

THE TOXIC PROPERTIES OF SERUM EXTRACTS OF HEMOLYTIC STREPTOCOCCI

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Although much work has been done in the search for a toxin of *Streptococcus hemolyticus* having lethal properties, no such toxin has, to date, been discovered. It is true that sterile filtrates of streptococcus cultures contain certain toxins, hemotoxins (1), leucocidins (2), Dick toxins (3), but none of these filtrates has been shown to be toxic for animals except in relatively enormous doses (4). Yet from the symptoms and pathology of streptococcus infections in man and in animals, there is every reason to believe that, *in vivo*, virulent strains of the streptococcus do produce a toxin or toxins with marked toxic properties which cause death. It was in the attempt to produce, *in vitro*, such a toxin or toxins that this work was undertaken.

In the course of some work on the production of powerful streptococcal hemotoxin, it was discovered that under certain definite conditions, the organisms themselves, if centrifuged down from broth cultures and taken up in the same volume of physiological salt solution or broth, were very hemolytic in minute amounts. That this hemolytic action had nothing to do with the growth of the organism in the red cell suspension was proved by plating the preparations, before, during, and after incubation and finding that there was no increase or only a slight increase of streptococci during the incubation. From these experiments it seemed probable that the streptococcal hemotoxin was bound to the surfaces of the organisms whence it was given off directly to the red cells.

Furthermore, the powerful hemotoxic effect of these streptococcus suspensions was obtained only from young cultures of 13 to 16 hours

growth. The hemotoxic content of suspensions of older cultures diminished directly with the age of the culture until practically no hemotoxin was demonstrable in suspensions of 2 days growth. Heating hemolytic suspensions to 56°C. for 20 minutes completely destroyed their hemotoxic properties.

It was next discovered that these hemotoxic streptococcus suspensions were extremely toxic for mice, causing death within 2 hours when injected intravenously. This lethal action of the suspensions appeared to be bound up in some way with their hemolytic content because suspensions of old cultures containing little hemotoxin or hemotoxic suspensions heated to 56°C. for 20 minutes were non-toxic for these animals.

In this work with streptococcus suspensions, the important question to be answered seemed to be what caused the death of these mice? Was death due to the hemotoxin *per se*, to some other toxic substance present with the hemotoxin, or to both, or was death brought about by some physical property of the preparation not present in suspensions of old organisms nor in heated suspensions? If death was caused by some toxic substance or substances attached to the surface of the organisms, it seemed possible that the toxic substance or substances might be removed by extracting streptococci with various materials. This we were successful in doing. By treating streptococci with serum, we obtained toxic extracts which were hemolytic and which, in relatively small amounts, killed mice. The following report deals with the methods used in the preparation of these toxic extracts and the experiments made to determine their nature.

Methods

Preparation of Media.—The medium used in this work which gave the best results was prepared as follows: 2 pounds of chopped veal in 850 cc. of distilled water were allowed to stand in the ice box for 2 or 3 days. Then in the meat juice recovered by squeezing through cheese-cloth, 40 gm. of Witte peptone were dissolved by heating the mixture to 45°C. for 20 minutes. After this the meat extract adjusted to a pH of 4.8 with N HCl, was poured into a 1 liter flask, tightly corked, and was boiled in a water bath for 30 minutes. Then to the clear meat extract supernatant recovered by centrifuging were added a sterile Berkefeld filtrate of 2 gm. of NaHCO₃ and 1 gm. of Na₂HPO₄·12H₂O dissolved in 100 cc. of distilled water. The whole preparation was next brought up to a pH of 8.1 with N NaOH, heated in a water bath at 70°C. for 15 minutes, cooled, and centri-

fuged. Finally the clear supernatant obtained by centrifuging was dispensed into centrifuge tubes 6 x 1 inch, 35 to 40 cc. to each tube, chilled, and heavy vaseline seals were added.

It is noted that in the preparation of this medium, the meat extract is heated at a high temperature only when adjusted to a low pH and that all filtration except in the instance of the buffers, is excluded.

When proper precautions are taken in the handling of the materials, the medium described above is sterile. In twenty-three consecutive batches no contaminations were encountered.

Source of Cultures.—Only 1 strain of *Streptococcus hemolyticus* was used in this work. This was an erysipelas strain obtained from Dr. Ada Clarke of the Department of Bacteriology, College of Physicians and Surgeons.

Sera Used.—Sheep, horse, and rabbit sera were employed in this work. The sera were inactivated at 56°C. for 40 minutes before use.

Throughout the extraction of streptococci with serum, the pH of a preparation is important, since if the pH is as high as 8.4 during the extraction process, only weak toxins are obtained. With sheep serum whose pH after inactivation is usually about 8.5, the pH was brought down to 8.2 with *N* HCl before use. Although the pH of both inactivated horse and rabbit sera may be as high as that of inactivated sheep serum, their pH's fall more rapidly than that of the sheep serum during extraction and so it was found unnecessary to adjust their reactions. To obtain the best results, the final pH of a preparation after extraction was found to be 8 to 8.1. When, after extraction, the pH of an extract was found to have fallen below pH 8, which often occurred when rabbit serum was used, it was raised to 8.1 with *N* NaOH. The reason for this last procedure is that although the toxins when freshly prepared with a final pH of 7.4 to 7.8 are exactly as toxic as those with a final pH of 8 to 8.1, they deteriorate more rapidly. Todd in his work on streptococcal serum hemolysin also noted that the hemolysin deteriorated faster at a pH of 7.4 than at pH 8 (8).

Preparation of Toxic Extracts.—Each tube of medium was inoculated through the vaseline seal with 0.1 cc. of a 1-50 dilution of streptococcus pleural exudate. After 13 hours growth, the tubes were chilled and centrifuged at high speed until the supernatants were clear. Then the supernatants were pipetted or poured off and the sedimented streptococci from each tube were taken up in 2 cc. of inactivated serum. The serum suspensions of streptococci were pooled, glass beads added, and they were shaken in a slow shaking machine for 1 hour. No growth of this strain of hemolytic streptococcus occurs in inactivated undiluted serum. After shaking, the material was chilled and centrifuged. Finally the clear supernatant was passed through a Chamberland-Pasteur filter L₂ for sterilization purposes. No poisonous properties were lost by such filtration. The recovered filtrate was the toxic extract used in this work.

The process of extraction of the same streptococci with untreated serum may be repeated twice if all 3 extractions are carried out on the same day. Under

these conditions, all 3 extracts are of approximately equal potency. More than 3 extractions have not been attempted.

Hemotoxin Tests.—Tests for hemotoxin were carried out in the usual way. Dilutions of the filtrate to be tested were made either with broth or with physiological salt solution. 1 unit of hemotoxin represented the smallest amount of filtrate which completely hemolyzed 2 cc. of 1 per cent well washed rabbit red cells in 1 hour at 37°C. in a water bath.

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 exsanguinated from the carotid artery, under ether anesthesia, and the pleural exudates procured from them were immediately centrifuged slowly for 3 minutes. Nine-tenths of the slightly clouded supernatant was discarded, leaving the leucocyte concentration approximately 10 times that of the original pleural exudate. Only those exudates showing no reddish color and containing very few if any red cells were used in the tests. The concentrated exudates were then titrated to determine the proper amount to use in the leucocidin tests. For this purpose, 0.1 cc. of 1–5,000 dilution of methylene blue was added in varying amounts to the leucocyte suspension in large precipitin tubes and the volume in each tube brought up to 0.5 cc. with saline solution. Vaseline seals were added to all the tubes and then they were put in a water bath at 37°C. The quantity of the leucocyte suspension used in the leucocidin tests was the amount that reduced the methylene blue completely in 20 minutes. The leucocyte suspension was diluted with broth so that 0.1 cc. contained the required amount.

The leucocidin tests were set up as follows: 0.1 cc. of leucocyte suspension was added to varying amounts of streptococcal serum extract and the volume of all tubes made up to 0.5 cc. with saline. All tubes were placed in the water bath at 37°C. for 1½ hours, during which time they were taken out and shaken at intervals of 10 minutes. Then 0.1 cc. of 1–5,000 dilution of methylene blue and a vaseline seal were added to each tube; and finally they were reincubated for 1 hour. 1 unit of leucocidin represented the minimum amount of a filtrate which completely prevented reduction of methylene blue by the leucocytes in 1 hour.

EXPERIMENTAL

Toxic Properties of Serum Extracts of Streptococci. Toxicity for Mice.—Mice were injected intravenously through a tail vein with the toxic filtrates. Generally 0.1 to 0.2 cc. of a filtrate brought about death within 24 hours. With larger amounts of a toxic filtrate, death occurred within 1 hour. The symptoms were dyspnea, weakness, and prostration. When the animals survived 1½ hours, deep red urine was passed. This appeared 50 minutes to 1½ hours after the inoculation.

At autopsy in those animals which died quickly, that is within 1 hour, nothing abnormal was seen macroscopically. In the majority of those which survived longer (2 to 8 hours) the bladder was large, tense, reddish black, and contained several cubic centimeters of very red urine. In the others, the bladder was small but almost always a slight amount of bloody urine was present. In animals which survived still longer (8 to 48 hours) generally all the organs and tissues were pale. Sometimes there was a slight amount of blood still present in the urine. The red color of the urines was shown to be due to the presence of hemoglobin. The pathology of mice dying of the serum extract toxin will be taken up in a later publication. M'Leod and M'Nee also noted hemoglobinuria and anemia in rabbits inoculated with large doses of streptococcal hemotoxic filtrates (4).

Normal inactivated serum (sheep, horse, or rabbit) had no toxic effect when injected intravenously into mice in 0.5 cc. amounts, nor had serum extracts of young cultures of other organisms (hemolytic *Staphylococcus aureus*, virulent Pneumococcus III, *B. typhosus*) produced by the same method as that used in the preparation of toxic extracts of streptococcus, any toxicity for mice. These latter preparations also contained no hemotoxin.

Experiment 1, Table I, gives one of our protocols on the effect of a toxic extract on mice.

This toxic extract was made with inactivated sheep serum. The pH of the filtrate was 8.1. It contained 200 hemotoxic units per cc.

The control serum was some of the same inactivated sheep serum as that used for the production of the toxic extract. Before use its pH was brought to 8.1 with N HCl and it was filtered through a Chamberland-Pasteur filter. Both the toxic extract filtrate and the control serum filtrate were sterile.

Hemotoxic Activity of Toxic Extracts.—All the serum extract filtrates were titrated for hemotoxin. Freshly prepared extracts which were toxic for mice in small amounts were always markedly hemolytic. These preparations contained between 150 and 330 hemotoxic units per cc. The question of the relationship between the lethal and the hemotoxic content of a filtrate will be taken up in a later part of this paper.

Leucocidic Activity of Toxic Extracts.—Relatively few of the toxic

extracts were titrated for leucocidin. However the results obtained in these were clear-cut. Usually a toxic filtrate contained 10 leucocidic units. An interesting phenomenon occurring in these tests was the clumping of the leucocytes in sticky masses in some of the tubes containing the toxic extracts. This necessitated frequent shaking of

TABLE I
Inoculation of Mice with a Serum Extract of Hemolytic Streptococci

| Mouse No. | Weight <i>gm.</i> | Material inoculated intravenously | Symptoms | Died or survived | Autopsy |
|-----------|----------------------|--------------------------------------|----------|---------------------|--|
| 1 | 16.5 | Control serum 0.5 cc. | 0 | S | Killed with ether 3 days after inoculation. Autopsy negative |
| 2 | 15.0 | Control serum 0.4 cc. | 0 | S | Killed with ether 3 days after inoculation. Autopsy negative |
| 3 | 14.5 | Toxic filtrate 0.4 cc. | ++++ | D 8 min. | Negative |
| 4 | 17.2 | Toxic filtrate 0.4 cc. | ++++ | D 8 min. | Negative |
| 5 | 17.2 | Toxic filtrate 0.3 cc. | ++++ | D 40 min. | Subcutaneous tissues and peritoneum deep pink. Intestines congested. Urine negative |
| 6 | 14.0 | Toxic filtrate 0.3 cc. | ++++ | D 1½ hrs. | In ice box overnight. Intestines congested. Urine negative |
| 7 | 17.0 | Toxic filtrate 0.2 cc. | ++++ | D 5 min. | Negative |
| 8 | 15.0 | Toxic filtrate 0.2 cc. | +++ | D 18 hrs. | Post mortem. Slight amount of red urine in bladder. Liver pale |
| 9 | 17.7 | Toxic filtrate 0.1 cc. | ++ | D 26 hrs. | Liver pale. Urine negative |
| 10 | 15.0 | Toxic filtrate 0.1 cc. | ++ | S | For 2 days very sick—then better. Killed with ether 3 days after inoculation. Liver pale brown. Few yellowish areas on surface. Kidneys dark brown |

the tubes during the first incubation in order to bring the leucocytes in contact with the toxin. In the tubes containing strong undiluted toxin, the leucocytes were dissolved and the tubes cleared.

Control tubes containing undiluted or diluted filtered inactivated serum brought to pH 8.1 had no effect or only a very slight effect in

delaying the reduction of the methylene blue by the leucocytes. Sometimes there was a slight sedimentation of the leucocyte suspensions, which, however, could be resuspended easily by shaking. The question of the relationship of the leucocidin to the other toxic properties of the toxic extracts is not taken up in this paper.

TABLE II

Titrations of a Serum Extract Filtrate for Leucocidin

Toxin A.—Horse serum extract filtrate, pH 8.1.

Toxin B.—Toxin A, to each cubic centimeter of which was added 0.05 cc. of hemolyzed red cells (0.1 cc. packed red cells, 2 cc. distilled water, centrifuged, used clear supernatant). Toxin B was prepared because of Evans' (2) work showing that washed red cells increase the production of leucocidin in broth culture filtrates of hemolytic streptococci.

| Tube No. | Toxin | Amount | | | | Amount of reduction in | | | | |
|----------|---------------------|--------|-------|-----------------------|---|------------------------|---------|---------|-------|---------|
| | | cc. | Broth | Leucocytic suspension | | 10 min. | 20 min. | 30 min. | 1 hr. | 1½ hrs. |
| 1 | A | 0.4 | — | 0.1 | 1½ hrs. at 37°C. in water bath—then added 0.1 cc. of 1-5,000 dilution of methylene blue and vaseline seals to each tube and reincubated | 0 | 0 | 0 | 0 | 0 |
| 2 | A | 0.3 | 0.1 | 0.1 | | 0 | 0 | 0 | 0 | 0 |
| 3 | A | 0.2 | 0.2 | 0.1 | | 0 | 0 | 0 | 0 | 0 |
| 4 | A | 0.1 | 0.3 | 0.1 | | 0 | 0 | ± | + | + |
| 5 | B | 0.4 | — | 0.1 | | 0 | 0 | 0 | 0 | 0 |
| 6 | B | 0.3 | 0.1 | 0.1 | | 0 | 0 | 0 | 0 | 0 |
| 7 | B | 0.2 | 0.2 | 0.1 | | 0 | 0 | 0 | 0 | 0 |
| 8 | B | 0.1 | 0.3 | 0.1 | | 0 | 0 | ± | + | + |
| 9 | Hemoglobin in broth | 0.4 | — | 0.1 | | 0 | ++ | +++ | +++ | +++ |
| 10 | — | — | 0.4 | 0.1 | | ++ | +++ | +++ | +++ | +++ |

± to +++ indicates amount of reduction of methylene blue.

+++ indicates complete reduction.

Experiment 2, Tables II and III, gives a leucocidin and a hemotoxin titration of a toxic extract filtrate.

After 10 minutes incubation (1st incubation) the contents of Tubes 1 and 5 had slightly cleared and suspensions in Tubes 2, 3, 6, and 7 had clumped. After 1 hour Tubes 1 and 5 were almost clear and the leucocytes in Tubes 2, 3, 4, and

6, 7, and 8 were clumped into sticky masses that were hard to break up by shaking. Tubes 9 and 10 showed only a slight sedimentation of the leucocyte suspension.

Smears were made from all the tubes after 1½ hours incubation (1st incubation) and stained with Jenner stain. In all the tubes excepting the controls (Tubes 9 and 10) the leucocytes had disintegrated or stained badly. The leucocytes appeared normal in Tubes 9 and 10.

In this experiment there seems to have been no difference in the amount of leucocidin in Toxins A and B; in other words, the presence of hemoglobin did not cause an increase of leucocidin. In another experiment, using a different extract, the addition of hemoglobin was also without effect.

TABLE III
Titration of Serum Extract Filtrates A and B for Hemotoxin

| Toxin | 30 min. | | | | | 1 hr. | | | | |
|-------|---------|-------|-------|-------|-------|-------|-------|-------|-------|-------|
| | 0.01 | 0.008 | 0.006 | 0.004 | 0.002 | 0.01 | 0.008 | 0.006 | 0.004 | 0.002 |
| A | +++++ | +++++ | +++++ | +++++ | ++ | +++++ | +++++ | +++++ | +++++ | +++ |
| B | +++++ | +++++ | +++++ | ++++ | ++ | +++++ | +++++ | +++++ | +++++ | +++ |

++ indicates hemolysis of approximately 1/2 to 3/4 of cells.

+++ indicates hemolysis of approximately 3/4 of cells to complete hemolysis.

++++ indicates complete hemolysis.

Biological Properties of the Streptococcus Toxic Filtrates

Stability and Preservation.—Heating a toxic extract to 56°C. for 30 minutes destroys virtually all the lethal and hemotoxic actions of a filtrate; and heating to a 56°C. for 2 hours almost completely destroys its leucocidic activity. The toxic properties of an extract of pH 8.1 usually remain stationary at ice box temperature for 24 hours, but if an extract is kept longer than this, it deteriorates rapidly, irrespective of whether or not it is covered with vaseline. Attempts were made to preserve the toxic activities of extracts by keeping them in jars of hydrogen or by reduction with 0.1 per cent Na₂S₂O₄ or by both these methods.¹ Our results indicate that the Na₂S₂O₄ did delay slightly the deterioration of the lethal and hemotoxic activities of a preparation but that the hydrogen had no effect in postponing the deterioration.

Immunology of Toxic Filtrates.—The question of the antigenicity of the toxic extracts was next considered. Numerous attempts to produce streptococcal antihemolysin have resulted in negative experi-

¹ We are indebted to Miss Ruth Pauli for setting up the hydrogen jars for us.

ments until the recent discovery of Todd (5) showing that the presence of serum in culture media for the preparation of streptolysin modifies the lysin, making it non-antigenic, whereas streptolysin prepared by growing streptococci in media without serum is an active antigen. From this work it was natural to expect that streptococcal serum extract filtrates also would not stimulate the production of anti-hemotoxin.

Our experiments along this line of work are only preliminary and therefore incomplete. We attempted to immunize 3 series of mice with serum extract toxins, using a toxin prepared with a different kind of serum for the test inoculation. These experiments were entirely negative; *viz.*, the immunized mice showed no more resistance to the toxin than the controls. We also tested several different antistreptococcal sera for the presence of neutralizing antibodies for the lethal toxin and the hemotoxin of streptococcal serum extract. Again our results were negative.

These experiments, so far as they go, appear to indicate that the lethal and the hemotoxic principles of the extracts are not antigenic. We expect to extend the researches on the immunology of streptococcal extract filtrates in a later work.

*What Substance or Substances in the Toxic Filtrates Cause Death
of Mice?*

A question of interest was: Is death of the mice due to the anemia produced by the hemotoxin *per se*, or to some condition resulting from the anemia it causes, or is death due to some other toxic principle or principles in the toxic filtrates?

In the first place, it was conclusively proved that the sickness and death of mice inoculated with toxic extracts, were not due to the toxic properties of the hemoglobin liberated during the hemolysis of the red cells. Large amounts of hemolyzed red cells (0.5 cc. of a 1-2 dilution of ether-hemolyzed packed rabbit red cells) were not toxic when injected intravenously into mice. Such an amount of hemoglobin caused hemoglobinuria similar to that obtained after inoculation of a toxic extract.

Secondly, it was demonstrated that extreme anemia is produced in mice inoculated with toxic extracts, by the red cell counts obtained in a series of 6 toxin-injected mice. Tables IV and V give the results of this experiment.

The toxin used in the experiments (Tables IV and V) was a comparatively weak sheep extract filtrate, both as to lethal and hemotoxic content, the latter being 150 units per cc.

The results of this experiment demonstrate that all the mice had severe anemia after the injection with the exception of Mouse 1, which became neither anemic

TABLE IV

Toxic Effect on Mice of Intravenous Injection of Streptococcus Serum Extract Filtrate

| Mouse No. | Weight | Amount of toxin injected | Symptoms | Died or survived | Remarks |
|-----------|--------|--------------------------|----------|------------------|-------------------------------------|
| | | cc. | | | |
| 1 | 17.5 | 0.2 | 0 | S | Never sick |
| 2 | 19.0 | 0.3 | ++++ | D 3 hrs. | |
| 3 | 19.5 | 0.2 | +++ | S | For 3 days very sick. June 26, well |
| 4 | 22.0 | 0.25 | ++++ | D 4 hrs. | |
| 5 | 21.5 | 0.25 | +++ | D 6 hrs. | |
| 6 | 18.0 | 0.3 | +++ | S | For 3 days very sick. June 26, well |

TABLE V

*Anemia Produced in Mice Inoculated with Toxic Extracts (See Table IV)**

R. B. C. counts in thousands per cubic millimeter

| Date | Time | No. 1 | Time | No. 2 | Time | No. 3 | Time | No. 4 | Time | No. 5 | Time | No. 6 |
|---------|------|-------|------|-------|------|-------|------|-------|------|-------|------|-------|
| 1933 | | | | | | | | | | | | |
| June 12 | | 8,950 | | 6,980 | | 7,870 | | 6,740 | | 7,210 | | 6,870 |
| June 13 | | 8,888 | | 6,688 | | 7,210 | | 7,195 | | 7,368 | | 6,970 |
| June 14 | | 7,880 | | 7,460 | | 7,930 | | 7,910 | | 6,970 | | 7,850 |
| June 17 | | 6,890 | | 6,410 | | 7,290 | | 7,320 | | 6,930 | | 7,050 |

Inoculated at 11 a.m. June 22. (See Table IV.)

| | | | | | | | | | | | | |
|---------|---------|-------|--------|----------|---------|-------|--------|----------|--------|----------------|---------|-------|
| June 22 | 3 p.m. | 7,610 | 1 p.m. | 1,860 | 1 p.m. | 3,850 | 1 p.m. | 2,170 | 3 p.m. | 2,760 | 1 p.m. | 2,610 |
| June 23 | 10 a.m. | 7,800 | | D 3 hrs. | 10 a.m. | 2,980 | | D 4 hrs. | | D about 6 hrs. | 10 a.m. | 2,300 |
| June 26 | 4 p.m. | 6,800 | | | 4 p.m. | 4,810 | | | | | 1 p.m. | 3,870 |

* We are indebted to Miss Margaret Prest for making the R. B. C. counts in this experiment.

nor sick. The most probable explanation of this discrepancy is that the inoculation was given subcutaneously instead of intravenously.

From these observations it appears that if the toxicity of the extracts for mice is due to the presence of hemotoxin in such extracts, death is brought about by the anemia itself or by some condition resulting from the anemia and not by the presence of hemoglobin *per se*.

But if death of the mice is caused by anemia, it would be logical to suppose that there would be a correlation between the hemotoxic and lethal properties of a preparation. Such, however, is not the case. In a series of 30 different streptococcal filtrates, titrated both for hemotoxic and lethal principles, 1 lethal unit of toxin contained anywhere from 11 to 100 units of hemotoxin. This series contained freshly prepared toxins which were usually strong in both lethal and hemotoxic properties, and weaker toxins, which had been deteriorated through standing or slight heating (37°C. for 1 hour or 56°C. for 5 minutes) and which were usually relatively stronger in lethal than in hemotoxic content per cubic centimeter. This last observation seems to indicate that the lethal principle is relatively more stable than the hemotoxic principle.

In connection herewith, it is interesting to note that the relationship between the lethal and hemotoxic content of a streptococcal serum extract preparation is in direct contrast to that obtained between the lethal and hemotoxic content of staphylococcal toxic filtrates. Here 1 lethal unit in a weak preparation contained usually twice as many hemotoxic units as in a strongly toxic preparation (6).

The preceding evidence on the lack of parallelism between the hemotoxic and lethal activities of streptococcal serum extracts appears to signify that there is some toxic substance other than the hemotoxin in such extracts which causes death; in other words, that the injected mice do not die solely of anemia or some condition resulting from the anemia. At present we have no experiments bearing on whether this other toxic substance is leucocidin.

Extraction of Hemolytic Streptococci with Diluted Serum and with Materials Other than Serum

Attempts were made to extract toxic substances from hemolytic streptococci with diluted serum and with various other materials.

Inactivated serum diluted 1-2 or 1-5 with broth is a fairly strong extracting agent for hemotoxin, the most potent preparation obtained containing 160 units per cc. Serum diluted 1-5 with distilled water, boiled for 10 minutes, and brought to isotonicity with NaCl also extracts hemotoxin from streptococci, in one experiment 80 units per cc. However, filtrates from the latter preparations (diluted serum and diluted boiled serum extracts) did not kill mice in 0.5 cc. amounts injected intravenously. Both egg white diluted 1-2 with broth, and milk heated to 56°C. for 40 minutes extracted about 60 units of hemotoxin per cc. from hemolytic streptococci. Broth was a weak extracting agent for hemotoxin (extracted 20 units per cc.) and dextrose 1 per cent, physiological saline, or gelatine 2 per cent in saline, extracted no hemotoxin from streptococci. These latter extracts were not tested intravenously in mice.

DISCUSSION

This work demonstrates that sterile filtrates of certain serum extracts of hemolytic streptococci are leucocidic and extremely hemolytic and cause death of mice when injected intravenously in comparatively small amounts. Whether the toxic properties of these extracts are due to one or to several substances has not as yet been conclusively proved.

It is interesting to note here how the origin of hemotoxin differs with various organisms. In the pneumococcus, hemotoxin is endocellular and is only liberated by the breaking up of the cells (7); in *Staphylococcus aureus* it appears not to be endocellular, nor is it on the surfaces of the organism (if our negative results with the serum extraction of the staphylococcus signifies this) but is given off during the growth in a suitable medium, perhaps either as a secretion product or as a derivative product of some substance in the medium; on the other hand, from this work, the hemotoxin of *Streptococcus hemolyticus* appears, in great part at least, to be easily extractable. It is possible that hemotoxin may be liberated in some way by the breaking up of the streptococci during the extraction process, but if there is a disintegration of the cocci, it must be slight, since in stained smears of the extracts after shaking, they appear to be intact.

CONCLUSIONS

1. A method is described whereby toxic substances may be extracted from hemolytic streptococci with inactivated serum.
2. Such extracts contain large amounts of hemotoxin and leucocidin.

3. Their intravenous injection into mice causes marked hemoglobinuria, anemia, and death.
4. There is evidence that this anemia is not the only cause of death of these animals.
5. Incomplete work seems to indicate that the hemotoxin and the lethal poisons are not antigenic.
6. Certain biological properties of the extract are described.

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