A TOXIC SUBSTANCE OBTAINED BY GROWING HEMOLYTIC STREPTOCOCCI IN A SPECIAL MEDIUM.*

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Invasion by the hemolytic streptococcus is responsible for a number of different diseased conditions. Thus, we have "septic sore throat," tonsillitis, bronchitis and broncho-pneumonia—to name but a few of the more common diseases in which the hemolytic streptococcus plays a prominent part. In addition, hemolytic streptococci are responsible, as secondary invaders, for grave complications in other diseases, of which measles may be taken as an example. The severity of the systemic reaction is a striking characteristic of all these infections and, where a fatal termination results, it is often difficult of explanation by the extent of the pathologic process or the apparent degree of the infection.

In addition to these clinical considerations, there are other points which suggest that at least a part of the pathogenic power of hemolytic streptococci is to be ascribed to specific intoxication. Thus, when mice are injected intraperitoneally with several times the lethal dose of a virulent strain, death usually takes place within a few hours. Whether death occurs in two hours or is delayed for twenty-four hours or longer, autopsy reveals little to explain the mechanism of the pathogenic action. The peritoneum contains little or no exudate. Organisms can be recovered from the heart's blood, but smears and cultures frequently reveal the absence of an overwhelming septicemia. Autopsy findings in man are correspondingly unsatisfactory in revealing the manner in which this organism produces its fatal effects.

The present study was undertaken, therefore, in the hope of determining more definitely the mechanism of the pathogenic action of the hemolytic streptococcus.

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Experimental.

The Organisms.—The source and laboratory numbers of the nine strains of hemolytic streptococci included in this study were as follows: Strains 4 and 64 were isolated from the sputa of cases of broncho-pneumonia, the first in January, 1920 and the second in November, 1918; strains 6 and 16 were recovered from throat cultures of cases of acute tonsillitis in February, 1920; strains 9, 34, 41 and 43 came from throat cultures of influenza patients, January, 1920; strain 170 was recovered from a throat culture of a case of acute bronchitis, October, 1918. All of these strains produced typical beta hemolysis on blood agar plates and their morphology, fermentation reactions and insolubility in bile placed them in the hemolytic streptococcus group. These particular strains were selected on account of their original virulence for mice and because it was found possible to raise their virulence by means of animal passage. The final virulence of all the strains was such that from 0.001 cc. to 0.0001 cc. of an 18-hour broth culture killed white mice within from twenty-four to forty-eight hours.

Experiment I illustrates the fact, already referred to, that streptococcus infections may be fatal with comparatively scant growth of the organisms within the host.

Experiment I.—Mouse 1: This mouse was given, intraperitoneally, 0.001 cc. of an 18-hour blood broth culture of strain 9. Death occurred in thirty hours. There was a slight exudate in the peritoneum. Films from the heart's blood showed only an occasional streptococcus.

Mouse 2: Five tenths cc. of a blood broth culture of strain 9 was injected into the peritoneal cavity. The mouse was dead four hours later. The peritoneum was dry. The mesenteric and visceral peritoneum was moderately congested. Films from the heart's blood showed very few organisms. Cultures made from the peritoneal cavity and heart's blood were positive.

The results obtained in Experiment I corroborate the statement made above, that infections by the hemolytic streptococcus may be fatal in the absence of an extensive multiplication of the organism in the tissues of the host. This was true in both instances here noted, one mouse receiving a 30-hour lethal dose and the other mouse 500 times this amount. It is indicated, therefore, that the organism produces a very active poison on multiplication within the tissues of the host, since only a slight growth was necessary to cause death, and since sterile filtrates from the infecting cultures were devoid of toxic action, 2 cc. not being fatal.

Evidence of the production of toxic substances in infected animals leads one to believe that the hemolytic streptococcus (as well as many other pathogenic bacteria) would do the same in vitro if it were grown under the proper conditions. An attempt to devise a medium in which this organism would produce filtrable toxic substances resulted in the preparation of the following medium which was used in the experiments described below: 1 per cent. peptone, 1 per cent. di-sodium phosphate and 0.5 or 1 per cent. glucose are added to an ordinary meat infusion made with distilled water, and the reaction adjusted to a PH of 8.0 to 8.2. The medium is tubed, about 10 cc. to a tube, and autoclaved at ten pounds' pressure for ten minutes. A fragment of sterile rabbit kidney and 1 cc. of defibrinated sheep or rabbit blood are now put in each tube. The medium is then ready for use and, to obtain the best results, should be inoculated at once, without incubation to determine sterility. Contaminated tubes can be detected and discarded as they appear.

In this medium the hemolytic streptococcus grows readily and abundantly. Cultures grown in it for a period of 18 to 36 hours were highly virulent for mice and killed fully as rapidly as when grown in plain meat infusion broth. Occasionally filtrates of such cultures incubated for this length of time will kill when injected in 1 or 2 cc. amounts, but not with any degree of certainty. After 48 hours' incubation there occurs a very marked increase in the toxicity of the centrifugalized or filtered culture and this increases up to 72 or 96 hours, when it begins to decrease. This time curve of the rise and fall of the toxicity of the cultures in this broth has been characteristic of the strains studied. Usually the maximum toxicity is obtained in about 72 hours, when from 0.1 to 0.2 cc. of filtrates from cultures kill white mice within 24 hours.

Properties of the toxic substance.

Effect of Filtration.—The passage of cultures through Mandler filters does not destroy the toxic action of the filtrate. While the supernatant fluids of cultures centrifugalized at high speed still contain a few organisms the number present has not been found to interfere seriously with the study of the toxic substance. This substance apparently lacks the characteristic aggressive action of most true toxins known at present and consequently the presence of a few streptococci in the fluid injected does not lead to a fulminating infection. This point will be referred to later in detail. The Mandler filtrates

were about one half as toxic as the supernatant fluid. This indicates that a portion of the toxic substance is held back or destroyed by filtration.

Relation of Acidity to Toxicity.—It is well known that streptococcus cultures produce large amounts of acid, and, while the initial reaction of the medium and the buffer action of the phosphate tend to control this acidity, most cultures in three days' time reach a PH of 6.5 to 6.0, and occasionally a higher degree of acidity develops. It was possible, therefore, that the toxic action might be due to the acidity, but neutralization of the fluid with sodium hydroxide does not alter the toxicity.

Specificity.—It was also possible that other organisms grown in this medium, or the medium alone, might display the same toxic effect. However, filtrates of the sterile medium incubated three days, and filtrates of cultures of non-hemolytic streptococci, pneumococci, staphylococci and typhoid bacilli grown in the medium showed no toxic action when injected, even in large amounts, into mice. This is in marked contrast to cultures of hemolytic streptococcus strains, where 0.1 cc. of the filtrate is often fatal to mice in less than twenty-four hours.

Pathogenicity.—As mentioned above, this product is toxic for mice when injected intraperitoneally. There appears to be a definite relation between the amount injected and the time of death. Following the injection of amounts as large as 1 to 2 cc. death sometimes occurs in as short a time as one hour. This is the shortest interval that has been observed with intraperitoneal injections. Following acutely fatal doses, death is apparently due to respiratory failure, the heart having been observed to beat for several minutes after respiration has ceased. When smaller amounts are injected and death is delayed the symptoms are more prolonged. Prostration occurs and the respirations become more and more difficult and labored until the time of death. Autopsy shows a remarkable absence of any gross pathological condition.

The intravenous injection of mice produces almost instantaneous death, the animal often being dead before it can be released following the injection. Even such small amounts as 0.01 cc. have sometimes caused death. If the injection is not immediately fatal the incubation period is variable. The intraperitoneal method gives more satisfactory results, the incubation period and the final results being more uniform.

A small number of rabbits and guinea pigs were injected, but the

effect of the toxic substance on these animals is irregular, some being apparently very susceptible and others relatively resistant. The intravenous injection into rabbits of 1 cc. per kilogram of body weight has caused death. The incubation period is usually short, as is the case with mice injected intravenously. The incubation period has been known to vary, however, from five minutes to eighteen hours, and many rabbits do not succumb to the injection. Death is preceded by convulsions and extreme respiratory distress.

Thermolability.—The toxic action of the filtrates is impaired by heating to 55° C. for 30 minutes and is completely destroyed by heating to 62° C. for 30 minutes.

Stability.—The filtrates rapidly lose their toxic properties even when kept sealed and in a cold dark place. Some specimens deteriorate as much as 50 per cent. in a few days' time in the ice box.

Relation of Virulence to Toxicity.—Roughly, the production of the filtrable toxic substance corresponds to the virulence of the strain of hemolytic streptococcus used. The increase of virulence of a strain by animal passage usually is accompanied by an increase in the production of the toxic product, although one strain (64) which was relatively avirulent for mice, produced a potent poison in vitro.

IMMUNIZATION EXPERIMENTS.

Immunization of Rabbits.—The injection into rabbits of sterile filtrates of cultures containing this toxic substance causes the production of neutralizing and protective substances in their serum. The rabbits respond whether injected intravenously or subcutaneously. The following protocols are illustrative of the immunization process.

PROTOCOL 1.

Immunization of rabbits with the filtrates.

Date.	Rabbit 1, strain 9, intravenous.	Rabbit 2, strain 41, intravenous.	Rabbit 3, strain 9, subcutaneous.	Rabbit 4, strain 41, subcutaneous.
4–27–20	0.25 cc.	0.25 cc.	0.5 cc.	0.5 cc.
4–28–20	0.25 cc.	0.25 cc.		
4–29–20	0.5 cc.	0.5 cc.		
4–30–20			0.5 cc.	0.5 cc.
5– 2–20	0.5 cc.	0.5 cc.		
5– 3–20	0.5 cc.	0.5 cc.	1.0 cc.	1.0 cc.
5– 4–20	1.0 cc.	1.0 cc.		
5– 7–20	0.5 cc.	0.5 cc.	1.0 cc.	1.0 cc.
5– 8–20	1.0 cc.	1.0 cc.		
5– 9–20	1.0 cc.	1.0 cc.		
5–10–20			2.0 cc.	2.0 cc.
5–17–20	Bled	Bled	Bled	Bled

The sera obtained from the rabbits of Protocol 1 were tested with respect to their neutralization and protection properties, both against the toxic filtrates and infection with the cultures. Protocols 2, 3, 4 and 5 illustrates the type of experiment used.

PROTOCOL 2.

Neutralization of the poison (Strain 41) in vitro.

Mouse.	Filtrate, strain 41.	Immune serum, rabbit 2.	Normal rabbit serum.	Results.
1.....	0.1 cc.	0	0	Dead in 26 hrs.
2.....	0.1 cc.	0	1.0 cc.	Dead in 28 hrs.
3.....	0.5 cc.	0.5 cc.	0	Survived.
4.....	0.5 cc.	0.25 cc.	0	Survived.

In Protocol 2 the serum and filtrate were mixed and kept at 37° C. for one hour before injection. The mixtures were injected into the peritoneal cavity. It is seen that 0.25 cc. of serum neutralized five lethal doses and that the same amount, or even twice the quantity of normal rabbit serum, had no neutralizing effect upon the toxic filtrate.

Protocol 3 illustrates the protective action of the serum of the immunized rabbits when the serum and the filtrates are separately injected. The serum was injected 8 hours prior to the filtrate.

PROTOCOL 3.

Neutralization of the filtrate in vivo.

Mouse.	Filtrate, strain 9.	Immune serum, rabbit 3.	Normal rabbit, serum.	Results.
1.....	0.2 cc.	0	0	Dead in 20 hrs.
2.....	2.0 cc.	0	1.0 cc.	Dead in 4 hrs.
3.....	2.0 cc.	1.0 cc.	0	Survived.

Protocol 3 shows that 1 cc. of the immune serum protected against ten lethal doses of the filtrate and that the same amount of normal rabbit serum was without effect. Other similar experiments showed that from 0.2 cc. to 0.05 cc. of the sera of the immunized rabbits protected against a lethal dose of the homologous filtrate.

Proportional Neutralization.—In view of the fact that 3 cc. is about the maximum quantity that can be injected into a mouse at one time and since this amount of filtrate would contain hardly more than twenty lethal doses, the following experiment was devised in order to test the proportional neutralizing action of the immune serum

on multiple lethal doses of the toxic substance. Thirty cc. of filtrate 9, representing 200 fatal doses, was mixed with 8 cc. of serum 9, 200 neutralizing doses. This mixture was allowed to stand at room temperature for one hour and then 3 cc. were injected intraperitoneally, into each of six mice. The results are shown in Protocol 4.

PROTOCOL 4.

Neutralization of proportional multiple doses of the filtrate.

Mouse.	Amount of Mixture.	Results.
1.....	3.0 cc.	Survived.
2.....	3.0 cc.	Survived.
3.....	3.0 cc.	Dead in 36 hrs.
4.....	3.0 cc.	Survived.
5.....	3.0 cc.	Dead in 48 hrs.
6.....	3.0 cc.	Survived.

It is seen that four of the six mice survived and that the other two lived more than 24 hours in spite of the fact that the mixture which each mouse received contained more than fifteen lethal doses of filtrate. This seems to show that multiples of the toxic filtrate up to at least 200 are quite effectively neutralized by equal unit proportions of the immune serum.

Protection Against Infection.—The following protocol was devised to determine the effect of the serum on infection with cultures of the different strains of hemolytic streptococci included in the study. In this experiment all injections were intraperitoneal and the serum was given 18 hours prior to the culture.

PROTOCOL 5.

Protection against infection.

Mouse.	Culture, strain 9.	Immune serum, strain 9.	Results.
1.....	0.0005 cc.	0	Dead in 20 hrs.
2.....	0.0005 cc.	0	Dead in 18 hrs.
3.....	0.05 cc.	0.5 cc.	Survived.
4.....	0.05 cc.	0.5 cc.	Suvrived.

This protocol shows that the serum of rabbits immunized with the toxic filtrate protected mice against at least fifty fatal doses of the whole culture. This seems to point to a definite specific relationship between the toxic substance and the organisms.

The Relation of Protection and Serological Grouping.—By means of agglutination and protection experiments with an antibacterial

serum produced with strain 9 it was found that at least two serological groups existed among the strains included in this study. Thus, the strain 9 serum agglutinated and protected against strains 4, 6 and 9, while it neither agglutinated nor protected against strains 41, 64 and 170. It was, therefore, of interest to see if this grouping extended to the toxic substances and their immune sera. For this purpose rabbits were immunized with the filtrates from strains 6, 9 and 41 respectively and their sera used in protection tests with filtrates from cultures 4, 6, 9, 41 and 64. The sera and filtrates were mixed in vitro and kept at room temperature one hour before they were injected. The details of the experiment are given in Protocol 6.

PROTOCOL 6.

Cross protection tests with the toxic filtrates and their respective immune sera.

Mouse.	Filtrates.	Immune serum.	Results.
1.....	0.5 cc. strain 9	0.5 cc. strain 9	Survived.
2.....	0.5 cc. strain 6	0.5 cc. strain 9	Survived.
3.....	0.5 cc. strain 4	0.5 cc. strain 9	Survived.
4.....	0.5 cc. strain 6	0.5 cc. strain 6	Survived.
5.....	0.5 cc. strain 4	0.5 cc. strain 6	Survived.
6.....	0.5 cc. strain 9	0.5 cc. strain 6	Survived.
7.....	0.5 cc. strain 41	0.5 cc. strain 41	Survived.
8.....	0.5 cc. strain 64	0.5 cc. strain 41	Survived.
9.....	0.5 cc. strain 9	0.5 cc. strain 41	Dead in 16 hrs.
10.....	0.5 cc. strain 41	0.5 cc. strain 9	Dead in 8 hrs.
11.....	0.5 cc. strain 4	0.5 cc. strain 41	Dead in 16 hrs.
12.....	0.5 cc. strain 41	0.5 cc. strain 6	Dead in 8 hrs.

The results of Protocol 6 show that the sera of the rabbits immunized with filtrates from the strains (two strains) of one serological group protected against filtrates from all the strains of this group but not against filtrates from two strains of the other group. In like manner the serum from a rabbit immunized with the filtrate from a strain of the other group protected against its own filtrate and the filtrate from another strain of this group and failed to protect against filtrates from the first group. It appears then that filtrates from different serological groups have correspondingly different antigenic properties. This part of the work, however, needs to be extended.

Discussion.

The development of the poisonous substance described above has been found to depend upon the cultivation of the hemolytic streptococcus in a special medium. The use of ordinary media of various

kinds leads to negative results. The use of special media for the demonstration in vitro of toxic products of the growth of pathogenic bacteria has been found to be of value in a number of instances. Clark and Felton (1) (1918) have suggested that a filtrable toxic substance is produced by the growth of hemolytic streptococci in blood diluted with Locke's solution. Bull and Pritchett (2) (1917) found that fresh tissue was an important constituent of the medium in demonstrating the production in vitro of the toxin of *B. welchii*, and Robinson and Meader (3) (1920) have found that such a procedure enhances the toxicity of the broth in which *B. diphtheriae* is grown.

At present only conjecture can be made concerning the purpose served by the different constituents of our medium. Certain precautions are, however, necessary. Distilled water should be used in the preparation of the meat infusion, since it has been found that certain specimens of tap water have an unfavorable action on the production of the poison. No sodium chloride is added since this salt seems also to have an unfavorable influence upon the production of the toxic substance. The addition of di-sodium phosphate is apparently necessary, but its influence is not entirely clear. It probably acts as a buffer in limiting the amount of free acid present in the culture fluid, since the toxicity of the medium decreases as the acidity rises. The presence of blood is not absolutely essential to the maximum production of the poisonous product, but a medium containing 10 per cent. of defibrinated sheep or rabbit blood has given more constant results than the plain medium. The use of fresh tissue is important. The tissue must be fresh and the medium used immediately.

In describing this filtrable toxic substance care has been taken to avoid the use of the term "toxin." The word "toxin" has come to be regarded as defining specific substances, generally of bacterial origin, which conform to definite criteria, such as high toxicity, a definite and characteristic relation between the so-called Lo and the L+ doses, definite antigenic properties and certain other characteristics of equal definiteness. While the toxic product which has been described for the hemolytic streptococcus possesses certain of the properties of a true toxin, such as the neutralization of large amounts by proportionate amounts of immune serum, and the production of immune sera which protect against infection with the cultures, it has been impossible to demonstrate other characteristics of true toxins. The toxic substance of the hemolytic streptococcus lacks high potency, although it compares favorably in this respect with the toxin of

B. welchii. It causes no local lesion and consequently it has been impossible to determine an Lo dose with any consequent differences between the Lo and the L + doses. Furthermore, it apparently lacks the typical agressin action which is a striking characteristic of all other known toxins. The injection of the toxic substance together with, or preceding, the injection of the streptococci themselves, has no marked influence on the course of the infection, aside from that which can be ascribed to the toxic action of the fluid itself.

The specificity of the toxic filtrates seems to have been established. As antigens they stimulate the formation of antibodies which neutralize their toxic action and also the homologous cultures. Further evidence of specificity is afforded by the fact that immune sera produced with filtrates from one serological group do not neutralize the filtrates from another group.

It is impossible to state definitely at the present time what part, if any, this poisonous product plays in natural infections with hemolytic streptococci, but both clinical and experimental evidence seems to point to the action of some such product as at least a part of the process of natural as well as experimental infections with this organism.

SUMMARY.

A special medium has been described in which a specific toxic substance has been produced during the growth of certain strains of hemolytic streptococci.

This toxic product is filtrable and the filtrates have a definite pathogenic action when injected into mice, rabbits and guinea pigs.

The poison possesses definite antigenic properties and the sera of rabbits immunized with such toxic filtrates gives protection both against infection with the cultures and against intoxication with the filtrates.

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