THE PRODUCTION OF AN EXOTOXIN BY CERTAIN STRAINS OF STAPHYLOCOCCUS AUREUS.

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PLATE 33.

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Following Dick and Dick's (1) recent discovery that scarlet fever strains of *Streptococcus hæmolyticus* produce a dermotoxic poison for man, the possibility of demonstrating that strains of *Staphylococcus aureus* produce a similar poison presented itself. It is not necessary to review here the literature on the various poisons found in filtrates from broth cultures of *Staphylococcus aureus.* The hemolysins (which can be demonstrated in broth cultures of *Staphylococcus aureus*) probably have no bearing in the present relation, as many of the non-poison-producing strains, ineffective in rabbits, were markedly hemolytic. On the other hand, the leucocidins appear to resemble the above mentioned dermotoxic poison in many respects (2–4), such as thermolability and neutralization by immune and non-neutralization by normal rabbit serum.

After many unsuccessful attempts, we succeeded in demonstrating in sterile filtrates of broth cultures of certain strains of *Staphylococcus aureus* a powerful poison, with a selective action for the skin. The poisonous filtrates have not been examined for destructive action on leucocytes, and therefore there is no evidence as to whether they do or do not contain leucocidins. The following report gives our results to date with the action of this poison on the skin of rabbits. Its effect on the human skin has not as yet been worked out.

Sources of Cultures.—Although twenty-one strains of *Staphylococcus aureus,* isolated from various conditions, were tested, from only four of these were filtrates obtained, which were effective on the skin of rabbits. We have no evidence as to whether, by varying experi-

mental conditions or by concentration of filtrates of the other seventeen strains, similar skin-poisoning products could be demonstrated. This statement applies only to the effects on rabbits, as one of these rabbit-negative strains produced an effect in human skin similar to the Dick reaction.

TABLE I.

Staphylococcus aureus Strains Studied for Poison Production.

No.	Date of isolation.	Condition isolated from.	Amount of skin poison demonstrated in broth filtrates.
1	Oct., 1923	Boil.	0
2	1924	Dermatitis.	0
3	1924	Carbuncle.	0
4	Apr., 1924	Dermatitis.	0
5	1924	"	0
6	1924	Boil.	++++
7	1924	Dermatitis.	+++
8	1924	Abscess.	0
9	1924	Dermatitis.	++
10	1924	Abscess.	0
11	1924	"	0
12	1924	Dermatitis.	0
13	1924	"	0
14	1924	"	0
15	Apr., 1924	"	0
16	1924	Boil.	0
17	1924	Septicemia.	0
18	1924	Dermatitis.	0
19	1924	"	0
20	Nov., 1922	Carbuncle.	+
21	1924	Dermatitis.	0

In the above table + signifies slight yield of poison; ++ signifies moderate yield; +++, large amount; ++++, very large amount.

The sources of the *Staphylococcus aureus* strains studied and the dates of isolation are given in Table I.

Preparation of Media.—The poison is produced by the growth of *Staphylococcus aureus* strains, presented in Table I, on any well buffered broth medium containing only a small amount of glucose.

It was found that the addition of 1 per cent glucose prevented poison production and that in media containing traces of glucose toxicity appeared late—probably

after the sugar was destroyed. This may be explained by the protein-sparing action of glucose demonstrated by Kendall (5), or by the fact that it was impossible to buffer sufficiently to prevent much acid formation and resulting inhibition of growth. Poison production was also dependent on the maintenance of a hydrogen ion concentration between 6.8 and 8.2. In broths allowed to become more acid or alkaline no substance toxic for rabbit skin was found. The medium, from which our most powerful poisons were obtained, was prepared by adding an equal volume of M/15 phosphate buffer solution, pH 7.4, to ordinary sugar-free broth containing 4 per cent Witte peptone. The resulting buffered broth was brought to a boil, filtered through paper to clear, distributed in Ehrlenmeyer flasks, and autoclaved for 15 minutes at 15 pounds pressure.

After inoculation with a suitable strain of staphylococcus, the poison can be demonstrated in the sterile Berkefeld filtrates of this broth after 24 hours growth, but the most toxic poisons were regularly obtained after 4 to 6 days growth. If growth is allowed to continue longer than 6 days, the filtrates become progressively less poisonous. As there is no change in H ion concentration even after 8 days growth on this medium, the deterioration of the poison cannot be related to any change in pH. The lessened toxicity of the older filtrates may be due to several factors,—diminished growth with resulting smaller amount of poison produced, deterioration of formed poison by prolonged incubation at 37°C., and destruction of poison by autolytic enzymes set free from disintegrating organisms. We have found that whenever there is autolysis in broth culture as evidenced by finding Gram-negative organisms in smears, the filtrate is comparatively atoxic.

Inoculation of Animals.—The toxicity of the filtrates was tested by intracutaneous inoculation in rabbits.

When white, gray, or black rabbits with thin white skins were used, there was practically no variation in the reaction which any one poison produced. Throughout this work, 0.1 cc. of the filtrate to be tested was inoculated intradermally on the back or side of the animal. At the same time a control of 0.1 cc. of uninoculated broth was always injected into the same rabbit. Six to fourteen tests can be made in one animal.

Characteristic Appearance of the Reaction.—The reaction produced by a toxic filtrate is striking and follows a definite course. As compared with control broth or non-toxic filtrate, a toxic filtrate diffuses rapidly, the wheal disappearing immediately after the inoculation. In many rabbits there is a definite blanching zone 1 to 2 hours after the injection of poisonous filtrate, which spreads beyond the original wheal raised by the injection. 3 to 4 hours after the inoculation there is in all rabbits, both in those that do and those that do not

give the blanching reaction, a dark bluish purple circumscribed area of 2 to 5 cm. in diameter, depending on the toxicity of the filtrate. The next day the purple color assumes a yellow tinge and there is added a wide deep red zone of 0.5 to 3 cm. surrounding the yellowish area. At this time, with strong poisons, there is much edema spreading down towards the abdomen. By the 3rd or 4th day, the yellowish area of 3 to 5 cm. has become progressively yellower, as if necrotic, and is slightly raised as compared with the surrounding border of red. By about the 5th day, brown patches appear in the yellow area and increase in size, until, at about the 20th day, the whole lesion has become a dark brown dry scab with an encircling rim of red. 4 to 8 weeks later, the scab falls off, leaving an ulcer. If a large amount (2 to 3 cc.) of poison is injected intradermally and immediately subcutaneously, the reaction is similar to the one just described, except that the area covered is proportionally larger.

Control broth or filtrates of non-poison-producing cultures give no reaction of any kind, except occasionally a slight redness on the area of inoculation, which disappears in 24 to 48 hours. Large amounts of control broth or non-toxic filtrates (3 to 5 cc.) injected intradermally and immediately subcutaneously may produce a slight redness on the area of inoculation, but this redness could not be mistaken for even a very slight reaction caused by the injection of a poisonous filtrate.

Whether or not a skin reaction is obtained when a poison is injected subcutaneously, under the superficial fascia, seems to depend entirely on the strength of the poison. The subcutaneous inoculation of even a large amount (2 to 3 cc.) of a weak poison produces no reaction or only a slight redness over the area of inoculation, whereas the subcutaneous injection of 0.1 cc. of a potent poison produces a reaction which is only slightly less than that obtained from the intradermal inoculation of the same amount of powerful poison.

On the other hand, the reaction caused by any one poison is the same, whether it is injected intradermally or immediately subcutaneously; that is to say, between skin and superficial fascia. This is an important point because it makes possible the injection of large amounts of poison and the obtaining of reactions corresponding to the quantity and strength of poison inoculated.

Histological Changes in the Skin Reactions.—I am indebted to Dr. William C. Johnson for the histological work.

While no attempt has been made to carry out a complete or detailed study of the tissue changes in the skin at the site of injection of the toxic filtrate, sections have been made from a few rabbits, showing various stages of the reaction.

In the section of a lesion taken ½ hour after injection no pathological alteration is discernible except slight edema, which extends through the whole corium, and a scattering infiltration with a very few polymorphonuclear leucocytes and large mononuclear cells. Section of a lesion 24 hours old shows marked evidence of inflammation and beginning necrosis. Through a circumscribed area over the site of injection the epidermis is thin, the nuclei of the epithelial cells are shrunken and pycnotic, and the staining reaction of the cytoplasm of the cells has become more acidophilic. The corium, especially just beneath the epidermis, shows a marked infiltration with polymorphonuclear leucocytes, and a few mononuclear cells. Scattered through the corium are many foci where the connective tissue fibers appear to be fragmented, and infiltrated with leucocytes. The cellular exudate shows a marked amount of pycnosis and nuclear fragmentation. In the superficial layer of the corium the capillaries are slightly congested. In the deepest part are several small arteries and veins whose walls show necrosis and infiltration with leucocytes. A few of the vessels are thrombosed. In the layer of cutaneous muscle beneath the corium many fibers show necrosis, fragmentation, and invasion by leucocytes.

Later stages of the lesions, as seen in sections 3, 4, 5, and 21 days after intradermal injection of the toxin, show more marked infiltration with leucocytes, and complete necrosis of a mass of tissue including a sharply circumscribed area of epidermis, together with the underlying corium, extending about half way down to the cutaneous muscle layer. As the necrotic tissue dries up, forming a scab, it becomes very sharply demarcated from the underlying living tissue by the accumulation within it of a broad dense zone of disintegrating leucocytes.

In the remaining portion of corium beneath the necrotic mass, reparative activity is shown by a proliferation of fibroblasts. The walls of some of the small arteries and veins in this layer are partly necrotic, and are infiltrated with polymorphonuclear leucocytes and mononuclear cells.

Evidence of Systemic Poisoning.—Only irregular results were obtained by the intravenous injection of the poison into rabbits. Usually there was no effect even when comparatively large doses (3 to 8 cc.) of a powerful skin poison were given to rabbits of over 1,300 gm. On the other hand, two very young rabbits, weighing 1,030 and 710 gm. respectively, died less than 2 hours after the intravenous

inoculation of a strong poison. 2 cc. of control broth were innocuous to two rabbits, each weighing 630 gm.

The poison appears to be definitely toxic when given intradermally. Usually the animal seems sick and drowsy, and will not eat. This condition begins ½ to 2 hours after injection and lasts for several days. About 25 per cent of the rabbits used, when a relatively large amount of poison was injected, died 3 to 5 days later. At autopsy nothing could be seen macroscopically to account for these deaths except extreme emaciation.

M croscopically, in one of these rabbits dying on the 2nd day after injection, the heart muscle showed numerous areas of focal necrosis. A few focal necroses were present in the liver. An interstitial nephritis present in this rabbit was thought to be probably independent of the experiment. No other visceral lesions were noted.

Properties of the Poison.—The poison is extremely labile, being partly destroyed if heated to 50° for 1 hour and completely destroyed if heated to 55° for 1 hour. The poison may be preserved with only slight deterioration for at least 2 months, if kept in the dark at 4°C.

Antiserum to the Poison.—A question of importance to determine was whether a neutralizing antibody could be produced for our poison, and, furthermore, whether such neutralization, if it occurred, would take place in multiple quantities. Although to date we have immunized no animals systematically with the poison, we find that the serums of many of the rabbits used previously in this work in testing out filtrates for skin-injuring substances easily neutralize in small amounts our most powerful poisons. Normal rabbit serum, even undiluted, incubated with equal amounts of even a weak poison has no detoxicating effect.

That an antiserum produced against our poison neutralizes it in multiple quantities is evident from the following experiment.

Experiment 1.—Preliminary titration demonstrated that 0.02 cc. of a certain toxic filtrate produced a marked reaction (4 by 4 cm.) when inoculated intradermally into a normal rabbit. Following this, it was found that 0.002 cc. of Immune Rabbit Serum 18 when incubated with 0.02 cc. of the poison completely neutralized it, while 0.001 cc. of Serum 18 had only slight neutralization effect. Table II gives the results obtained by incubating 0.002 cc. of Serum 18 with 0.02 cc. of the poison and multiples of both the reagents, and inoculating these mixtures along with controls into a rabbit. The volume injected in each case was 0.2 cc.

Normal rabbit serum has no neutralizing effect on the poison except when incubated with it in comparatively large amounts. This is shown in Table III in which the same poison was used as in Table II.

From the experiments recorded in Tables II and III, we believe that there is only one interpretation of our results; namely, that our poison is a true toxin because its injection into rabbits stimulates the production of antitoxin.

TABLE II.

No.	Serum 18.	Poison.	Result. Reaction of rabbit.
	cc.	*cc.*	
1	0.001	0.02	++
2	0.002	0.02	0
3	0.01	0.1	0
4	0.5	5	0
5		0.02	++++
6		0.1	+++++

In Tables II and III, 0 signifies no reaction in rabbit; ±, red reaction of 2 by 2 cm. lasting at least 3 days; ++, reaction of 3 by 3 cm.; +++, reaction of 3.5 by 3.5 cm.; ++++, reaction of 4 by 4 cm.; +++++, reaction of 4 by 6 cm.

TABLE III.

No.	Normal rabbit serum.	Poison.	Result. Reaction of rabbit.
	cc.	*cc.*	
1	0.1	0.005	+++
2	0.5	0.005	±
3	2	0.005	0
4		0.005	+++
5		0.02	++++

The Toxin Is Identical from the Three Poison-Producing Strains Studied.—There is considerable variation in the potency of the filtrates produced by the four different strains (see Table I), but no variation in the character of the reaction, which is strikingly alike in all toxic filtrates.

By using the serum of a rabbit which had been inoculated with toxic filtrates from our Strain 7 only, and testing it against the poisons produced by two other strains (Nos. 6 and 20), we have found that it

neutralizes all three toxins equally well; and we therefore conclude that the toxins are identical in the strains tested. The fourth strain (No. 9) has not as yet been tested against our Serum 7.

TA

Immunization of rabbits.				Skin reactions.	
1	2	3	4	5	6
Rabbit No.	Approximate total No. of skin units injected.	Method of injection.	Dates of immunizing injections.	Dates of obtaining serums and doing skin tests on rabbits.	Reaction of rabbits with 1c. test of 50 skin units.
1				May 26	++++
2				" 26	++++
3				" 31	
4				" 31	
5				" 31	
6				" 31	
7				" 31	
8	5	Ic.	May 9	" 26	+
9	5	"	" 5	" 26	++++
10	5	"	" 5	" 26	+++-
11	10	"	" 1	" 26	++-
12	10	"	" 1	" 26	++-
13	5	"	Apr. 25, 30	" 26	++++
14	120	"	Mar. 17, Apr. 25, 30, May 13	" 26	0
15	80	50 ic., 25 iv.	Apr. 17, 25, May 13	" 26	±
16	75	5 " 20 subc., 50 iv.	" 15, 25, 30, May 2	" 26	+++
17	110	60 ic., 50 ip.	" 15, 25, May 13	" 26	−
18	80	Ic.	" 15, 25, 28, May 13	" 26	*
19	155	"	" 15, 17, 22, " 13	" 26	±
20	155	"	" 15, 17, 25, 28, 30, May 13	" 26	0
21	200	"	May 23	" 31	++++
22	150	"	" 20	" 31	++++
23	60	"	" 15	" 31	++++
24	550	"	" 21	" 31	++
25	1,500	"	" 14	" 31	++

0 indicates no reaction; ±, red reaction 2 by 2 cm. lasting at least 3 days; +, ye[illegible] erythema; ++, yellow center 3 by 3 cm. to 4 by 4 cm.; ++++, yellow center [illegible]

Ic. indicates intracutaneous inoculation; iv., intravenous inoculation; ip., intraperitone

* Rabbit 18 gave a 0 reaction to 50 skin units injected intradermally on May 13 and w

Effect on the Skin Reaction of Circulating Antitoxin.—That the skin reactivity to the toxin of any treated rabbit is dependent on the amount of antitoxin which its serum contains appears probable from

VIV.

Titration of serums for neutralization capacity.

7	8	9	10	11	12	13	14
Reaction of normal rabbit with 0.01 cc. of serum + 50 skin units.	Reaction of normal rabbit with 0.02 cc. of serum + 50 skin units.	Reaction of normal rabbit with 0.04 cc. of serum + 50 skin units.	Reaction of normal rabbit with 0.1 cc. of serum + 50 skin units.	Reaction of normal rabbit with 0.1 cc. of serum + 10 skin units.	Reaction of normal rabbit with 0.1 cc. of serum + 2.5 skin units.	Reaction of normal rabbit with 0.1 cc. of serum + 1 skin unit.	Approximate No. of antitoxin units in 1 cc. of serum.
++++	++++		++++	+++	++	+	0
+++	++++		++++	+++	++	+	0
					++	+	0
					++	+	0
					+	±	?
					++	+	0
					++	+	0
++++	++++			±	0		100
++++				±	0		100
++++				+++	0		40
++++				0	0		100
++++				++	0		40
++++				++	0		40
++	±						2,500
+++	++	0					1,250
++++	++++		++++	++	+	±	?
+++	++	0					1,250
0							5,000
+++	++						1,250
++	±						2,500
				+++	+	0	10
				+++	+	0	10
				+++	+		?
			++	±			100
			±				500

2 by 2 cm. surrounded by erythema; ++, yellow center 3 by 3 cm. surrounded by m. to 6 by 6 cm.
ulation; subc., subcutaneous inoculation.
d to death on May 21.

Table IV, in which we have correlated the results of skin tests on previously treated rabbits with the amount of antitoxin which the serums of these same rabbits contain.

The rabbits were taken at random from those which had been used previously for the determination of toxicity of the filtrates. They were bled from the ear and inoculated intracutaneously in a fresh area with 0.1 cc. of a powerful toxin on the same day. The results of the skin tests on these rabbits are given in Table IV, Column 6. The titration of their serums for antitoxin is shown in Columns 7 to 14; the figures in the last column show the approximate number of antitoxin units contained.

The same toxin was used throughout the experiment, both for retesting the treated rabbits (Column 6) and for determining the antitoxin content of their serums. The potency of the toxin was such that 0.1 cc. of 1:50 dilution (0.002 cc.) produced a slight but typical reaction (yellow area 2 by 2 cm. surrounded by erythema) when inoculated into a normal rabbit. In the table we arbitrarily designate this amount of toxin as one unit, and the reaction obtained with it as a + reaction.

The titration of the antitoxin was carried out as follows: 0.1 cc. of varying dilutions of serums was mixed with 0.1 cc. of varying dilutions of toxin and incubated in the water bath at 37°C. for 1 hour. Simultaneously control mixtures were made with toxin alone diluted with salt solution and also toxin plus normal rabbit serum incubated at the same time. After incubation, normal rabbits were injected intradermally with 0.2 cc. of the mixtures and of the control toxin. One antitoxin unit was taken to be that amount of serum which would neutralize one unit of toxin.

The reactivity of every normal rabbit was controlled with at least one intracutaneous inoculation of the toxin dilution alone.

In Table IV, in Columns 2, 3, 4, and 5 are given the approximate number of skin units with which the rabbits had previously been injected, the method of injection, the dates the immunizing doses were given, and the dates of obtaining the serum and carrying out the skin tests recorded in Column 6. In Columns 7 to 13 inclusive are given the results of the titration of antitoxin. In Column 14 is given the approximate number of antitoxin units in 1 cc. of serum as calculated very roughly from the results of Columns 7 to 13. The antitoxic content of the serums was estimated by taking the amount of serum which neutralized a definite number of toxin units (produced ± or 0 reaction when inoculated together in a normal rabbit after incubation) and calculating from this the approximate number of toxin units which 1 cc. of the serum would neutralize.

The points worthy of note in Table IV are the following.

(*a*) The skin reactivity of any one rabbit to the toxin was found to be inversely proportional to the amount of antitoxin its serum con-

tained. There was one exception, Rabbit 8 which gave only a + reaction although its serum contained little antitoxin. Two possible explanations may be offered. This animal was much emaciated and its weak condition may have lessened its capacity to react to the poison. It was also an unfavorable subject for tests on account of its reddish brown color. It had also reacted unsatisfactorily to the immunizing injection (May 9).

(*b*) Under the conditions of this experiment, normal rabbit serums do not neutralize the toxin.

TABLE V.

No.	Approximate total No. of skin units injected.	Method of injection.	Dates of immunization.	Dates of obtaining serums.	Skin reaction with 25 skin units on June 24.
1	375	Iv.	May 29, June 2, 5, 12	June 21	++++
2	435	"	June 2, 5, 9, 12	" 21	++
3	375	"	May 29, June 2, 5, 12	" 21	++++

Titration of Serums for Neutralization Capacity.

No.	Reaction with 0.05 cc. of serum plus 25 skin units.	Reaction with 0.1 cc. of serum plus 25 skin units.	Reaction with 0.1 cc. of serum plus 5 skin units.	Reaction with 0.1 cc. of serum plus 1 skin unit.	Antitoxin units in 1 cc.
1	++++	++++	+++	+	0
2	++++	++++	±	0	50
3	++++	++++	+++	+	0

(*c*) Although there were two instances (Rabbits 24 and 25) in which a definite amount of antitoxin was produced after previous intracutaneous injections of toxin carried out all on one day, the most potent serums were those obtained after a series of inoculations spread out over a period of time.

Immunization by the Intravenous Route Elicits the Production of Very Little, If Any, Antitoxin.—From Table IV, it is evident that the stimulation of antitoxin is easily brought about by three or more intradermal injections of toxin. The next question, one of both practical and theoretical importance, was whether the intravenous injection of toxin would also elicit the production of antitoxin. Table V records

the results obtained in investigating this point. For details of this experiment the reader is referred to the explanation of the previous experiment, Table IV.

Although only three rabbits were immunized by the intravenous route, the results appear convincing as far as they go. Antitoxin could only be demonstrated in the serum of one of the three rabbits immunized, and in this serum the amount of antitoxin present was very small. This experiment (Table V) indicates that, for the production of antitoxin, the intradermal method of injection is far superior to the intravenous.

CONCLUSIONS.

1. The sterile Berkefeld filtrates of broth cultures of certain strains of *Staphylococcus aureus* have a selective poisonous action for the skin of rabbits.

2. The poison is thermolabile, being completely destroyed when heated to 55°C. for 1 hour.

3. The poison when injected intradermally into rabbits stimulates the production of antitoxin. We therefore conclude that the poison is a soluble toxin.

BIBLIOGRAPHY.

1. Dick, G. F., and Dick, G. H., *J. Am. Med. Assn.*, 1924, lxxxii, 265.
2. Denys, J., and Van der Velde, H., *La Cellule*, 1895, xi, 357.
3. Bail, O., *Arch. Hyg.*, 1898, xxxii, 133.
4. Neisser, M., and Wechsberg, F., *Z. Hyg. u. Infectionskrankh.*, 1901, xxxvi, 299.
5. Kendall, A. I., *Endocrinol. and Metabol.*, 1922, iii, 663.

EXPLANATION OF PLATE 33.

FIG. 1. Reaction in a normal rabbit 2 days after the intradermal injection of 0.1 cc. (25 toxin units) of *Staphylococcus aureus* toxin. $\times\frac{1}{2}$.

FIG. 1.

(Parker: Exotoxin produced by *Staphylococcus aureus*.)