

AN EXAMINATION OF THE MECHANISM OF PNEUMOCOCCUS IMMUNITY BY MEANS OF BACTERICIDAL MEASUREMENTS

I. THE REACTION BETWEEN THE ANTICARBOHYDRATE ANTIBODY AND THE PURIFIED SPECIFIC CARBOHYDRATE

By HUGH K. WARD, M.B.

(From the Department of Bacteriology and Immunology, Harvard University Medical School, Boston)

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In a previous communication (1) the author described in some detail the bactericidal effect of defibrinated human blood on the three types of pneumococci and confirmed the work of Sia (2) on the antibactericidal action of the specific carbohydrate. In a second paper (3) the neutralization of this action of the carbohydrate by the corresponding antiserum was studied quantitatively in bactericidal tests. The reaction between the specific carbohydrate and the antiserum has been investigated with great accuracy by Heidelberger and Kendall (4), using chemical methods. This work centered round the specific precipitate between the two reacting substances. The present writer was more interested however in what may be termed the "functional neutralization" of the carbohydrate by the antiserum and this extends far beyond the precipitation zone. For example, a reference to Table I of the second paper (3) mentioned above will show that the Type III carbohydrate has a strong antibactericidal effect in a concentration of 1/75,000, but this effect is specifically neutralized by an antiserum concentration of 1/80,000. It need hardly be said that this concentration of antiserum (1/80,000) would give no visible precipitate with the carbohydrate, indeed the concentration of the antiserum would have to be raised to about 1/150 before the faintest precipitate appeared. The same table shows further that when precipitation does occur with the stronger concentrations of antiserum, the precipitate actually hinders the bactericidal action of the blood. Cromwell and

Centeno (5) have shown that specific precipitates are ingested by leucocytes and this perhaps interferes with the efficiency of the leucocytes in taking up the pneumococci.

We have not sufficient knowledge at the present time to make any definite assertions about the factors which determine virulence and resistance in the case of the pneumococcus, but the facts strongly suggest: (1) That the capsule is the armour of the virulent pneumococcus, and when this armour is intact, the organism defies phagocytosis. And as far as we know the body can kill the pneumococcus in no other way. (2) That for the virulent pneumococcus the most vital constituent of the capsule is the specific carbohydrate. (3) That the only weapon at the disposal of the body to overcome this carbohydrate defence is the anticarbohydrate antibody (carbohydrate precipitin) which neutralizes the carbohydrate and lays the organism open to phagocytosis. (4) That if there is any free carbohydrate present in the body, it will combine with the anticarbohydrate antibody, leaving so much less free antibody to neutralize the carbohydrate capsules of the living pneumococci.

Since it is possible to measure accurately the carbohydrate-neutralizing power of the anticarbohydrate antibody (3), this theory can be tested to see if it fits quantitatively with the known data of pneumonia serum therapy. But before this is attempted, reference must be made to the recent work of Sabin (6), which has thrown some doubt on the above hypothesis that the anticarbohydrate antibody is the only essential antibody in pneumococcus immunity. Sabin absorbed a Type I pneumococcus antiserum by precipitating the antiserum with Type I carbohydrate. The antiserum after this precipitation gave no further precipitation with the carbohydrate, and Sabin inferred from this that the absorbed antiserum had been deprived of all its anticarbohydrate antibody. However, when this absorbed antiserum was tested on mice, it was found that about 30 per cent of its protective titre was still present. The natural conclusion to be drawn from this experiment, if the premises were correct, was that the protective action of the serum depended mainly on the anticarbohydrate antibody, but partly also on another unknown antibody, which was left untouched by absorbing only with the specific carbohydrate. It seemed inherently unlikely to the present writer that 70 per cent of

the protective power of an antiserum should depend on the presence of one antibody, and that when this antibody was completely removed, the remaining 30 per cent should depend on another antibody. Accordingly the premises were examined more closely. It has been generally assumed that when one can no longer detect precipitation on adding a precipitinogen to an antiserum there is no precipitin left in the serum. If this were the only test for the presence of precipitin, it is self-evident that this would be true, as far as we could tell. But in this particular case the antibody which reacts with the carbohydrate in the form of a precipitate can be detected in another way; *viz.*, by its power to neutralize the antibactericidal effect of the carbohydrate in a bactericidal test. It has already been pointed out in this paper that this neutralization test is far more delicate than the precipitation test. If, then, it can be shown that a carbohydrate-absorbed antiserum, which no longer forms any precipitate with the carbohydrate, is still able to neutralize the antibactericidal effect of the carbohydrate, it is clear that it is incorrect to assume that the anticarbohydrate antibody has been completely removed by absorption. It only shows that the precipitation test is not delicate enough to detect the residuum of antibody. Accordingly an experiment was planned to determine whether the carbohydrate-absorbed antiserum had any neutralizing effect on the carbohydrate.

EXPERIMENTAL

The experiment was carried out with the Type III organism instead of Type I because Type I carbohydrate was not available in sufficient quantity at the time. There is however no reason to believe that the behaviour of Type III antiserum differs from that of Type I antiserum in this respect and indeed former experiments indicated that carbohydrate-absorbed Type I antiserum still had a neutralizing effect on Type I carbohydrate. These Type I protocols are not given here because the complete experiment (the ordinary bactericidal as well as the carbohydrate-neutralizing effect) was not done on the same specimen of absorbed antiserum.

To 3.0 cc. of a strong Type III antiserum were added 2.0 mg. of Type III carbohydrate. The mixture was incubated for 2 hours at 37°C. and was then placed in the ice box for 48 hours. A heavy precipitate had formed and was removed by centrifugation. To the supernatant serum was added 0.1 mg. of the carbohydrate. After incubation for 2 hours and a further 48 hours in the ice box, a very small precipitate had formed. This was removed and the supernatant serum tested for precipitins. It gave no precipitate by the ring test with concentrations of the

carbohydrate ranging from 1/100 to 1/1,000,000. This specimen of serum will be referred to as the absorbed antiserum.

A bactericidal experiment was carried out according to the slightly modified Todd (7) technique described by the present writer in a previous paper (1). To two series of tubes each containing 0.5 cc. of defibrinated human blood were added a certain number of Type III pneumococci (120,000 in this experiment). Decreasing concentrations of the unabsorbed Type III antiserum were added to one series of tubes, and decreasing concentrations of the absorbed antiserum were added to the other series of tubes. The tubes were then sealed, incubated in a rotating machine for 18 hours, the tubes broken open, the contents plated out, and the plates incubated.

TABLE I

No. of Type III diplococci in tube	Concentration of unabsorbed antiserum	Growth	Concentration of absorbed antiserum	Growth
120,000	1/250	+	1/32	0
120,000	1/500	++	1/64	0
120,000	1/1,000	++	1/128	0
120,000	1/2,000	++	1/250	0
120,000	1/4,000	+	1/500	0
120,000	1/8,000	0	1/1,000	0
120,000	1/16,000	0	1/2,000	0
120,000	1/32,000	++++	1/4,000	+++
120,000	1/64,000	++++	1/8,000	++++
120,000	1/128,000	++++	0	++++

Here and in the following tables + + + +, + + +, + +, + = degrees of growth and 0 = sterility.

Table I shows the result of the bactericidal experiment with the unabsorbed and absorbed antiserum. In the case of the unabsorbed antiserum there is a well marked prozone where the pneumococci are not all killed, but the end-point is clearly seen at a concentration of 1/16,000. In the case of the absorbed antiserum there is no prozone, and the end-point is reached at a concentration of 1/2,000. The prozone is thus seen to be associated with the presence of precipitins in the unabsorbed antiserum. But it is quite obvious that the absorption of the precipitins does not rob the antiserum of all its bactericidal powers, the absorbed antiserum retaining some 12 per cent of its original bactericidal strength. This *in vitro* experiment parallels and confirms Sabin's *in vivo* experiment.

It now remained to test the carbohydrate-neutralizing power of the

absorbed antiserum, and the results of this experiment are seen in Table II.

Every tube contains 0.5 cc. of defibrinated human blood. To one set of these tubes were added a constant amount of the absorbed antiserum, a varying amount of the specific carbohydrate, and a varying number of organisms. The other set of tubes is exactly the same as the first set, save that there was no absorbed antiserum added.

In the last column of Table II is seen the bactericidal effect of the blood alone, and it will be noted that all the concentrations of specific carbohydrate that have been used show an antibactericidal effect in the tubes where there is no absorbed antiserum present. In the tubes where the absorbed antiserum is present, it will be seen that it completely neutralizes the antibactericidal effect of the carbohydrate up to a carbohydrate concentration of 1/15,000.

These experiments indicate, in the writer's opinion, that it is impossible to absorb the whole of the anticarbohydrate antibody out of an antiserum by precipitation with the specific carbohydrate and therefore it is unnecessary to postulate another antibody to account for the fact that such an absorbed antiserum has a definite, though diminished mouse protection titre. This anticarbohydrate antibody appears to account satisfactorily for the bactericidal action of the antiserum in test-tube experiments, for the protective action of antiserum in animal experiments, and is at any rate one of the main factors in determining the crisis in pneumonia.

Turning now to the problem of infection and resistance in pneumonia, it naturally occurred to the writer, as no doubt to many others, that the ultimate outcome in pneumonia might be explained in terms of the specific carbohydrate and the anticarbohydrate antibody in the following manner: If the carbohydrate was still in excess at the time when the patient's vitality was at the critical point, the pneumococci continued to multiply and death was the result. If on the other hand the anticarbohydrate antibody was produced in sufficient quantity to be in excess at this point, the pneumococci were phagocytosed, and recovery by crisis was the result. Further, if an amount of antibody which would result in excess were introduced artificially into the circulation, an artificial crisis and recovery should follow.

TABLE II

No. of Type III diplococci in tubes	Concentration of absorbed antiserum																											
	1/16	0	1/16	0	1/16	0	1/16	0	1/16	0																		
	Concentration of specific carbohydrate																											
1,400,000	+	+	+	+	+	+	+	+	+	+	1/400,000	+	+	+	+	+	+	1/400,000	+	+	+	+	+	0	+	+	+	
140,000	+	+	+	+	+	+	+	+	+	+	1/135,000	0	+	+	+	+	+	1/400,000	0	+	+	+	+	+	0	+	+	+
14,000	+	+	+	+	+	+	+	+	+	+	1/45,000	0	+	+	+	+	+	1/135,000	0	+	+	+	+	+	0	+	+	+
1,400	+	+	+	+	+	+	+	+	+	+	1/15,000	0	+	+	+	+	+	1/135,000	0	+	+	+	+	+	0	+	+	+
140	+	+	+	+	+	+	+	+	+	+	1/5,000	0	+	+	+	+	+	1/135,000	0	+	+	+	+	+	0	+	+	+
14	+	+	+	+	+	+	+	+	+	+	1/15,000	0	+	+	+	+	+	1/45,000	0	+	+	+	+	+	0	+	+	+

TABLE III

No. of Type I diplococci in tubes	Concentration of specific carbohydrate																															
	1/1,600	1/1,600	1/1,600	1/1,600	1/1,600	1/1,600	1/1,600	1/1,600	1/1,600	1/1,600																						
	Concentration of antiserum																															
400,000	+	+	+	+	+	+	+	+	+	+	1/250	0	+	+	+	+	+	1/4,000	+	+	+	+	+	0	+	+	+					
40,000	+	+	+	+	+	+	+	+	+	+	1/128	+	+	+	+	+	1/2,000	+	+	+	+	+	1/1,600	+	+	+	+	0	+	+	+	
4,000	+	+	+	+	+	+	+	+	+	+	1/64	+	+	+	+	+	1/1,000	0	+	+	+	+	+	1/1,600	+	+	+	+	0	+	+	+
400	+	+	+	+	+	+	+	+	+	+	1/32	+	+	+	+	+	1/500	0	+	+	+	+	+	1/1,600	+	+	+	+	0	+	+	+
40	+	+	+	+	+	+	+	+	+	+	1/1,600	+	+	+	+	+	1/250	0	+	+	+	+	+	1/4,000	+	+	+	+	0	+	+	+
4	+	+	+	+	+	+	+	+	+	+	1/1,600	+	+	+	+	+	1/128	+	+	+	+	+	1/2,000	+	+	+	+	1/1,600	+	+	+	

It was quickly seen however that this explanation of infection and resistance in pneumonia was not true. Perhaps the clearest way to demonstrate the inadequacy of such a hypothesis is to examine those cases of Type I pneumonia which die after the injection of 200 cc. of antiserum, a result which is not uncommon if the antiserum is given after the 3rd day of the disease. It is a simple matter to ascertain how much specific carbohydrate would have to be produced in the body so that it would be functionally in excess after the 200 cc. of antiserum had been administered. Table III shows a careful titration of the neutralizing effect of an unconcentrated Type I antiserum on the Type I carbohydrate, the bactericidal technique already described being employed.

In the last column of Table III the bactericidal effect of the blood alone may be observed, and in the preceding column the antibactericidal effect of a 1/1,600 concentration of the carbohydrate. The usual prozone in such experiments is seen with the stronger concentrations of antiserum, then a zone of obvious neutralization, and only when the concentration of antiserum is lowered to 1/4,000 is the 1/1,600 concentration of carbohydrate functionally in excess. In other words there has to be two and a half times more carbohydrate than antiserum if the carbohydrate is to show clearly its antibactericidal effect. Thus if an excess of specific carbohydrate was the critical factor leading to the death of the patient after 200 cc. of antiserum had been injected, there would have been produced in the body $2.5 \times 200 = 500$ gm. of specific carbohydrate. Heidelberger, Sia, and Kendall (8) have shown that when the Type I pneumococcus is grown and allowed to autolyze in broth, 20 litres of this broth contain only about 0.8 gm. of the specific carbohydrate. Accordingly 500 gm. of carbohydrate would be the yield from 12,500 litres of broth. It is obvious that neither 500 gm. of carbohydrate nor anything approaching this amount could be produced in the body. This simple explanation, therefore, of the struggle between the parasite and the host in pneumonia is reduced to an absurdity. Indeed the antibactericidal effect of the specific carbohydrate is so weak when one takes into account the small amount that is found in an autolyzed broth culture of the pneumococcus, that one begins to doubt whether the carbohydrate is an important factor at all. And yet the facts suggest so

strongly that the carbohydrate is associated with virulence, and the antibody, which neutralizes its effect, with resistance, that one hesitates to discount the carbohydrate as a factor in pneumonia, before examining the premises of the theory more closely. This will be done in the second paper of this series (9).

CONCLUSIONS

1. Type III antipneumococcus serum, after absorption with the specific carbohydrate, no longer forms a precipitate with the carbohydrate, but still has a definite, though diminished bactericidal action on virulent pneumococci in a bactericidal test.

2. Such an absorbed antiserum still retains some of its power to neutralize the antibactericidal effect of the specific carbohydrate in a bactericidal test, showing that absorption with the carbohydrate does not remove all the anticarbohydrate antibody from an antiserum.

3. This carbohydrate neutralization test is a very much more delicate method for detecting the anticarbohydrate antibody (precipitin) than the precipitin test.

4. There is therefore no necessity to predicate another antibody to explain the bactericidal action of a carbohydrate-absorbed antiserum, or a similar result in a mouse protection test.

5. The specific carbohydrate has a definite antibactericidal action, but it is demonstrated that, were it present in this form in the body during pneumonia, it could not conceivably be produced in sufficient quantity to influence the disease.

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