DIFFERENTIATION BETWEEN CERTAIN TOXIC PROPERTIES OF FILTRATES OF HEMOLYTIC STAPHYLOCOCCUS AUREUS

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That sterile filtrates of hemolytic staphylococci are toxic for the cells of the body has been shown by their destructive action (1) on leucocytes (the leucocidins (1-4)), (2) on erythrocytes (the hemotoxins (5-8)), (3) on the skin (the dermatoxins or necrotoxins (9-14)), and (4) by their quick killing effect when injected intravenously in rabbits ("acute killing poison" (15-17)).

The evidence, however, is contradictory as to whether these various toxic effects are due to one or several distinct substances. The view that they are due to the same substance is based upon the following observations: (a) The poisons are all exotoxins with roughly the same heat stability. (b) The relative proportions of the different toxins in a toxic filtrate are approximately the same. (c) The antibodies produced by various methods against the live organisms or against the toxic filtrates, show equivalent neutralizing activities against the various toxic actions (hemotoxic, necrotoxic, or quick killing actions).

Not all observers are willing to accept the idea that the toxic actions described are due to one and the same substance. For instance, several observers have not been able to demonstrate the same relative proportion of the different poisons in the toxic filtrates while Neisser and Wecksberg (4), using leucocytes, were able to adsorb the leucocidin but not the hemotoxin from the filtrates, though they do not give the protocols of their experiments.

In previous papers (12, 13), one of us has reported the results of our studies of the necrotoxin in staphylococcus filtrates. Although in those papers no experiments were described which bear directly on the question of the unity of the necrotoxin and hemotoxin, our impression was that the necrotoxic and hemotoxic effects were due to separate constituents in the filtrates, because many of the cultures investigated were extremely hemolytic when streaked on blood plates and yet produced no demonstrable necrotoxin by the method used. At that time very few attempts had been made to study the killing properties of the toxic filtrates when given intravenously. However, we had observed that two young rabbits (weights 1030 and 710 gm.) died 2 hours after receiving intravenous injections of 2 cc. of the necrotizing poison, whereas large rabbits were insusceptible to it, even in large
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doses. Later, we obtained several filtrates which killed large rabbits (2000 gm.) in less than 24 hours in small amounts: viz., 0.1 to 0.2 cc., but these filtrates lost their toxicity after standing in the ice box for 7 days. No work was done to determine if a quantitative relationship existed between the killing, the necrotoxic, or the hemotoxic effects in these latter filtrates.

Recently Burnet (14) has studied the relationship which the necrotoxin, hemo-
toxin, and killing poisons of the staphylococcus bear to one another. He found that there was a quantitative relationship between the killing and necrotizing effects and a fairly quantitative relationship between the hemotoxic and killing effects in all filtrates from the three strains studied; and that in five antitoxins which had been produced by different methods, all showed a constant relationship in antitoxin for the three toxic activities. Based on these observations, he concludes that these three toxic activities are all manifestations of a single antigenic substance; in other words, that the staphylococcus produces a single exotoxin which destroys erythrocytes, skin, and capillary endothelium.

We wish to report some experiments which seem to establish the fact that there are at least two, and probably more, antigenically distinct exotoxins in toxic filtrates of the Staphylococcus aureus. The experiments to be described may be divided into two groups; first, those dealing with the relative proportions of hemotoxin, necrotoxin, leucocidin, and the "acute killing poison" in filtrates of hemolytic Staphylococcus aureus cultures; and second, those dealing with the selective adsorption of the toxins from the filtrate by erythrocyte stroma or by leucocytes.

EXPERIMENTAL

Preparation of Media.—A modification of Walbum's (18) medium was used throughout this work. This consisted of a sugar-free veal infusion broth to which 1 per cent Witte peptone, 1 per cent Difco peptone, 0.2 per cent KH2PO4, and 0.03 per cent MgSO4 7H2O were added.

Sources of Cultures.1—Four strains of the hemolytic Staphylococcus aureus were used. These strains were virulent for rabbits, death occurring within 1 to 3 days after receiving intravenous inoculations of 0.05 to 0.2 cc. of a broth culture. The virulence of a strain was found to be roughly proportionate to the quantity

1 The sources of the strains used were as follows:
779. 7/10/30 from a pustule obtained from Dr. Galbreath in Porto Rico.
814. 12/1/30 from a blood culture obtained from Dr. McKinley in Porto Rico.
805. 12/1/30 from a blood culture from a case of acute endocarditis at the Presbyterian Hospital.
782. 12/17/30 from a skin lesion from the Vanderbilt Clinic.
of hemotoxin it produced. Since our previous work indicated that the virulence of a strain disappeared rapidly when subcultured on agar, in these experiments inoculations of the broth for toxin production were made only from the original blood agar slants.

_Growth of the Staphylococcus._—The staphylococci were grown aerobically in Erlenmeyer flasks two-thirds full of the medium. Growth was allowed to continue until the pH, which always dropped to 6.4 to 6.8 for the first few days of growth, had risen to 7. This required from 10 to 16 days, depending on the strain and batch of medium used. After incubation, the cultures were distributed in tubes, chilled, vaseline seals added, and centrifuged. The clear chilled supernatant fluids were then passed through Berkefeld V filters. The filtrates were preserved in the ice box under vaseline seals. With these precautions to prevent oxidation, the filtrates retained all their toxic properties for at least 4 months.

For testing the action of the filtrates to kill rapidly, young rabbits weighing between 700 and 1000 gm. were used. The reaction of the small rabbits to the same dose of filtrate is remarkably regular. For skin tests, rabbits weighing from 1500 to 2000 gm. were used. From eight to sixteen tests with various dilutions of toxins may be inoculated into one animal.

_Hemotoxin Tests._—Tests for hemotoxin were carried out in the usual way. One unit of hemotoxin was taken as the smallest amount of a filtrate which completely hemolyzed 2 cc. of 1 per cent well washed rabbit red cells in 40 minutes.

_Necrotoxin Tests._—One unit of necrotoxin was taken as the smallest amount of filtrate or diluted filtrate which, when injected intradermally, will cause definite necrosis of an area of 5 mm. in diameter.

_Leucocidin Tests._—The leucocyte suspensions for the leucocidin tests were obtained from rabbits following the intrapleural injections of aleuronat. 18 hours after injection, the animals were bled to death from the carotid, and the pleural exudates taken up in one-third their volume of 0.5 per cent sodium chloride solution containing 2 per cent sodium citrate. Only those exudates showing no reddish color and containing very few, if any, red cells, were used. The exudates were then titrated to determine the proper amount to use in the leucocidin tests. For this purpose, 0.05 cc. of 1:10,000 solution of methylene blue was added to varying amounts to the leucocyte suspension in precipitin tubes, and the volume in each tube brought up to 1 cc. with saline solution. Vaseline seals were added to all the tubes, and they were put in a water bath at 37°C. The volume of the leucocyte suspension used in the leucocidin tests was the amount that would reduce the methylene blue completely in 20 minutes.

The leucocidin tests were set up as follows: the previously determined quantity of the leucocyte suspension was added to various amounts of the staphylococcus filtrate to be titrated, and the volume of all tubes brought up to 1 cc. with saline. One control tube containing the largest dose of the filtrate used in the experiment was heated at 60°C. for 20 minutes, and a second contained the leucocytes without the filtrate. All tubes were placed in the water bath at 37°C. for 1 hour, then 0.05 cc. of 1:20,000 of methylene blue and a vaseline seal were added to each; and
finally they were reincubated for 1 hour. One unit of leucocidin represented the minimum amount of a filtrate which completely prevented the reduction of methylene blue by the leucocytes in 1 hour.

"Acute Killing" Tests.—One unit of the "acute killing" toxin represented the smallest amount of filtrate which, when injected intravenously, would kill a 700 to 1000 gm. rabbit within 1 hour. The symptoms of rabbits which die soon after the intravenous injection of staphylococcus filtrates have been described by others. For some time after receiving the injection the animals appear normal, but then become unsteady, fall over, breathing becomes rapid and shallow, and they become progressively weaker until death, which usually occurs in less than 1 hour after injection. At autopsy nothing abnormal is to be seen. If the dose of toxin is so adjusted that the rabbits survive for 12 to 48 hours, a very different picture presents itself. 1 to 3 hours after injection they flatten out on their abdomens, appearing weak, and they become progressively weaker till death.

At autopsy marked changes are always present in the kidneys. In the animals which die in 12 to 24 hours, the kidneys are mottled deep red against a pale background or are a deep purplish red throughout. In those which survive longer, viz., 24 to 48 hours, the kidneys are a deep red, mottled with yellow areas varying from 1 to 10 mm. in diameter. The histological changes in these kidneys will be taken up in a later publication.

A. Experiments Dealing with the Relative Proportions of the Various Toxins in the Staphylococcus Filtrates.—Our first experiments were devised to determine the accuracy of Burnet's claim that the three toxic activities—(hemotoxic, necrotoxic, or "acute killing")—bear constant ratios to one another. A large number of filtrates from our four toxic strains were titrated for these three toxins, some also being tested for leucocidins.

Our results may be briefly summarized as follows: In general, filtrates which were strong in one toxic activity were strong also in the others. In the strongly hemolytic filtrates, viz., those containing 100 to 200 hemotoxic units per cubic centimeter, there appeared to be a fairly definite ratio between the hemotoxic, necrotoxic, and "acute killing" activities. This same quantitative relationship held for all strong hemotoxic filtrates from the four strains studied. Roughly, one "acute killing" unit was equivalent to 25 hemotoxic and to 80 to 100 necrotoxic units. In filtrates with less hemotoxic strength this quantitative relationship did not hold, especially as regards the ratio of the hemotoxic to the necrotoxic units in a filtrate. There appeared to be no definite relationship between the leucocidins in a
It is clear that no definite conclusions as to the unity or plurality of poisons in staphylococcus filtrates can be drawn from these experiments. More convincing results were obtained in the following experiments.

B. Experiments Dealing with the Adsorption of Staphylococcus Toxic Filtrates with Erythrocyte Stroma or with Leucocytes.—In a previous paper, it was proved conclusively by adsorption of pneumococcus toxic autolysates with red cells in the cold, that the necrotoxin and the hemotoxin of the pneumococcus are separate entities. On analogy, one would suppose that the staphylococcus hemotoxin and necrotoxin are likewise different substances.

Neisser and Wecksberg (4) were able to remove staphylococcus hemotoxin from the toxic filtrates by treatment with red blood cells in the cold. We have made many attempts to confirm their findings, both with sheep and rabbit red cells, but always with negative results. It appeared that under the conditions of our experiments, staphylococcus hemotoxin and red cells combined only at higher temperatures, where hemolysis also occurred and obscured the results.

We next attempted to bind the hemotoxin with erythrocyte stroma, a procedure which could be carried out at 37°C. By this method, we were successful in obtaining almost complete selective adsorption of the hemotoxin with either rabbit or sheep red cell stroma, leaving the necrotoxin undiminished in the filtrate. When the stroma-adsorbed filtrates were tested for the presence of leucocidin it was found that most of this toxin had also been removed from the treated filtrates. Very little of the leucocidin could have been removed by the relatively small amounts of leucocytic substances present in the stroma prepara-

<table>
<thead>
<tr>
<th>Filtrates</th>
<th>Killing dose</th>
<th>&quot;Acute killing&quot; unit</th>
<th>Hemotoxin</th>
<th>Necrotoxin</th>
<th>Leucocidin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Strongly toxic</td>
<td>0.1 to 0.25 cc.</td>
<td>1</td>
<td>25</td>
<td>80-100</td>
<td>5-20</td>
</tr>
<tr>
<td>Weakly toxic</td>
<td>0.5 to 2 cc.</td>
<td>1</td>
<td>40-60</td>
<td>80-300</td>
<td>5-20</td>
</tr>
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tions, for in our later experiments in which we used leucocyte sus-
pensions as the adsorbing agent, very thick leucocyte suspensions
were required to remove this toxin. As noted before, Neisser and
Wecksberg also found that red cells adsorbed leucocidin as well as
hemotoxin from staphylococcus filtrates. Because of insufficient
material, only two young rabbits, weighing 610 and 835 gm., were
injected intravenously with 1 and 0.75 cc. respectively of the stroma-
adsorbed filtrates, and both survived. These rabbits were killed 3
days after receiving the injections and were found to have no lesions
in the kidneys. The untreated filtrate diluted one-half killed rabbits
in 0.2 and 0.3 cc. amounts respectively. The survival of these two
rabbits and the absence of kidney lesions at autopsy, appear to indicate
that the substances responsible for sudden death and the kidney le-
Table II

<table>
<thead>
<tr>
<th>Filtrate</th>
<th>Hemotoxin</th>
<th>Necrotoxin</th>
<th>Leucocidin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stroma treated</td>
<td>0.15</td>
<td>0.0035</td>
<td>0</td>
</tr>
<tr>
<td>Untreated</td>
<td>0.015</td>
<td>0.0035</td>
<td>0.05</td>
</tr>
</tbody>
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and therefore cannot have been associated with the necrotoxic action
of the filtrates. A typical protocol of an experiment with a stroma-
treated filtrate is given below:

Experiment.—To 1 cc. of packed stromata obtained from 50 cc. of rabbit red
cells, was added 2 cc. of a staphylococcus filtrate plus 2 cc. of normal salt solution,
and the preparations well mixed. The control tube contained 2 cc. of the same
filtrate plus 2 cc. of normal salt solution. After adding paraffin oil to both tubes,
they were placed in the water bath at 37°C. for 2 hours and then in the ice box
overnight. The following morning the tube containing the stroma was centri-
fuged and the clear, slightly reddish supernatant fluid tested for the various toxic
effects along with the control. While being centrifuged, the tube was kept im-
mersed in ice water.

The results are summarized in Table II.

The experiment recorded in Table II seems to show that the red cell shadows have removed nine-tenths of the hemotoxin and the
leucocidin, leaving the necrotoxin undiminished in the filtrate.
The purpose of our next experiments was to find out if the leucocytes selectively adsorb one or more of the toxins from a toxic filtrate. In their work on this point, Neisser and Wecksberg (4) state that in the cold, leucocytes remove the leucocidin and not the hemotoxin from staphylococcus toxic filtrates.

The results of our experiments along this line have not been conclusive and therefore will not be given in detail. We found that the leucocytes sometimes did, and sometimes did not, remove the hemotoxin from the filtrates. Only very thick suspensions of leucocytes without a trace of red cells were used, so that the removal of the hemotoxin could not have been due to the presence of red cells in the preparation. However, in contrast to this, the leucocidin was invariably almost completely removed by the leucocytes; whereas the necrotoxin remained undiminished in the leucocyte-treated filtrates. Taken as a whole, these last experiments with leucocyte-treated filtrates bring out nothing new but confirm the points brought out in the red cell stroma experiments; viz.: that the necrotoxin is a different toxic principle from either the hemotoxin or the leucocidin.

CONCLUSIONS

1. Sterile filtrates from certain hemotoxic strains of Staphylococcus aureus have several toxic properties, of which the most important are the hemotoxic, the necrotoxic, the leucocidic and the property of killing rapidly.

2. The necrotoxic action appears to be caused by a constituent in the filtrates different from either the hemotoxic or the leucocidic one.

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