

THE TOXINS AND ANTITOXINS OF SYMPTOMATIC ANTHRAX.

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QUARTER evil, or *charbon symptomatique*, which causes such mortality among young cattle and other susceptible animals, has been very fully investigated by Arloing, Cornevin, and Thomas. Their work, the first of any importance on the subject, was published in 1887;¹ since then several other investigators, notably Kitasato, Roger, Roux, Nocard, and Duenschmann, have studied the disease, and added considerably to our information on the subject.

When Behring and Kitasato, in 1890, announced the results of their investigations on the valuable antitoxic qualities of the blood serum of animals vaccinated against tetanus and diphtheria, a new field was opened up for research.

The properties of the serum of animals immunised against many other diseases have been investigated with very marked success, so that now, in addition to the antitoxic serums of the above-named diseases, we know something of the serum of animals immunised against cholera, typhoid fever, pneumonia, abrine, ricine, and snake venom.

Until the year 1894 the investigations on quarter evil were chiefly upon the growth and virulence of the organism causing this disease (*B. chauvæi*), and the conditions which influence them; but in that year the first step was taken by Duenschmann to determine whether the blood serum of rabbits immunised against the disease had any antitoxic or therapeutic properties. This, no doubt, was a step in the right direction, considering the course which investigators are pursuing at the present time, and, although his results were not very complete, they furnished valuable data for further research.

¹ Arloing, Cornevin, et Thomas, "Le charbon symptomatique du bœuf," Paris, 1887. Kitasato, "Ueber den Rauschbrandbacillus und sein Kulturverfahren," *Ztschr. f. Hyg.*, Leipzig, Bd. vi. und viii. Roger, "Contrib. à l'ét. du charbon symptomatique," *Rev. de méd.*, Paris, 1891, tome xi. Roux et Nocard, *Ann. de l'Inst. Pasteur*, Paris, tome i. p. 257. Duenschmann, *ibid.* tome viii. No. 6, Juin 1894.

The present investigation was undertaken with the object of testing the results obtained by Duenschmann ; of obtaining, if possible, a stronger antitoxic serum ; and to endeavour to keep alive the experimental animals which Duenschmann had been unsuccessful in doing.

During the progress of the work many results were obtained which showed a distinct improvement on Duenschmann's methods, and gave promise of still further advance.

The results of the present experiments tend to confirm the fact that the serum obtained possesses antitoxic properties, although considerable difficulty was experienced in preserving alive the injected animals from which it was prepared. The animals at first received only small doses of toxins ; these were gradually increased ; and, after a time, the treated animals were able to withstand fairly large doses of virulent bacilli. The method chiefly used by Duenschmann was to inject virulent bacilli directly into a vein, or into the peritoneal cavity ; he considered that intramuscular injection was very dangerous, although he made use of it in several of his experiments.

The results obtained in my investigation do not confirm this view, as the animals received frequent doses of toxin and bacilli into the muscles without any dangerous symptoms appearing ; they received similar injections into the peritoneal cavity, and subcutaneously ; and always kept up their weight, although, towards the end of the investigation, several succumbed from being too strongly "forced" with bacilli. Intravenous injection was never attempted, as it was considered much too dangerous a method. Almost all Duenschmann's animals, which had received intravenous injections, gradually lost weight, became cachectic, and died ; while those in my experiments that received intramuscular injections were not nearly so seriously affected.

The *B. chauvæi*, being an anærobic organism, can be conveniently grown under hydrogen, in bouillon or nutrient gelatine, to which has been added 1·5–2 per cent. of glucose. After about 48 hours the colonies become visible, and soon assume characteristic features, bubbles of gas making their appearance. No growth occurred in distinctly acid media ; but it was abundant in alkaline or neutral media. A most remarkable feature connected with the cultivation of this organism is the rapid attenuation of the growth, which, after a short time, does not prove fatal, and gives rise to no symptoms of the disease.

The experiments given below show that, within a comparatively short time, the cultures will cause no more than a slight sickness. But it is remarkable that the presence of even a trace of lactic acid will restore the original virulence of the culture ; a fact some time ago pointed out by Arloing.

Every thing which diminishes the vitality of the tissues into which the bacilli are injected, will facilitate their development by enfeebling the concurrence of cells, and will appear thus to restore

their virulence. The lactic acid has not a specific action; and, as Roux and Nocard have shown, acetic acid, chlorate of potash, and lactate of potash are also efficacious, but none of them are so good for the purpose as lactic acid.

EXPERIMENT 1.—Four colonies of symptomatic anthrax from glucose gelatine (one week old) were rubbed up with 2 c.c. distilled sterile water. Guinea-pig, 420 grms., received this emulsion by intramuscular injection. It was ill for a few days, but ultimately recovered.

EXPERIMENT 2.—Guinea-pig, 415 grms., received the same quantity of the same growth with the addition of a trace of lactic acid. The animal died within 48 hours.

EXPERIMENT 3.—Guinea-pig, 500 grms., received 3 c.c. of a growth (two months old) with no acid. The animal had a slight swelling, but recovered.

EXPERIMENT 4.—Guinea-pig, 400 grms., received 1 c.c. of the above growth, to which had been added a trace of lactic acid. The animal was dead at the end of 48 hours.

The animals which died showed extensive emphysematous swelling at the seat of inoculation, with shedding of the hair. On incision into the tumour, the muscles were found to be dark red, or even black, and contained a blood-stained serum in which bacilli were very numerous. No spore-bearing forms were found until some-time after death; but the characteristic spindle spore forms of *B. chauvæi* could be found in the heart's blood which had been kept for 24 hours in a sealed sterile pipette in the incubator at 37° C.

TO INCREASE THE VIRULENCE OF THE VIRUS.

The method used for this purpose was the one in use at the Pasteur Institute, and followed by Duenschmann.

A small quantity of the modified virus, or vaccine, in use for vaccinating animals was made into an emulsion with 1 c.c. of distilled water to which a trace of lactic acid had been added; this was injected into the muscles of the thigh of a guinea-pig, death occurring in 48 hours. Some of the blood of the heart was taken, and either kept 24 hours at 37° C. in sterile pipettes, or after dilution 1:5, injected immediately into another guinea-pig.

In this manner the virulence of the organism after four or five passages became very great, proving fatal in 18–20 hours. In some cases dried sheep or ox muscle was used instead of the vaccine of Arloing.

In order to preserve the virulent material, small sterile capillary pipettes were made; the thorax of a recently dead guinea-pig having been opened with proper precautions; the pericardium was cut open with sterilised scissors and forceps; a spot on the heart was sterilised with a red-hot spatula; the point of a pipette pushed into the ventricle and sufficient blood drawn off; and the pipette sealed carefully at both ends, leaving as little empty space as possible.

This was incubated at 37° C. for 24 hours; and, at the end of that time a copious formation of gas bubbles had taken place, and numerous spindle-shaped spore-bearing forms of the bacillus could be found.

PREPARATION AND PRESERVATION OF TOXIN.

In order to obtain the toxin formed by the microbe, the method used by Roux, and subsequently followed by Duenschmann, was tried.

Several guinea-pigs were inoculated with very strong virus, obtained from an animal which had succumbed to the disease. These animals died in about 18 hours with the characteristic symptoms of symptomatic anthrax.

The abdomen of each animal was opened with strict antiseptic precautions, and the intestines taken out after a ligature had been placed above the stomach and another on the rectum, the cavity being washed out with sterilised water.

These precautions were taken to prevent the intestinal microbes from finding their way into the abdominal cavity and tissues after death. The animals were rolled in a sterilised cloth, enclosed in gutta-percha tissue, and kept at 37° C. for 24 hours. In this way the microbes of the disease, which had already possession of the tissues, were allowed to grow and produce their products, so as to use up any remaining pabulum in the body of the animal.

When the animals were taken out of the incubator at the end of 24 hours there was a distinct smell of rancid butter, due to the presence of butyric acid, which has been shown to be a very characteristic product of the organism; there was no putrefactive smell.

The muscles and other tissues were removed from the bodies, and found to weigh about 1000 grms.; these were cut up into small pieces, 520 c.c. of distilled water were added, the muscles well pounded in a mortar and left overnight in an ice safe. The muscle juices were squeezed through a cloth, and 25 c.c. distilled water added to the residue, which was again squeezed; the fluid was filtered through four layers of fine muslin, then through filter paper, and finally passed through a Pasteur-Chamberland filter.

The filtrate had a clear red-brown appearance; and, when tested on guinea-pigs, was found to have no toxicity, except in doses which are quite too large for such small animals.

It was reduced, by careful evaporation, to half its original bulk; but even then did not show great toxicity. Reduced to a fourth of its original bulk, 2 c.c. were found to cause illness in a guinea-pig of 455 grms. for a few days, but it ultimately recovered.

Another method used by Duenschmann was now tried.

Sterilised flasks of about 700 c.c. capacity were filled about one-third full with finely minced beef; about 4-6 c.c. of a 1 per cent. carbonate of soda solution was added, and the whole sterilised in the autoclave for 20 minutes at 120° C. These were inoculated with blood from the heart of an animal which had recently died; and hydrogen gas was passed into the flasks after exhaustion with a water-pump. The flasks, having been sealed while the hydrogen was passing, were placed in the incubator at 37° C.; and in about 2 days the growths began to have the brick-red appearance of which Duenschmann speaks.

The flasks incubated for 14 days gave a product, after filtration through a Pasteur-Chamberland filter, which smelled distinctly of butyric and acetic

acids, and proved fatal to guinea-pigs in doses of 4 c.c. ; $1\frac{1}{2}$ c.c. causing sickness for a few days.

Thinking that possibly the microbes had been unable to gain access to all the particles of meat in the flask, the medium being no doubt too dry, it was deemed advisable to add a certain quantity of water or broth to the cooked meat, and to get the latter in as small particles as possible; accordingly the following method was tried:—

1600 grms. of minced meat were cooked in the autoclave; and the almost solid mass was now rubbed up and broken down with the hands in 25 c.c. of a 1 per cent. soda solution, until the meat was reduced to a state of very fine division. Sterilised conical flasks were filled, each about one-third full, with the above minced meat; to each was added 20 c.c. of distilled water; glucose broth (1 per cent.) was also added until the flasks were a little more than half full. These were sterilised in the autoclave for 20 minutes, at 120° C.; the flasks exhausted by means of a water pump and hydrogen passed through; they were then sealed and incubated at 37° C.

After 7 days' growth the filtered liquid showed the following toxicity:—

4 c.c. killed guinea-pig, 480 grms., in 24 hours.

$1\frac{1}{4}$ c.c. did not affect guinea-pig, 390 grms.

After 16 days' growth:—

$3\frac{1}{2}$ c.c. killed guinea-pig, 440 grms., after some days.

4 c.c. sickened guinea-pig, 410 grms., but it ultimately recovered.

So we see that the 16-day toxin had not any advance in toxicity over the 7-days' growth.

The previous toxins not proving satisfactory, peptone broth was used instead of glucose broth; it was added to the finely pounded meat, and the material made into a thick cream, sterilised, inoculated, after which hydrogen was passed through it in the usual manner.

After 14 days' growth—

5 c.c. killed guinea-pig, 410 grms., in 27 hours with blackening of muscles and swelling.

$1\frac{1}{2}$ c.c. sickened guinea-pig, 325 grms., for several days.

During the preparation of the foregoing toxins the evolution of gas was very great; and on several occasions the flasks were broken or the tightly-fitting rubber corks blown out. In order to avoid this, and thinking that the pressure of the evolved gas might hinder the proper development of the organism, it was considered advisable to use a bent tube with a mercury valve, so as to admit of the escape of the evolved gases.

This was found to be of great advantage, as not only was the danger of breakage and other damage avoided, but the toxins produced were considerably stronger.

The two following methods will be given in detail, as they yielded the best results as regards toxicity; and it was mostly with these that the immunising experiments on rabbits were carried out:—

Minced meat was cooked, and worked into fine particles, as in the previous methods; enough of a 1 per cent. glucose broth was then added to make a fairly thin cream, and introduced into a clean carefully sterilised glass bottle of about one litre capacity; this was filled to about half its extent.

A rubber cork was inserted into the neck, and through it passed two glass tubes, one going to the bottom of the bottle, the other just passing through the cork, and not reaching the level of the liquid; this latter tube was conducted into a bent U-shaped piece, which contained a small quantity of mercury as a valve.

This valve was tested previous to use, and found to act admirably.

The whole apparatus was sterilised at 100° C. on three separate days, and after inoculation, and the passage of hydrogen was kept at 37° C. for 14 days.

The liquid, after filtration through a Pasteur-Chamberland filter, gave the following results:—

4½ c.c.	killed guinea-pig,	380 grms.,	in 24 hrs.—blackening and falling of hair.
2 c.c.	„ „	470 „	in 18 hrs.—falling of hair.
2 c.c.	„ „	300 „	in 40 hrs.
½ c.c.	sickened „	300 „	but it ultimately recovered.

The toxin became rapidly weakened, and after 14 days 2 c.c. and 4 c.c. failed to prove fatal.

The excellent results obtained at the laboratories of the College of Surgeons in the preparation of diphtheria toxins were such as to suggest the possibility of obtaining an active toxin after the methods there in use. Accordingly, the following method described by Cartwright Wood¹ for the preparation of diphtheria albumoses was tried. A large bottle of a litre capacity, fitted with the mercury valve apparatus, was filled with 500 c.c. of a decalcified 2 per cent. peptone broth, to which was added 100 c.c. of sterile blood plasma.

The broth was first put into the sterile bottle and sterilised in the usual way, the carefully preserved and sterile plasma being added after the broth had cooled down. This was inoculated with the heart blood of an animal recently dead from symptomatic anthrax. Hydrogen gas was then passed through the liquid by means of the glass tube, properly plugged with cotton wool; and it might be mentioned that, in all the experiments, the most scrupulous care was taken to prevent the entrance of foreign organisms into the culture fluids. The toxins were preserved in conical flasks tightly closed with parchment paper covers, and when not in use were kept in an ice safe. After incubation for 9 days at 37° C., it was found that the growth had stopped; no further evolution of gas took place, and there was a good growth of bacilli.

The filtered liquid gave the following results:—

1 c.c.	killed guinea-pig,	345 grms.,	in 18 hours.
2 c.c.	„ „	345 „	in 18 hours, with slight swelling.

This toxin also became weakened, and in 10 days—

2 c.c. sickened guinea-pig, 365 grms., but it recovered in 3 days.

¹ A method for rapidly producing diphtheria antitoxins, *Proc. Roy. Soc. London*, 20th February 1896.

After one month the toxin gave the following results:—

3 c.c. did not affect guinea-pig,	375 grms.
4 c.c. " "	420 "
5 c.c. killed guinea-pig,	385 grms., in 30 hours.
6 c.c. also proved fatal to an average guinea-pig.	

It is seen from the above experiments that the best results, as regards the preparation of an active toxin, were given by the last two methods, and especially by that where blood plasma was used; yet the preservation of the toxicity of the liquids was a question of considerable difficulty, and no doubt was a considerable hindrance to the further progress of the work. However, a sufficient amount of active toxin was obtained to enable me to proceed a considerable length in the investigation. The question of the best means of preserving the toxins from attenuation will be the subject of a further research.

In guinea-pigs the toxins when introduced into the muscular tissues, or subcutaneously, produce the same changes as are seen when the parts are attacked by the disease:—serous exudation stained with blood, and, when severe, necrosis and sloughing of the skin.

REINFORCEMENT OF THE NATURAL IMMUNITY OF RABBITS.

Since the experiments of Arloing, it is well known that rabbits possess a marked immunity against symptomatic anthrax, although there are always found a few which will succumb to the disease.

An attempt was now made to increase this natural resistance of rabbits by the injection of virulent blood and toxins, prepared from pure artificial cultures as described in the preceding section.

The rabbits used were of an average weight of 2000 grms., and the method adopted was as follows:—The animals were first injected with small doses of toxins almost daily, or on alternate days, generally about 11 A.M.; the reaction extended over one or two degrees of temperature, and was highest in the evening, but had generally returned to about normal on the following morning; the injections were repeated if the animals seemed all right and were not losing in weight.

No intravenous injections were used, the intraperitoneal or subcutaneous method being made use of in every case; it was found that the injections could be increased considerably without much inconvenience to the animals; nevertheless, on several occasions diarrhoea was caused by very large doses. In the course of the experiments the quantity of toxins introduced was gradually increased; and, when the animals had become accustomed to them, injections of diluted virulent blood were tried after the method of Duenschmann.

Blood from the heart of an animal which had recently succumbed was, with antiseptic precautions, aspirated into a sterile pipette and

into the muscles of the thigh. In a few of the latter cases a small abscess formed, and it was found that injections during the persistence of the abscess were attended with very great danger. (In the accompanying charts the temperature reactions following the injections of the toxins are graphically represented. The weight of each animal is recorded at intervals below the chart.)

Another fact was noticed during the experiments on guinea-pigs, namely, that a guinea-pig which had recovered from a strong dose of toxin was afterwards (8 days) not susceptible to the action of a strong fatal dose of virus.

EXPERIMENT.—Guinea-pig, 390 grms., received $1\frac{1}{4}$ c.c. of 7-day toxin—recovered completely, tested after 8 days with a very strong dose of virus (2 c.c. of a mixture of Lyons virus with a trace of lactic acid)—on the following day it was quite lively, and evidently resisting, ultimately showing no ill effects. The control guinea-pig, 490 grms., which received 2 c.c. of the same mixture, died in 30 hours with characteristic signs of the disease.

The above does not confirm Duenschmann's statement, that animals which have received non-fatal doses of toxin remain for a long time peculiarly sensitive to the living virus. But the following fact noticed by him was confirmed, namely, that a guinea-pig which had received toxin was for a long time less resistant to the action of this toxin than fresh guinea-pigs.

PROPERTIES OF THE SERUM OF IMMUNISED ANIMALS.

During the investigation some strongly immunised animals died, and the serum of rabbits Nos. 2 and 3 only could be examined.

To supplement this supply for experiment, it was considered advisable to rub down the clots of blood in a mortar with normal saline solution, and filter all through a Pasteur-Chamberland filter, so that the serum was considerably diluted by this process; nevertheless, a sufficient amount was obtained for experimental purposes.

The effect of the serum was first tried against a fatal dose of toxin. Plasma toxin of 9 days was used, and proved fatal in doses of 5 c.c.; the quantity used to test the diluted serum was 6 c.c.

Serum No. 2.

Guinea-pig, 400 grms., received 6 c.c. plasma toxin, with 4 c.c. serum in the peritoneum—no symptoms. Guinea-pig, 350 grms., received 6 c.c. plasma toxin, with 2 c.c. serum in the peritoneum—dull, 24 hours—dead, 48 hours.

From the above it will be seen that in doses of 4 c.c. the diluted serum prevents, while in 2 c.c. it retards slightly, the action of the toxin.

The toxin was next injected 24 hours after the serum.

Guinea-pig, 405 grms., received 7 c.c. of serum, and 24 hours later was tested with 6 c.c. toxin—dead, 20 hours.

A mixture of vaccine with a trace of lactic acid was then tried.

Guinea-pig, 310 grms., received 2 c.c. serum, with 1 c.c. vaccine mixture—dead, 20 hours.

The same mixture was tried 24 hours after the serum.

Guinea-pig, 410 grms., received 5 c.c. of serum in the peritoneal cavity, and 24 hours after the mixture as for the last animal was given—no symptoms.

As a test of the strength of serum No. 2, these experiments are not so satisfactory and definite as one might wish, owing to the fact that only a limited amount of serum was obtainable. Yet it may be definitely said that it possesses, even in its diluted state, a certain amount of antitoxic power when introduced along with the toxin. Injected 24 hours previously, it is useless against the toxin, but will prevent the bacilli from developing in the tissues. This would seem to point to the tissue reaction being effected against the bacilli, but not against the toxin produced by them.

The animal which received the serum and bacilli simultaneously, and died, in all probability did not receive a sufficient amount of serum.

Serum No. 3.

The experiments with serum No. 3 were as follows:—

Guinea-pig, 380 grms., received 5 c.c. toxin, with 4 c.c. serum—dull, 24 hours—dead, 30 hours. Guinea-pig, 390 grms., received 5 c.c. toxin, with 2 c.c. serum—dull, 24 hours—dead, 27 hours.

Here we see no prevention at all. An explanation of this result is suggested below.

The vaccine mixture with acid was then tried.

Guinea-pig, 355 grms., received 1 c.c. vaccine mixture (with acid) and $2\frac{1}{2}$ c.c. serum—dull, 24 hours—recovered, 48 hours. Guinea-pig, 320 grms., received 5 c.c. of serum, and 24 hours afterwards 1 c.c. of above mixture—no symptoms. Control guinea-pig, 325 grms., received 1 c.c. of vaccine mixture as above—dull, 24 hours—dead, 48 hours.

Here we have a distinct preventive action of the diluted serum against the bacilli, not only when the bacilli are inoculated simultaneously with the serum, but also when the serum has been introduced 24 hours previously.

In comparing this serum with No. 2, it should be noticed that this animal had received neither so many injections nor so great a quantity of toxins as No. 2; and it is suggested that this explains to some extent the peculiar results observed in the first experiment. Probably also a greater amount of diluted serum than 4 c.c. would have been required to neutralise the amount of toxin used, as the animal could not have been considered so strongly immunised as No. 2.

The relation which these results bear to the number of injections of toxin and bacilli which the animals respectively received is a

matter for further investigation and one of considerable interest. The solutions of virus (·05 c.c. of a sol. of virus 1:20) used by Duenschmann to test the preventive powers of his serum were, as far as one could judge, too weak to be consistently successful.

The results may be summarised as follows:—

1. Blood plasma and peptone broth in the proportion of one to five gave the best toxins.

2. These toxins became rapidly attenuated.

3. Guinea-pigs which had received toxin appeared less sensitive to the bacilli, but more sensitive to the toxins, than fresh animals.

4. Rabbits which had received progressive doses of toxin for a considerable time, and finally of bacilli, furnish a serum which, mixed with fatal doses of toxin, prevent the action of the latter.

5. The serum has also a decided preventive action on the bacilli when introduced into the tissues.

The investigator wishes to express his indebtedness to Dr. Woodhead, for many valuable suggestions during the progress of the work; also to Professor Arloing, of Lyons; and to Professor M'Fadyean, of the Royal Veterinary College, for their kindness in supplying material.