work of Pfeiffer and his colleagues in the case of cholera demonstrated a close parallelism between immunity to infection and the occurrence of bacteriolysis both *in vivo* and *in vitro*.

Malone *et al.* (1925) correlated the well-known difference in susceptibility to *B. pestis* of Bombay and Madras rats with a difference in the bactericidal potency of their blood towards this organism. Recent work of Schütze *et al.* (1936) also brings similar evidence for correlation between the presence of antibodies and resistance to disease. The sera of four different lines of mice were found to vary in their bacteriostatic effect on the growth of certain *Salmonella* organisms, as the lines themselves varied in their resistance to infection with these bacteria.

Whether this anti-gonococcal bactericidal mechanism, so universally distributed among mammals, can by itself alone ensure to the mucous membranes absolute protection against natural or experimental infection, or whether it is helped in this respect by other factors, is still to be elucidated. It is difficult to believe that it represents only an adventitious accompaniment to the immune state and does not participate in any way in protection.

Human sera and mouse sera in the *in vitro* reaction both gave negative results; but whereas human serum lacks the bactericidal antibody and is rich in complement, mouse serum contains the bactericidal antibody but its complement is deficient in end-piece.

The origin of natural antibodies in sera has stimulated controversy and the postulation of at least two well-known theories, viz. (*a*) that these antibodies are the result of repeated, subinfective doses of the organism in question, and (*b*) that they are purely developmental in origin.

The fact that, though the gonococcus is a strict human pathogen, man is the only mammal encountered whose serum is deficient in this natural bactericidin, would lend little support to the theory maintaining that external stimuli in every case play a fundamental role in the production of natural antibody. On the contrary it supports Hirszfeld's (1926) theory of a genetic origin. Even the observed rise in the bactericidin titre after injection with anticatarrhal vaccine cannot be regarded as weakening the evidence. That rise was slight and temporary; the serum returned to its natural level within 3 months. It is the maintenance of a certain level of antibody throughout the life of the mammal that strongly supports the theory of some inborn regulating mechanism.

It was easy to demonstrate the presence of immune bactericidins in immune serum even when of low agglutinin titre. Such sera exerted their maximal effect on the homologous strains and left some heterologous strains unaffected. This behaviour of immune bactericidins is an additional proof of the serological heterogeneity of the gonococcus group and is explained by the various strains sharing or failing to share antigenic constituents. The bactericidal test suggests itself as a method of serological classification for such closely allied bacteria as the meningococci and one that surmounts the difficulty of dealing with non-agglutinable strains.

Consideration of the results obtained with bactericidins produced by infection shows that in the first 15 days of illness bactericidins are almost absent from the sera of patients. As the disease advances, a steady rise in the percentage of sera that will react positively—as well as a marked increase in the titre of the reaction—is obtained. Some sera, however, even in the 5th month of illness possessed no bactericidins against one or another of the three strains used. Infection with a strain entirely unrelated to either of these three strains may have been the cause of this negative result. This is supported by the fact already mentioned that bactericidins resulting from inoculation acted selectively on their homologous strains.

The scanty observations on these infection bactericidins do not justify comment on their significance. However, in view of the universally acknowledged fact that phagocytosis occurs readily in the patient without cure or immunity being established, it may be important to consider the relationship between an increase in the bactericidal power of a patient's serum and his convalescence from the disease.

SUMMARY

1. Normal human sera have no natural bactericidins active against strains of gonococci.

2. Normal sera from eleven other mammalian species showed a powerful bactericidal action against various strains of gonococci.

3. The serological heterogeneity of the gonococcus group revealed by agglutination and complement fixation is again demonstrated in the bactericidal test with immune antiserum.

4. There is a steady rise in the incidence of positive bactericidal action in the sera of patients following the progress of infection with the gonococcus.

5. The probable significance of these bactericidins, natural and immune, discussed.

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