THE PRODUCTION OF SKIN NECROSIS BY CERTAIN AUTOLYSATES OF PNEUMOCOCCUS (TYPES I AND II).

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Many studies have been made on the various toxic substances obtained from pneumococci. Rosenow (1) and Cole (2) investigated the anaphylactic-like reactions produced in guinea pigs by the intravenous inoculation of autolysates of pneumococci and of the peritoneal exudates of animals dead of pneumococcus septicemia. The hemotoxin of the pneumococcus has been studied by Cole (3), Avery (4), and Neill (5), and the purpura-producing principle of the autolysates by Julianelle and Reimann (6). The effect caused by these various products of the pneumococcus when injected into the skin of man and animals has also been studied (7). No mention, however, has been made of the production of skin necrosis by sterile pneumococcus products, except by Zinsser (8), who was able to produce necrosis in the skin of large normal guinea pigs and in small guinea pigs which had been previously sensitized to the pneumococcus, by the intracutaneous inoculation of certain heat-stabile aerobically produced autolysates of this organism.

When rabbits or guinea pigs are given virulent pneumococci (Type I) intradermally in doses so adjusted that the animals die in 24 to 48 hours, there usually develops at the site of inoculation an area of greenish necrosis several cm. in diameter. Except for its greenish color, the appearance of such a lesion is very similar to that produced by the intracutaneous inoculations of the staphylococcus exotoxin (10), and, therefore, it seemed possible that the pneumococcus lesion might be caused by the same kind of poison. The experiments to be described deal with some investigations made to determine the nature of this necrotizing agent.

EXPERIMENTAL.

The strain of Pneumococcus I used in this work was isolated in November, 1924, from the blood of a patient with lobar pneumonia at the Presbyterian Hospital. Since then it has been passed through rabbits or guinea pigs at least once in every 2 weeks and is now of such virulence that 1-10,000,000 cc. regularly kills rabbits. In mice the lethal dose is 1-1,000,000 cc. No higher dilutions were tried.

Numerous preliminary experiments were conducted to find out whether the necrotizing toxin could be obtained from filtrates of various aerobic and anaerobic broth cultures, exudates, and the expressed fluids of organs and tissues of rabbits and guinea pigs which had died of pneumococcus infection, but the results were uniformly negative.

We next began a study of the autolysates of pneumococci for the presence of this hypothetical necrotizing poison. In general, the method used was to take up the sedimented pneumococci from well centrifuged broth cultures in saline solution, pneumococcus broth, or other fluid, and to allow the suspensions to autolyze, with and without a vaseline seal, at room temperature for different periods of time. It was soon observed that a slight necrotizing action was sometimes obtained with the preparations sealed with vaseline, while the preparations kept without the vaseline seal never possessed this property. From this we inferred that the poison either was formed only in the absence of oxygen, or if formed, was destroyed by oxidation. Various methods of protecting preparations from oxidation were tried, with methylene blue as an indicator. It was found that the addition of sodium hydrosulfite (5), a reducing agent, interfered with the formation of the poison; and that the addition of other reducing agents, such as cysteine (9), live *B. coli*, or yeast, was of no apparent advantage in obtaining an active autolysate. Below I have described in some detail the method which is now being used for the preparation of this toxin.

Preparation of the Necrotizing Poison.

The pneumococci are grown on double strength veal infusion broth containing 4 per cent Witte peptone and 0.5 per cent salt. Flasks are filled nearly to the top with the medium which has been brought to a pH of 7.8. The medium is sterilized in the Arnold sterilizer by the intermittent method.

Each flask containing 200 cc. was inoculated with 1 large loopful of blood from the heart of a guinea pig or rabbit which had died of a pneumococcus infection. After 18 to 24 hours in the incubator, the broth cultures were chilled and then centrifuged at high speed. After centrifuging, they were chilled, the supernatant fluid poured off carefully, and the pneumococci taken up in a quantity of freshly boiled and chilled broth equal to that of the sediment with the small amount of supernatant fluid remaining. For pneumococcus sediment from 100 cc. of broth culture, there should be in all approximately 1.5 cc. of fluid. The pH of the pneumococcus suspensions is brought to 7 or 7.2 and cultures taken to make sure that only pneumococci are present in the preparation. The pneumococcus suspension is distributed into narrow test-tubes, which are then chilled for at least 30 minutes. After this, any bubbles present on the surface of the suspension are gotten rid of with a hot platinum loop and heavy vaseline seals added to all the tubes. The tubes are left at room temperature in the dark at 22–24°C. for 6 to 8 days and then placed in the ice box until used. Immediately before use, the autolysates are centrifuged, iced, the seals opened, and the clear supernatant fluid filtered through a well iced Berkefeld apparatus. This filtrate contains the necrotizing poison. It is necessary to keep the preparations chilled when they are exposed to the air, otherwise they become oxidized and the toxicity disappears.

Young guinea pigs weighing from 250 to 350 gm. were inoculated intradermally on the side with 0.1 cc. of sterile pneumococcus necrotizing poisons. The reaction to the intracutaneous inoculation in these animals is as follows: 10 to 30 minutes after the inoculation, a small purplish area, which gradually increases in size, appears at the site of inoculation. With strong poisons, this area covers a zone of 2 to 3 cm. in diameter within 2 to 4 hours. At this time the area usually appears purplish black, with an encircling border which is bright red in color and which appears to be hemorrhagic. 18 to 24 hours after the inoculation, the central purple zone has become definitely yellowish and necrotic. Several days later, this necrotic area has become a brown scab.

Properties of the Necrotizing Poison.

When filtration of an active autolysate is carried out rapidly through a new N or V Berkefeld filter, with every precaution taken to have the apparatus and autolysate well chilled, there is practically no loss of its activity. Such a filtrate will remain active for several weeks if it is sealed with vaseline immediately after filtration and preserved in the ice box. If opened, even when kept packed in ice, it quickly deteriorates.

The necrosis-producing principle of an autolysate is completely destroyed when heated under vaseline seal at 60°C. for 5 minutes. Under these conditions, with a pH of 6.8 to 7, there is usually a slight clouding of the filtrate.

Are the Pneumococcus Hemotoxin and Necrotizing Poisons Related?

There is no doubt that our necrotizing autolysates contain other pneumococcus products such as the poison which produced the anaphylactic-like symptoms, the hemotoxin, and the purpura-producing principle. Is, then, the necrotizing toxin identical with one of these previously recognized poisonous substances which has been demonstrated by other methods? Apparently, the only toxic product of the pneumococcus which need concern us is the hemotoxin which, as regards thermolability, sensitiveness to oxidation, and ability to be neutralized by immune serum prepared against it, appears to be very similar to the necrosis-producing poison. During the course of this work we have titrated many of our autolysates for the presence of the hemotoxin, and have found, as would be expected, that they always contained more or less of the hemolytic substance, but there was no parallelism between the amount of hemotoxin and of the necrotizing substances present in an autolysate. Moreover, the extracts produced by the freezing and thawing method of Cole and Avery, while rich in hemotoxin, contained little or no necrotic activity. Furthermore, we have been able to separate the hemotoxic and necrotizing substances by treating the autolysate with the red cells of rabbits or guinea pigs. By this procedure the hemotoxin is entirely removed by the red cells, while the necrotizing poison remains unaffected in the supernatant fluid. These last experiments seem to prove conclusively that the hemotoxin and necrotizing poison are different entities. An experiment showing the selective adsorption of the hemotoxin is described below.

This autolysate caused complete hemolysis of 2.5 cc. of a 1 per cent suspension of washed rabbit red cells in a dilution of 1–200 and 50 per cent hemolysis in a dilution of 1–1000. 0.4 cc. of washed rabbit red cells was thoroughly mixed with 2 cc. of chilled necrotizing filtrate. The mixture was allowed to stand for 4 minutes at 2°C. and then centrifuged at high speed for 5 minutes, care being taken to have ice water in the centrifuge cups. After centrifuging, the cold clear slightly green-

ish supernatant fluid was pipetted off and used along with the original filtrate in the tests for hemotoxin. Table I shows the results of this experiment.

That the hemotoxin was removed by a specific combination with the red cells was proved by washing the centrifuged red cells in cold salt solution and resuspending them in warm salt solution. Hemolysis of the red cells took place promptly.

Very recently, by a slightly different method, Neill and Fleming (11) have also been able to completely remove the hemotoxin from pneumococcus extracts by adsorption with red cells.

Antigenic Properties of the Necrosis-Producing Principle.

Three rabbits were injected intracutaneously at weekly intervals with 1 cc. doses of sterile necrotizing filtrates from Pneumococcus I. Six injections were given

TABLE I.

Action of Hemotoxin-Free Autolysate.

Autolysate	Hemotoxic action		Necrotizing action	
	Before adsorption	After adsorption	Before adsorption	After adsorption
0.1 cc.	++++	0	++++	++++
0.02 cc.	++++	0	+	+

in all. The last two inoculations caused no reaction at the sites of injection. The rabbits were bled 10 days after the last inoculation and their serums titrated for the presence of neutralizing antibodies.

These tests were carried out as follows: 0.9 cc. of a well chilled necrotizing filtrate was placed in each of three narrow test-tubes. To the first tube was added 0.1 cc. of the antiserum; to the second tube 0.1 cc. of normal rabbit serum; and to the third the same amount of broth. The contents of the tubes were well mixed, and a heavy vaseline seal was then added to each tube. After standing at room temperature for 1 hour, the tubes were again chilled, the vaseline seals removed, and the preparations in amounts of 0.1 cc. were injected intracutaneously into a small guinea pig. The results are summarized in Table II.

Numerous similar experiments have been made, and it has been found that our immune serum in dilution of 1–10 neutralized the necrotizing poison, whereas normal rabbit serum in the same dilution had no detoxicating effect. The immune serum diluted 1–20 only par-

tially neutralized the toxin. Occasionally slight inactivation occurred with normal serums diluted 1–5.

These experiments seem to show that the immunization of rabbits with necrotizing filtrates causes the production of neutralizing antibodies. Interpretation of these results, however, is complicated by the fact that considerable cloudiness develops in the mixtures of immune serum and toxin, probably due to the action of the antiprotein precipitins, which the antiserums contain, on the pneumococcus protein which is present in the filtrates. From these experiments alone, it would be impossible to say whether the neutralization of the necrotizing poison by this immune serum is really a neutralization, or whether the active substance is merely carried down with the precipitated particles of protein. The same criticism applies to our experiments with Pneumococcus II autolysates as described below.

TABLE II.

Dilution of normal serum	Reaction on guinea pig	Dilution of Type I serum	Reaction on guinea pig
1–5	++	1–5	0
1–10	+++	1–10	0
1–20	+++	1–20	++

Is the Necrosis-Producing Principle Type-Specific or Species-Specific?

It is of considerable interest to know if the necrotizing autolysates prepared from other types of pneumococci can be neutralized by the serums produced against our preparations from Pneumococcus I. To investigate this point, we tested the neutralizing action of our Pneumococcus I antiserum against the necrotizing autolysates prepared from a Pneumococcus II strain which was isolated in October, 1927, from the blood of a patient with pneumonia at the Presbyterian Hospital. It was found that the Type I antinecrotizing serums in dilutions of 1–10 completely neutralized the Type II poison.

As far as they go, these experiments appear to indicate that the necrotizing principles of Pneumococcus I or II are antigenically similar and are probably, therefore, like the pneumococcus hemotoxin, species- and not type-specific.

CONCLUSIONS.

1. Certain pneumococcus autolysates produce necrosis when injected into the skin of guinea pigs.

2. The necrosis-producing principle can be filtered through a Berkefeld N filter, is extremely thermolabile, and is very sensitive to oxidation.

3. The necrotizing poison can be separated from the pneumococcus hemotoxin by adsorption with red cells. This removes the hemotoxin and leaves the necrosis-producing principle unaffected.

4. The necrotizing substances obtained from Pneumococcus Types I and II are neutralized by the antiserum prepared with Pneumococcus I.

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