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

II. The Reaction between the Anticarbohydrate Antibody and Type-Specific Products of the Organism

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(Received for publication, January 8, 1932)

In the first paper of this series (1), an examination was made of the theory that the outcome in pneumonia largely depended upon (1) the quantity of specific carbohydrate which was produced by the pneumococcus growing and autolyzing in the body and which by its antibactericidal action protected the pneumococcus from phagocytosis; and (2) the quantity of carbohydrate-neutralizing antibody either produced naturally in the body as a result of infection or artificially introduced by injection.

On this theory, excess of the carbohydrate would result in death, excess of the antibody in recovery. When the theory was tested quantitatively, it broke down at once. For it was found that the antibactericidal action of the carbohydrate was so easily neutralized by the specific antibody that the introduction of 200 cc. of antiserum at any stage of the disease would be more than sufficient to neutralize any conceivable amount of carbohydrate that could be present in the body. According to the theory then, no serum-treated patient could possibly die. Unhappily this is not the case.

Up to this point in the investigation, the author had made the natural assumption that the pure carbohydrate (isolated by Avery and Heidelberger (2) in their researches on type specificity) is present as such in the washings of the organisms, in culture fluids, and in the body during pneumonia. And in favour of this assumption is the well known fact that the washings of the organisms, the culture fluids in

which they have grown, and often the urine in the course of the disease contain a substance which precipitates specifically with the antiserum in the same manner as does the purified carbohydrate. This suggests that the substance in the washings of the pneumococcus, in the culture fluids, and in the urine, is identical with the purified carbohydrate, but does not prove it beyond doubt. The possibility remains that the reacting substance in the washings of the pneumococcus, culture fluid, or urine contains the carbohydrate, but is more complex and less stable than the carbohydrate itself. Whether the carbohydrate is isolated from the pneumococci themselves or from the broth in which the organisms have grown and autolyzed, many rather severe chemical manipulations have to be carried out before it can be separated from other substances. If there were a parent substance, which was less stable than the carbohydrate, there would be ample occasion for its breakdown. That a parent substance exists in the pneumococcus itself is suggested by the fact that the carbohydrate is not antigenic, type-specific antibodies being produced only by injection of intact pneumococci or of carbohydrate artificially synthesized with a protein (3). However a pneumococcus autolysate resembles the carbohydrate in that it does not give rise to type-specific antibodies on injection, but again this does not prove beyond doubt that the type-specific precipitinogen in the autolysate and the carbohydrate are indentical. The possibility that the reacting substance in the autolysate is more complex and less stable than the carbohydrate—perhaps a substance intermediate between the antigenic carbohydrate compound in the intact pneumococcus and the carbohydrate itself—was forced on the author as the most likely explanation of some experiments which will now be presented.

When working with a Berkefeld filtrate of a culture of Type III pneumococcus in 0.5 per cent rabbit blood broth, which had been incubated for 5 days, it was found that this filtrate had a strong antibactericidal effect, which was only neutralized by strong concentrations of Type III antiserum in a bactericidal test. This was in marked contrast to the antibactericidal effect of Type III carbohydrate, which in far stronger concentration than could be present in the filtrate is neutralized by a considerably weaker concentration of antiserum. These bactericidal experiments, the results of which are shown in

Tables I and II, were carried out in the manner already described in Paper I (1).

In Tables I and II the last column shows the bactericidal action of the blood alone, and in the immediately preceding columns it will be noted that in the concentrations employed the broth filtrate and the

TABLE I

No. of Type III diplococci in tube	Concentration of Type III broth filtrate						
	1/16	1/16	1/16	1/16	1/16	1/16	0
	Concentration of Type III antiserum						
	1/16	1/160	1/1,600	1/16,000	1/160,000	0	0
800,000	++	++	++++	++++	++++	++++	++++
80,000	++	++	++++	++++	++++	++++	0
8,000	++	++	++++	++++	++++	++++	0
800	+	++	++++	++++	++++	++++	0
80	0	++	++++	++++	++++	++++	0
8	0	0	++++	++++	++++	++++	0

TABLE II

No. of Type III diplococci in tube	Concentration of Type III carbohydrate						
	1/16,000	1/16,000	1/16,000	1/16,000	1/16,000	1/16,000	0
	Concentration of Type III antiserum						
	1/16	1/160	1/1,600	1/16,000,	1/160,000	0	0
400,000	++	++	+	++++	++++	++++	++++
40,000	++	+	0	++++	++++	++++	+++
4,000	+	+	0	++++	++++	++++	0
400	+	++	0	++++	++++	++++	0
40	0	+	0	0	++++	++++	0
4	0	+	0	0	++++	++++	0

carbohydrate have completely inhibited this bactericidal action. In Table I a prozone with antiserum concentrations of 1/16 and 1/160 is seen. The prozone in these neutralization experiments can always be recognized by the partial growth of the organisms. It is always associated with the formation of a specific precipitate, and if the two

reacting substances are mixed and the precipitate removed before adding the mixture to the blood, a sterile series of tubes results, showing that the prozone is really a zone of neutralization, which is masked by the inhibiting action of the precipitate. The third column of Table I shows that an antiserum concentration of 1/1,600 has no neutralizing effect on the filtrate.

In Table II, a prozone with antiserum concentrations of 1/16 and 1/160 is followed by a series of tubes where an antiserum concentration of 1/1,600 has obviously neutralized the antibactericidal action of the carbohydrate. And a certain amount of neutralization is even seen with a 1/16,000 concentration of the antiserum.

From a comparison of the two tables, it is clear that it requires at least ten times more antiserum to neutralize the broth filtrate than it does to neutralize the carbohydrate. Naturally the first explanation to account for this result would be that the concentration of specific carbohydrate in the broth filtrate is stronger than that used in the experiment shown in Table II. As a matter of fact however, if it is the carbohydrate in the filtrate, titration shows it to be very much weaker. When the broth filtrate is titrated out against the antiserum the end-point is reached at 1/40. A similar titration of the pure carbohydrate gives an end-point of 1/8,000,000. A simple calculation shows that the concentration of the carbohydrate in the filtrate, if it is the pure carbohydrate, cannot be higher than 1/200,000, which means 1/200,000 $\times$ 1/16, or 1/3,200,000 in the actual tubes used in the experiment. And this figure has to be compared with the carbohydrate concentration of 1/16,000 used in the experiment shown in Table II. It can, moreover be shown experimentally that a concentration of 1/3,200,000 of the pure carbohydrate has little or no antibactericidal effect on the blood, presumably being neutralized by the natural antibody in the serum.

The second obvious explanation is that the antibactericidal effect is due to a non-specific substance present in the broth filtrate. But this cannot be the case, because the filtrate of Pneumococcus Type III has no antibactericidal effect at all, if Type I or Type II pneumococci are used in the test.

A third explanation is that there is another type-specific antibactericidal substance in the filtrate, which is quite distinct from the

carbohydrate. Enders (4) has recently shown that there is a second type-specific substance in the autolysate of pneumococci, and that this substance has no relation to the carbohydrate. Experiment has shown however (see Table IV) that if a carbohydrate-absorbed antiserum is used in a filtrate neutralization test, such an antiserum is quite definitely weaker in its neutralizing power than the unabsorbed antiserum. This would indicate that the substance in the filtrate which is responsible for the antibactericidal effect is related to the carbohydrate and not to the second type-specific substance, because Enders has demonstrated that the precipitation titre of the antibody against the second type-specific substance is not affected by absorbing antiserum with the specific carbohydrate.

There remains the fourth possibility that the active substance in the filtrate is an intermediate substance between the antigenic carbohydrate complex in the pneumococcus itself and the pure carbohydrate. It is admitted that the only evidence—and that indirect—connecting the filtrate substance with the pure carbohydrate is the above mentioned experiment with the carbohydrate-absorbed antiserum. This hypothetical intermediate substance would be more complex, less stable, and as has been seen, much more difficult to neutralize than the pure carbohydrate. Actually, if a comparison is made on the basis of the precipitinogen content of the filtrate and the pure carbohydrate it requires at least one thousand times as much antiserum to neutralize the antibactericidal effect of the filtrate as it does the same effect of the carbohydrate. The reason for this is quite obscure at present. It may lie in the nature of the union with the antibody, perhaps irreversible in the case of the filtrate substance, and reversible under certain conditions in the case of the carbohydrate. A closely related problem is the explanation of why it is so difficult—or rather impossible—to remove the protective power from an antiserum by absorbing with the specific carbohydrate, and so easy if whole pneumococci are used as the absorbing agent. In both these problems, considerable evidence has already been advanced in these papers that only one antibody is concerned—the anticarbohydrate antibody.

The relation between the active substance in the Type III broth filtrate and Type III pneumonia would be largely theoretical unless it could be shown that a substance with similar characteristics was

present in the lesion of Type III pneumonia. Through the courtesy of the Boston City Hospital, a pneumonic lung was obtained from an autopsy of a case of Type III pneumonia. The purulent fluid was expressed from the lung as thoroughly as possible, diluted with an equal part of saline solution, and centrifuged. The supernatant fluid was passed through a large Berkefeld filter, and the filtrate stored in the ice box. Table III shows the result of testing the antibactericidal action of the lung filtrate, and also the neutralizing effect of Type III antiserum on this action. A fairly weak concentration of the lung filtrate is used, because with a stronger concentration, one cannot

TABLE III

No. of Type III diplococci in tube	Concentration of lung filtrate					
	1/320	1/320	1/320	1/320	1/320	0
	Concentration of Type III antiserum					
	1/16	1/160	1/1,600	1/16,000	0	0
600,000	++	+++	++++	++++	++++	++++
60,000	++	+++	++++	++++	++++	0
6,000	++	+++	++++	++++	++++	0
600	++	+++	++++	++++	++++	0
60	+	+++	++++	++++	++++	0
6	0	+++	++++	++++	++++	0

show clearly that the antiserum has any neutralizing effect at all. The bactericidal technique already described is used.

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 the lung filtrate. The partial growth in the first column indicates that the lung filtrate has been neutralized by this concentration of antiserum. The greater growth in the second column indicates partial neutralization. But the third and fourth columns show that weaker concentrations of antiserum have no neutralizing effect. This experiment, then, parallels the one conducted with Type III broth filtrate.

The antibactericidal action of the lung filtrate cannot be due to any non-type-specific substance, because even with a ten times stronger

concentration of lung filtrate, there is no antibactericidal effect if Type I or Type II pneumococci are used in the test. The antibactericidal action of the lung filtrate cannot be due to the presence of pure carbohydrate, because, by precipitinogen titration, it was found that the concentration of carbohydrate, if it were carbohydrate, could be no higher than 1/320,000, and this concentration of pure carbohydrate is neutralized by a weaker concentration of antiserum even than was used in this test.

The neutralizing effect of Type III antiserum which had been absorbed with Type III carbohydrate was then investigated, with the result shown in Table IV. As a control, the effect of the unadsorbed serum was again tested, and also the effect of removing the precipitate

TABLE IV

No. of Type III diplococci in tube	Absorbed antiserum 1/16	Unabsorbed antiserum 1/16	Supernatant fluid of mixture of unabsorbed antiserum 1/16 and lung filtrate 1/320	0	0
	Lung filtrate 1/320	Lung filtrate 1/320		Lung filtrate 1/320	0
700,000	++++	++	++	++++	++++
70,000	++++	+++	0	++++	0
7,000	++++	++	0	++++	0
700	++++	++	0	++++	0
70	++++	++	0	++++	0
7	0	0	0	++++	0

between the filtrate and the unadsorbed serum before adding the mixture to the tubes. It may be noted here that the specific precipitate formed on adding undiluted antiserum to the undiluted lung filtrate is so bulky that even after centrifugation, it is difficult to obtain any supernatant fluid. No precipitate was however seen on adding the carbohydrate-absorbed antiserum to the lung filtrate.

The third column in Table IV shows clearly that the effect of this concentration of lung filtrate is really neutralized by the 1/16 concentration of antiserum, the partial growth seen in the second column being due to inhibition of the bactericidal power of the blood by the specific precipitate present. The first column shows that when the anticarbohydrate antibody is weakened by absorbing the antiserum

with the carbohydrate, the neutralizing effect on the lung filtrate is very definitely weakened also. As with the Type III broth filtrate, this evidence points to the active substance in the lung filtrate being a type-specific substance related to, but not identical with the specific carbohydrate.

Assuming that none of the active substance was lost in filtration, it would require about four times the quantity of a strong Type III antiserum to completely neutralize the antibactericidal effect of a given amount of the fluid in this particular lung. But, although the lungs in this instance contained at the very least half a litre of this fluid, we have no means of ascertaining during life the amount of the concentration of the active substance in the lesion, and therefore it is impossible to say whether the presence of a large amount of this powerful substance at autopsy explains why the Type III antiserum is ineffective therapeutically. However the behaviour of this substance revives the hope that was dimmed by the examination of the behaviour of the specific carbohydrate—the hope that some day the outcome in pneumonia may be largely explained in terms of a known product of the pneumococcus and a known antibody which neutralizes that product. The evidence presented here is suggestive that the antibactericidal substance in the Type III broth filtrate and the Type III lung filtrate is the sought for product of the pneumococcus, but the evidence is not strong enough to justify a more definite claim than that.

While these experiments were in progress, Dr. Sutliff of the Boston City Hospital kindly supplied a specimen of defibrinated blood from a convalescent case of Type III pneumonia, the blood being drawn about 5 or 6 days after the crisis. It was thought that the action of convalescent blood on the specific carbohydrate, on the broth filtrate, and on the lung filtrate might throw some light on the natural mechanism of recovery and perhaps give a hint as to the method to be attempted in serum therapy. Table V shows the result of this experiment. The same technique was employed with the convalescent blood as with the normal blood.

In Table V, the last column shows that the convalescent blood is strongly bactericidal, and the third and fourth columns demonstrate that the blood was able in addition to neutralize a considerable con-

centration of Type III carbohydrate. The complete sterility indicates that the concentration of precipitin (anticarbohydrate antibody) was weak, because had it been strong, a precipitate would have formed on the addition of the carbohydrate, and all the organisms would not have been killed—see Table II. Yet despite this weakness in precipitin content, the first and second columns of Table V show that the convalescent blood was able to neutralize the lung filtrate fairly well, and the broth filtrate very well. The only way that it is possible to parallel these results with a mixture of normal blood and antiserum is to use a much stronger concentration of antibody (well within the precipitation zone) and remove the precipitate between the antibody and the lung filtrate (or broth filtrate) before adding these substances to the blood—see third column, Table IV.

TABLE V

No. of Type III diplococci in tube	Type III lung filtrate 1/320	Type III broth filtrate 1/16	Type III carbohydrate 1/16,000	Type III carbohydrate 1/160,000	0
600,000	++++	+	0	0	0
60,000	+++	0	0	0	0
6,000	0	0	0	0	0
600	0	0	0	0	0
60	0	0	0	0	0
6	0	0	0	0	0

The action of the convalescent blood on these filtrates is difficult to explain. It is weak in precipitin (anticarbohydrate antibody) but neutralizes the filtrates, whereas a similar concentration of precipitin in an artificial mixture of normal blood and dilute antiserum (see Column 3 in Tables I and III) has no neutralizing action. This would point to the presence of another unknown antibody in the convalescent blood, were it not that in stronger concentration the antiserum has a neutralizing antibody for the filtrates, which is presumably the anticarbohydrate antibody, since its neutralizing action is weakened by absorbing the antiserum with the carbohydrate. The problem is complicated and puzzling as it is left in this stage, but it is possible that the clue to Type III serum therapy lies hidden in the maze. The tentative hypothesis upon which the author is working at present is

that the neutralizing antibody present in the convalescent blood differs in some subtle way from the neutralizing antibody present in the antiserum. As an explanation, this is admittedly weak and unsatisfactory. However, it must be borne in mind that the stimulus to the production of antibodies is different in the two cases. In the artificially produced antiserum, the stimulus is first dead pneumococci and later living pneumococci, which are no doubt soon killed after they are injected into the horse. In the actual disease the stimulus is the organism growing, multiplying, and later autolyzing in the lesion. Obviously the first point to determine is whether the active substance in the lung filtrate is antigenic. All previous experience is against this possibility, type-specific antibodies being apparently produced only by intact pneumococci. Nevertheless this question is being investigated once more, although with no great optimism, and so far with no success.

Two other specimens of Type III convalescent blood neutralized the Type III broth filtrate in a similar manner.

A few experiments have been carried out with Type I pneumococcus. The filtrate of a 5 day broth culture of the Type I organism behaves more like the Type I carbohydrate, its antibactericidal action being neutralized by the weaker concentrations of antiserum. Several attempts have been made to grow the organism in fresh undiluted human serum. It did not always grow very well, but whenever there was good growth the filtrate had a strong antibactericidal effect, and like the Type III lung filtrate was not neutralized by the weaker concentrations of antiserum. Up to the present, no filtrate from a lung of an untreated Type I pneumonia case has been available for testing.

DISCUSSION

Despite the great progress that has been made during the last decade in our knowledge of the pneumococcus, the mechanism of infection and resistance is not yet clearly understood. In any theory of immunity, proof or disproof must of course in the end rest on animal experimentation, but this method of investigation has disadvantages in attempting an analysis of the mechanism of immunity, because the various factors cannot be separated or their quantitative relationship studied, save with the greatest difficulty. It is in this field of analysis

that the bactericidal technique can be used with advantage, if its obvious limitations are borne in mind.

One can hardly doubt that the problem of virulence and resistance in the case of the pneumococcus centers round the specific carbohydrate and its specific antibody. Accordingly it is very desirable to understand how these substances react with one another in pneumococcal infection, and in defibrinated human blood the conditions are fairly similar, though of course not identical with those that pertain in the living body. It has been possible to demonstrate by this method the following facts.

1. That a serum which gives a negative precipitin test (using the specific carbohydrate as the precipitinogen) may still have a considerable antibody content against the specific carbohydrate. Accordingly it is unjustifiable to assume that an animal or human being has no type-specific immunity against a type pneumococcus because no specific precipitins can be demonstrated in the serum.

2. That the antibactericidal action of the specific carbohydrate, though definite, is seen to be very weak, when the small amount of the polysaccharide that is present in an autolyzed culture is taken into account. If the actual carbohydrate is present in the body in pneumonia, it is difficult to see how there could possibly be sufficient to influence the course of the disease.

3. That in the filtrate of an autolyzed broth culture of Type III pneumococcus, and more significant still, in the filtrate of a lung obtained at autopsy from a patient dead of Type III pneumonia, there could be demonstrated a type-specific substance which has a far more powerful antibactericidal action than the purified carbohydrate. This substance appears to be related to the specific carbohydrate, and its nature and possible influence on the disease have been discussed in the text.

4. That as far as the evidence from three specimens of convalescent blood goes—and that is admittedly not far—the blood just after crisis appears to be more efficient in neutralizing the autolysate than a corresponding mixture of normal blood and antiserum. If this can be confirmed, it may eventually throw some light on the problem of serum therapy. No adequate explanation has yet been offered of why serum therapy is only effective in producing a crisis in the early stages

of Types I and II pneumonia, is ineffective at any stage in Type III pneumonia, while the natural crisis occurs much later in the disease.

In attacking the baffling problem of pneumococcal immunity by investigating the functions of the pneumococcal products and the neutralizing action of the antiserum on these products, one is but doing in pneumonia what was done many years ago in diphtheria. The details in the two problems are very different, but broadly speaking the products of each organism are its defences, as the respective antiserums are the main defences of the host. Unfortunately we cannot attack the toxemia of pneumonia directly as we can in diphtheria, but the experiments with the lung filtrate indicate that the products of the pneumococcus have to be reckoned with, even though they are not themselves toxic to the body cells.

CONCLUSIONS

1. There is in the filtrate of a 5 day broth culture of Type III pneumococcus a type-specific substance which has a very powerful antibactericidal action. If the precipitinogen content of the broth filtrate and the specific carbohydrate is taken as the basis of comparison, it requires approximately one thousand times as much antiserum to neutralize the broth filtrate as is necessary to neutralize the specific carbohydrate. The active substance in the broth filtrate appears to be related to the specific carbohydrate. Its possible nature is discussed.

2. A similar substance, but in stronger concentration, was found in the filtrate of a lung from a Type III pneumonia autopsy. The influence of this substance on the disease is discussed.

3. One specimen of Type III convalescent blood, though comparatively weak in anticarbohydrate antibody (precipitin) was better able to neutralize the broth filtrate and the lung filtrate than a corresponding mixture of normal blood and antiserum. Two other specimens of Type II convalescent blood neutralized the Type III broth filtrate efficiently.

REFERENCES

1. Ward, H. K., *J. Exp. Med.*, 1932, **55**, 511.
2. Avery, O. T., and Heidelberger, M., *J. Exp. Med.*, 1925, **47**, 367.
3. Avery, O. T., and Goebel, W. F., *J. Exp. Med.*, 1931, **54**, 437.
4. Enders, J. F., *J. Exp. Med.*, 1930, **52**, 235.