THE ENDOTOXIN OF THE MENINGOCOCCUS

By G. F. PETRIE

From the Serum Department, Lister Institute, Elstree, Herts.

CONTENTS

https://doi.org/10.1017/S002217240003480X Published online by Cambridge University Press

I. INTRODUCTION

RESEARCHES into the pathogenic action of the meningococcus have been carried on during the past thirty-five years. The outbreaks of cerebrospinal fever among the troops in this country during the years 1915–19 afforded abundant material for the investigations that were made by Gordon *et al.* (1920). Their work on the serological classification of the prevalent strains, their observations on the characters of the endotoxin, and their efforts to improve the serum therapy of the disease gave an impulse to further enquiries into the refractory problems of meningococcal immunity. Gordon was convinced that an anti-endotoxin was an essential constituent of an efficient therapeutic serum, although this antibody was virtually absent in most of the numerous samples of serum he examined and was present only in low concentration in those samples which passed the test he employed. The test consisted in mixing and injecting into mice 0·5 c.c. of the sample of serum together with one lethal dose of a dried preparation of endotoxin. Gordon has stated that none but the clinically effective batches of serum neutralized this amount of endotoxin. Later workers have not been successful in obtaining sera of a higher order of potency. An illustration given by Malcolm & White (1932) may be cited; they found that anti-meningococcus serum protected mice against 1 lethal dose of endotoxin and sometimes 2·5 lethal doses; amounts greater than 0·5 c.c. of some samples of normal horse serum neutralized one lethal dose. Murray (1932) reviewed the subject and concluded that the endotoxin does not readily provoke an immunity response, since the evidence for its neutralization by immune sera is not wholly convincing.

https://doi.org/10.1017/S002217240003480X Published online by Cambridge University Press

Within recent years a variation in the experimental technique has been employed, namely, the introduction of meningococci or their products by intracisternal injection or by suboccipital injection into the subarachnoid space of the rabbit and the guinea-pig (Zdrodowski & Voronine, 1932; Belkina, Krestownikowa & Lasowsky, 1933; Branham & Lillie, 1932, 1933; Tschaika, 1936). We may infer from the results obtained by these workers: (1) that meningitis is produced in this way; (2) that killed cocci or culture filtrates are capable of giving rise to the same set of symptoms and post-mortem signs as those which are seen after the administration of a dose of living cocci; and (3) that the toxin in the material injected probably plays an important part in the production of the clinical and pathological effects.

The writer with his colleague Dr Douglas McClean (1932; unpublished) endeavoured to obtain preparations of endotoxin, as judged by the lethal dose when given intraperitoneally to the mouse, of such potency as would ensure that the test dose in serum-toxin experiments contained a reasonable number of lethal doses in a suitable volume. Despite many variations in the experimental methods it was not found possible to prepare consistently endotoxin solutions of which less than 0·5 c.c. was an average lethal dose. After this work was concluded a few preliminary experiments by the present writer showed that it was not difficult to obtain an endotoxin, the intracerebral lethal dose of which for the guinea-pig was 0·01 c.c., an amount which made it easily possible to inject at least five lethal doses in the test dose. Accordingly horses, rabbits and guinea-pigs were immunized in various ways with the object of obtaining anti-endotoxic sera which could be tested by the intracerebral method of injection. The results are given in the following pages.

II. The characters of the endotoxin

Methods used in preparing the endotoxin

During the course of this work seven batches of meningococcal endotoxin were prepared in the following way:

An agar medium was made from a meat-infusion peptone broth which contained 0·5 per cent glucose. The meat used was either Bacto veal, or fresh veal or horse meat; a mixture of Difco proteose peptone, Bacto peptone and Witte peptone—0·7 per cent of each—was dissolved in the meat infusion. The medium was distributed into plates or flat bottles and the surface was sown with the culture. The growths were incubated for 48–72 hours and then washed off with distilled water or normal saline or hormone broth or horse-meat broth, so that the density of the resulting suspension was about $10{,}000 \times 10^6$ cocci per c.c. as estimated by the opacity method. The reaction was adjusted to *p*H 8·2–9·0 in four of the preparations and to *p*H 7·0–7·2 in three of them. Autolysis was then allowed to proceed at 36° C. for 5, 6 or 7 days in the presence of toluol, which was well shaken into the suspension at fairly frequent intervals. The autolysed suspension was filtered through paper and afterwards through a Seitz disc; the first portion of the final filtrate was discarded and the remainder, which contained the endotoxin, was stored under toluol in the cold room. In the experience of the writer, a method of this kind yields satisfactory preparations of endotoxin from *Bact. dysenteriae* (Shiga). There was no indication that any of the variations in the process of

https://doi.org/10.1017/S002217240003480X Published online by Cambridge University Press

extraction described above was of special advantage for the production of a potent endotoxin. The cultures of meningococcus used were "G 827", a Type I strain received from Dr W. M. Scott; and "Pringle" and "Robinson", both of them Type I–III strains received from Dr B. G. Maegraith.

The toxicity of meningococcal products for the guinea-pig when given by the intracerebral route

The technique of intracerebral injection used in the tests.

After clipping the fur, a solution of iodine in chloroform was applied over the site of inoculation. The endotoxins and the coccal suspensions from which they were derived by autolysis were injected, usually in a volume of 0·1 c.c. of an appropriate dilution in saline, directly into the cerebral hemisphere by means of a short needle introduced behind the posterior border of the orbit, as near as could be judged to the midpoint of the left fronto-parietal suture. After the injection was made the needle was kept in situ for 25–30 sec. in order to allow time for diffusion of the inoculum and thus to minimize the leakage that might result from the increased intracranial pressure.

The intracerebral lethal dose of various preparations of endotoxin.

The lethal dose depends upon the age of the guinea-pig chosen for the test, since young animals of 200–250 g., and even animals below 100 g. are more resistant to the toxic action of the endotoxin than those between 400–800 g. Thus, for guinea-pigs weighing from 75 to 100 g., the lethal volume of a particular toxin was 0·1 c.c., and for guinea-pigs from 600–700 g. it was 0·0025 c.c. When graduated doses contained in a constant volume were prepared from serial dilutions and given to a group of heavy guinea-pigs, irregularities in the results were not uncommon. For young guinea-pigs the lethal dose of each of the seven endotoxin preparations was: $>0·1$, 0·1, 0·1 0·1, 0·02, 0·01 and 0·01 c.c.; and the corresponding amounts for heavy guinea-pigs were: 0·002, 0·0025, 0·0025, 0·00167, 0·002, 0·00125 and 0·001 c.c.

The intracerebral lethal dose of meningococcal suspensions.

The endotoxin of the meningococcus exists in the highest concentration within the coccus, for it has never been found to be practicable to recover the whole of the toxin that is present in a suspension after this has been extracted and filtered. The following observations on the pathogenic action of meningococcal suspensions in saline were made. These produced toxic and lethal symptoms when injected intracerebrally in the guinea-pig, and the young animal again proved to be more resistant than the adult. Thus the lethal dose for light guinea-pigs, under 300 g., varied from 6 million to 20 million cocci, whereas for heavy guinea-pigs, above 400 g., the corresponding doses varied from 700,000 to 3,800,000 cocci. Maegraith (1935) observed a similar difference in the susceptibility of light and heavy guinea-pigs to meningococcal exotoxin. The present writer has confirmed this observation and has noted in both groups of animals occasional irregularities in tests for determining the lethal dose of all the varieties of meningococcal preparations.

https://doi.org/10.1017/S002217240003480X Published online by Cambridge University Press

The intracerebral lethal dose of a specific polysaccharide isolated from a Group 1 *strain of meningococcus.*

This material was prepared by my colleague Dr W. T. J. Morgan with the co-operation of Dr L. A. Elson, and I am indebted to them for a sample of it; they informed me that it was a highly purified product.

On 1. v. 34, three guinea-pigs, each weighing 225 g., received an intracerebral dose of 10, 1 and 0·1 mg. respectively of the polysaccharide in saline. About 2 min. after the dose was given all showed signs of cerebral irritation, and the guinea-pig to which 10 mg. was given exhibited "circus" movements until its death 10 min. after the injection. The blood vessels on the surface of the brain were congested. Three and a half hours after the injection the animal that received 1 mg. was ill with similar symptoms to those of the first guinea-pig, but they were less severe; tremors, grinding of the teeth and dyspnoea were noted. When examined 4½ hours later this animal was in much the same condition, but next morning it had recovered. The guinea-pig with the smallest dose had no symptoms and remained normal. Next day the test was repeated with intermediate doses; four guinea-pigs (200–235 g.) received a dose of 5, 2, 2 and 1 mg. respectively of the polysaccharide. One hour and a half after the injection all the animals appeared to be quite unaffected. When observed 2 hours later the guinea-pig with the 5 mg. dose was lying on its side scarcely able to move and there were twitchings at short intervals. After another period of 2 hours fine muscular tremors were felt when the chest wall was loosely held and there were recurring twitchings; the animal was unable to move. After yet another interval of 2 hours it seemed to be moribund and showed convulsive movements of the limbs. Death occurred 19 hours after the injection. Post-mortem examination revealed hyperaemia of the blood vessels around the site of injection into the brain. The adrenals were congested. One of the guinea-pigs which received a dose of 2 mg. had similar symptoms to those of the last-mentioned animal and died 18 hours after injection of the material. Post-mortem, there was some congestion of the meninges and to a less degree of the brain. The lungs were markedly hyperaemic and so also were the adrenals. The other guinea-pig that received a dose of 2 mg. was dull and disinclined to move and had some dyspnoea, but it recovered; the guinea-pig that received a dose of 1 mg. was dull for an hour or two, and soon recovered. The lethal dose of this polysaccharide for a light guinea-pig was thus apparently about 2 mg. The small amount of the material available did not permit the determination of the lethal dose in heavy guinea-pigs. The cerebral symptoms and the post-mortem signs in the experimental animals were similar to those which resulted from the injection of meningococcal endotoxin and suspensions and which are described below. The significance of the experiments with the specific polysaccharide is referred to later, but it may be noted here that Krestownikowa & Rjachina (1934) believe that meningococcal toxin is a complex nitrogen-containing carbohydrate.

https://doi.org/10.1017/S002217240003480X Published online by Cambridge University Press

The symptoms produced in the guinea-pig by the intracerebral injection of meningococcal endotoxin or a suspension of living or dead meningococci

During the first 30–60 min. after the injection the animal appears to be normal, but later it becomes restless and shows the characteristic symptom of a recurring generalized muscular twitching, apparently of an epileptiform character.[1] The condition gradually becomes worse until the animal is found to be lying on its side and dyspnoea is then often a noticeable feature; associated with this the muzzle is sometimes covered with a frothy blood-stained fluid. The dyspnoea may be due to inco-ordinated contractions of the thoracic muscles from interference with their nervous control. Death usually occurs within 5–12 hours. Some of the animals which showed definite symptoms recovered and appeared to be quite well on the morning after the injection. Observations were carried out on the body temperature of light and heavy guinea-pigs after they had received a fatal intracerebral dose of an endotoxin; an equal number were inoculated intracerebrally with a dose of exotoxin, prepared according to the method described by Ferry *et. al.* (1931) (see Table I). Most of the animals in this experiment showed a fall of temperature which persisted till death. Flexner (1907) noted that a fall of temperature followed the intraperitoneal injection of a fatal dose of endotoxin in the young guinea-pig, and Gordon & Bell (*see* Gordon, 1918) found that, when a suspension of living meningococci or an aqueous extract of the dried coccus was given intravenously to the rabbit in a dose which caused rapid death of the animal, the temperature fell continuously till death occurred.

An experiment was planned in order to ascertain whether luminal might alleviate the epileptiform symptoms. A dose of 40 or 60 mg. of luminal sodium, when given subcutaneously, produced a sedative effect which lasted for at least 7 hours; the effect from 20 mg. was less obvious. Accordingly each of a group of ten guinea-pigs weighing 400–500 g. received 15 mg.; and then 20 mg. on each of six successive days; and finally 40 mg. $\frac{1}{2}$–1 hour before an intracerebral dose of 0·005 c.c. of an endotoxin solution was given to them. Ten untreated animals of similar weight formed a control group and received the same dose of endotoxin. During the period of treatment by luminal, the average increase of weight in the two groups was virtually the same. The twitchings that resulted from the dose of endotoxin were much less pronounced in the luminal-treated guinea-pigs, but otherwise there was no amelioration of the symptoms and most of the animals died between the fifth and eighth hour after the injection. There were seven deaths in each group and there was no significant difference in the death times.

[1] I am indebted to Dr W. Denny-Brown, to whom I demonstrated these symptoms, for giving me his opinion of their nature.

https://doi.org/10.1017/S002217240003480X Published online by Cambridge University Press

https://doi.org/10.1017/S002217240003480X Published online by Cambridge University Press

Table I. *Observations on the body temperature of the guinea-pig after intracerebral injection of meningococcus endotoxin. A preparation of meningococcus exotoxin was injected into an equal number of control animals*

Serial no. of guinea-pig	Weight (g.)	Sex	Temp. before injection (° F.)	Intracerebral dose	Symptoms at the end of: 1 hour	2 hours	4 hours	5 hours	6 hours	8 hours
1	475	M.	101·2	0·1 c.c. endotoxin	No symptoms	98·2 Nil	96·2 Tw. Dyspn. V. ill	96·6 Tw. Dyspn. V. ill	95·6 Tw. Dyspn.	+
2	450	F.	101·0	,,	,,	100·0 ,,	97·8 ,,	+		
3	240	M.	101·0	,,	,,	99·8 ,,	99·8 ? Ill	99·2 On side	95·4 Tw. V. ill	+
4	220	F.	101·2	,,	,,	96·0 Ill	96·6 ? Ill	98·4 Ill	99·0 Ill	+ 6.30 a.m. next day
5	425	F.	99·6	0·1 c.c. exotoxin	,,	100·6 Nil	97·6 Tw. Dyspn.	98·8 Tw. V. ill	99·0 Tw. V. ill	+
6	500	M.	101·8	,,	,,	101·0 ,,	100·4 ,,	+		
7	220	M.	102·4	,,	,,	100·8 ,,	99·4 Ill	99·6	97·2 Dyspn. on side V. ill	+
8	220	F.	100·8	,,	,,	101·0 Tw. Ill	99·0 Tw. on side	96·4 On side. V.ill	+	

Note: Tw. = muscular twitchings. V. ill = very ill.
Dyspn. = dyspnoea. + = death.

Post-mortem appearances.

The brain and its membranes show no marked abnormality, but in sections an excess of polymorphonuclear cells is apparent at the site of the injection. The lungs are distended and are often intensely congested; pleural haemorrhages are often seen. The adrenals are injected in varying degrees; they sometimes show an extreme degree of congestion and sections reveal an excess of polymorphonuclear cells, and even necrotic changes, in spite of the brief interval between the administration of the endotoxin and the time of death. The spleen contrasts with the lungs in appearing small.

Observations on the necrotizing action of meningococcal products

Rabbit. Successive subcutaneous doses of endotoxin—78 c.c. in all—were given to each of two rabbits, and thereafter a course of doses of a toluol-killed suspension totalling $560{,}000 \times 10^6$ cocci. One of the animals died on the day following a dose of 10 c.c. of suspension, and the other was bled to death in order to obtain serum for neutralization tests. Both showed extensive subcutaneous necrosis at the various sites of injection, but this did not involve the overlying skin.

The intracutaneous injection of a suspension in the rabbit produced a hyperaemic area with induration and central necrosis. These effects were noted also by Malcolm & White (1932), Krestownikowa *et al.* (1933) and Alissowa & Wygodschekoff (1933).

Guinea-pig. The subcutaneous injection of toluol-killed suspensions gave rise to persistent indurated nodules which sometimes broke down. Thus a group of guinea-pigs which had received successive doses subcutaneously were carefully examined for residual indurated swellings; when found, these were incised and caseous material was expressed from twenty out of twenty-four of them. When the suspension was injected intracutaneously, for example, in a volume containing 100×10^6 cocci, a small swelling was evident after 6 days; this showed superficial necrosis or a scab, the removal of which revealed an ulcer, or the scab might have already separated and disclosed an ulcer with alopecia of the surrounding skin; the loss of hair is an indication of superficial necrosis. Two guinea-pigs that received a dose of 10 c.c. of endotoxin subcutaneously in the course of an immunization with this material showed next day a severe local reaction which measured $8 \times 5{\cdot}5$ cm. A few days later some superficial ulceration of the skin appeared, and 14 days after the dose was given an area of alopecia, measuring 9×2 cm., was noted.

Each of a group of guinea-pigs which had been immunized with subcutaneous doses of toluol-killed suspensions received a series of intracutaneous doses of endotoxin, namely, 0·5, 0·25, 0·1 and 0·05 c.c. The three largest doses and, in one animal the smallest dose, produced, after about 1 week, small ulcers with induration and alopecia of the surrounding skin.

https://doi.org/10.1017/S002217240003480X Published online by Cambridge University Press

Horse. A horse "Endo" was immunized with subcutaneous doses of endotoxin. After it had received about twenty doses, a circumscribed oval swelling, 7·5 × 9 cm. in size, followed the dose of 35 c.c. given on 1. x. 34, and on 8. x. 34 it was found to be exuding some serous fluid. The collodion dressing was removed, and it was then seen that the skin was detached from the subcutaneous tissues and was necrotic so that it could be painlessly excised with scissors; its removal left a cavity which presented a clean appearance and the floor of which was formed by the subjacent muscle. A smear showed numerous polymorphonuclear leucocytes but no bacteria. The cavity was dressed with iodoform powder, iodoform vaseline, cotton wool and collodion, and on 19. x. 34 it was healing well. The lesion always appeared quite healthy and pus was never seen, although it took a month to heal. On 29. x. 34 another small swelling was found to be covered by a piece of dead skin measuring 2·5 × 4 cm.; this was removed and the cavity healed in the course of a week. Again on 4. ii. 35, a swelling broke down and took 18 days to heal. On 29. x. 35 an area of dead skin, 2·5 × 2·5 cm., was observed at the site of injection of a dose given to "Endococcus", a horse which was immunized at the same time as "Endo", but with intravenous doses of meningococcal suspension in addition to subcutaneous doses of endotoxin.

The occurrence in these horses of a dry aseptic gangrene, which was doubtless due to the endotoxin, reminded the writer of a striking instance of the necrotizing action of the endotoxin in a horse which, some years previously, was undergoing a routine immunization with meningococcal strains. A subcutaneous dose of 200 c.c. of a meningococcal suspension which contained 25 per cent glucose was given to the animal; the object of adding the glucose was to extract the endotoxin by means of the osmotic action of the sugar. As a consequence of the dose a dry gangrene spread from the site of injection so that the skin became detached from the underlying muscles. The process extended downwards along the foreleg as if by gravity, and although the horse seemed comfortable and in no pain with no rise of temperature or pulse-rate, it was thought best to destroy it in view of the prolonged period of healing that was likely to ensue. Experiments carried out later showed that the addition of an equal volume of 50 per cent glucose solution to a meningococcal suspension (Type I), or to an endotoxin preparation, did not materially enhance the resulting necrosis when the mixture was injected intracutaneously in the rabbit, guinea-pig, and horse; in control mixtures an equal volume of saline was substituted for the glucose solution. Moreover, intracutaneous injections in the rabbit and the guinea-pig of 50 per cent glucose caused necrosis in doses of 0·2–0·5 c.c., but the same doses of 25 per cent glucose had no necrotizing effect.

Necrosis of the skin in man.

Gangrene with sloughing of the skin has been observed as a rare complication of cerebrospinal fever in man. Elliott & Kaye (1917) have reported the following remarkable case.

https://doi.org/10.1017/S002217240003480X Published online by Cambridge University Press

A young soldier was admitted to hospital from duty in the trenches in winter with a mild attack of cerebrospinal meningitis complicated by the presence of extensive areas of purpura on the legs and nates and by failure of circulation in the feet due to the condition known as "trench feet". On the 4th day of the illness the purpuric rash showed central areas of a greyish purple tint which appeared as if they would soon become necrotic. On the 8th day the periphery of the necrotic areas on the thighs and legs had become almost coal black and on the 25th day the limbs showed large areas of ulceration from which the necrosed skin had sloughed away and laid bare the subcutaneous tissues; the ulcerated areas were covered with healthy granulations, and the skin at their edges was growing actively. And yet at this stage of his illness the patient had completely recovered from the meningeal attack and his general condition was good. Elliott & Kaye concluded that the necrosis was caused by obstruction of the capillary circulation in the skin, and that this may have been caused by a thrombosis due either to a toxin or to local chilling of the tissues. These authors refer to a case of cerebrospinal meningitis described by Gardner Robb (1916) in which purpuric and haemorrhagic symptoms were a prominent feature. The illness was a severe one, and during convalescence some patches of skin became necrosed and sloughed.

In the light of the experimental observations that have just been described, the ulceration of the skin in these patients may be attributed to the necrotizing action of the endotoxin liberated locally by autolysis of the coccal bodies.

The relative susceptibility of the mouse, the rat, the rabbit, and the guinea-pig to the intracerebral injection of meningococcal endotoxin

The mouse is much more resistant than the heavy guinea-pig to the endotoxin when this is administered intracerebrally. Thus 0·025 c.c. of a toxin, of which 0·0025 c.c. was the lethal dose for the heavy guinea-pig, did not cause much disturbance in mice. The rat, too, is probably less susceptible than the heavy guinea-pig and, even if death ensues, this species does not manifest the characteristic group of symptoms that have been described for the guinea-pig. The rabbit is much less sensitive to the intracerebral injection of the endotoxin than the heavy guinea-pig.

The relative toxicity of the endotoxin when administered by the intracerebral and the intravenous route

An endotoxin, of which the intracerebral lethal dose for heavy guinea-pigs was 0·00133 c.c., was injected intravenously in doses of 1, 0·1, 0·01, 0·002 and 0·001 c.c., into both light and heavy guinea-pigs. There were no deaths, and no severe symptoms were observed even with the larger doses. It thus appears that the endotoxin, when it is injected intravenously, does not readily pass through the haemato-encephalic barrier into the tissues of the nervous system but that, when injected intracerebrally, it quickly escapes into the systemic circulation and so produces morbid changes, particularly in the adrenals. An alternative possibility, however, is that the concentration of the endotoxin is reduced by its immediate dilution in the circulating blood to a lower level than that necessary to cause damage to the capillary vessels such, for example, as results from injection within the dense tissues of the skin in the form of a solution or of a coccal suspension.

https://doi.org/10.1017/S002217240003480X Published online by Cambridge University Press

III. Active immunization of experimental animals with the endotoxin and with suspensions of meningococci

The experiments detailed under this heading were carried out with the object: (1) of ascertaining whether it is possible to confer an active immunity to the endotoxin; and (2) of obtaining samples of serum that could be used for neutralization tests with the endotoxin in experimental animals.

Immunization of the guinea-pig by means of successive subcutaneous doses of toluol-killed meningococci

A group A of twelve guinea-pigs (seven males and five females) whose mean weight was 660 g., received on 30. vii. 34 a subcutaneous dose of 1 c.c. ($10{,}000 \times 10^6$ cocci); eight doses followed, all of them subcutaneous except an intracutaneous dose of 0·01 c.c. (100×10^6 cocci); the last dose of 1 c.c. ($10{,}000 \times 10^6$ cocci) was given on 1. xii. 34. Blood was taken by heart puncture on 8. xii. 34 or 10. xii. 34. Later, two further subcutaneous doses were given and then three small intraperitoneal doses; the last of these on 2. iii. 35 consisted of 5000×10^6 cocci. Each animal received in all $65{,}000 \times 10^6$ cocci before blood was taken by heart puncture; a total of $93{,}000 \times 10^6$ cocci subcutaneously; a total of $12{,}000 \times 10^6$ cocci intraperitoneally, and a combined total of $105{,}000 \times 10^6$ cocci. The mean weight on 11. iii. 35 was 880 g.

A group B of nine guinea-pigs (two males and seven females), whose mean weight was 425 g., received on 20. ix. 34 a subcutaneous dose of 1000×10^6 cocci, and thereafter a course of immunization which corresponded closely to that outlined above. Each animal received a total of $95{,}000 \times 10^6$ cocci. On 4. iii. 35 the mean weight was 850 g.

A group C of four female guinea-pigs were kept throughout under the same conditions as group B to serve as controls. The mean initial weight was 415 g. and the weight on 11. iii. 35 was 845 g. A comparison of the weights in this and the previous group B shows that the immunization did not bring about any serious toxic effect that predisposed to malnutrition.

A group D consisted of six male guinea-pigs whose mean weight on 20. ix. 34 was about 450 g. when the first subcutaneous dose of 1000×10^6 cocci was given. The course of immunization followed that of groups A and B as described above. After nine subcutaneous and one intracutaneous dose a blood sample was taken by heart puncture on 8. x. 34 or 10. x. 34. A series of intraperitoneal doses followed, and on 14. ii. 35 a subcutaneous dose of $20{,}000 \times 10^6$ cocci. The aggregate dosage for each animal was about $120{,}000 \times 10^6$ cocci. The mean weight on 14. ii. 35 was 780 g. The corresponding mean weights of a group E of six control animals (2 males and 4 females) were 410 and 800 g.

Immunity tests by means of intracutaneous and intracerebral injection of endotoxin.

Intracutaneous. On 15. iii. 35 the immunized guinea-pigs in groups A and B received intracutaneous doses of an endotoxin preparation, the intracerebral lethal dose of which was 0·001 c.c.; 0·5, 0·25, 0·1 and 0·05 c.c. were given to some and 0·025 c.c., and 0·0125 c.c., in addition, to others. The four control animals received the complete series of 6 doses. During the next 12 days the reactions in all the animals were measured and compared. Those caused by the three larger doses consisted of indurated swellings which tended to undergo necrosis, so that after a week cheesy material could be squeezed out; or the centre of the lesion was covered by a scab or was occupied by a small ulcer. On the third day the reactions on the control guinea-pigs were of the same character, but they were rather less severe since there was less induration.

On 19. iii. 35 four guinea-pigs of group B and the four controls (group C)

https://doi.org/10.1017/S002217240003480X Published online by Cambridge University Press

received intracutaneous doses of 0·0125, 0·00625, 0·00312 and 0·00156 c.c. of the same endotoxin. The area of the resulting reactions varied from 17 × 17 mm. to 11 × 11 mm., and took the form of slightly raised injected and indurated swellings which showed desquamation of the skin. There was no material difference between the reactions in the two sets of animals.

Thus no evidence emerged that any immunity had been produced against the endotoxin as the result of the inoculation of the guinea-pigs with doses of a meningococcal suspension, although these contained large amounts of endotoxin.

Intracerebral. On 21. ii. 35 an intracerebral dose of 0·00125 c.c. of the same endotoxin was given to each animal of groups D and E, with the result that all the immune guinea-pigs had typical symptoms and died after the usual interval; five of the six in the control group E died.

There was thus no evidence of any protection having been afforded by subcutaneous immunization with killed meningococci against the action of the endotoxin when this was injected intracerebrally.

Immunization of a guinea-pig by means of successive subcutaneous doses of endotoxin

On 8. vi. 34 guinea-pig no. 36 (male, initial weight 800 g.) received 0·000125 c.c. of a toluol-killed suspension, a dose which contained 1,250,000 cocci, and thereafter it received sixteen subcutaneous doses of from 2·5–10 c.c. of a saline solution of endotoxin. The immunizing course lasted for 189 days and the total amount of endotoxin solution injected was 107 c.c. Seventy days later this animal together with a control guinea-pig received doses of 0·5, 0·25 and 0·1 c.c., of a potent endotoxin solution intracutaneously. Both showed much the same kind of reaction as those in the groups of guinea-pigs described above, and again the reactions in the immune animal were rather more marked than those in the control. In both animals there was necrosis of the skin and at a later stage pus formation.

An attempt to immunize light and heavy guinea-pigs by inoculating them intracerebrally with successive doses of endotoxin solution

Light guinea-pigs. Thirty animals were used in this experiment: (1) a control group of three with a mean weight of 192 g., each of which received nine successive doses of 0·1 c.c. normal saline intracerebrally; (2) a group of seventeen guinea-pigs each with an initial weight of less than 300 g.; the mean weight for the first weighing was 221 g.; and (3) a group of ten guinea-pigs each with an initial weight of more than 300 g.; the mean weight for the first weighing was 312 g. The control guinea-pigs remained healthy and gained weight to the extent that at the twelfth weighing, about 90 days after the first dose, the mean weight was 522 g. The group of seventeen light guinea-pigs suffered casualties during the course of immunization as a direct result of the injections, so that in 90 days only six guinea-pigs had survived and the mean weight of these was 362 g. After 90 days the heavier group of ten guinea-pigs was reduced to two and the mean weight of these was 460 g. The continuous loss of weight of the guinea-pigs inoculated with endotoxin is an index of

https://doi.org/10.1017/S002217240003480X Published online by Cambridge University Press

nutritional disturbance caused by the injections. The first dose given to some of the guinea-pigs varied because they were survivors from previous tests, but each animal received 0·005 c.c. as a second dose. Not more than ten doses in all were given to any one animal and 0·04 c.c. was the last dose; the total volume given to each guinea-pig was about 0·25 c.c. The intolerance to the injections in spite of carefully graduated increments of the doses is evidence that an active immunity was not produced: the repetitions of the intracerebral doses did not mitigate the symptoms caused by the endotoxin.

Definite post-mortem signs in the brain or organs were not found in any of the animals as the result of the treatment except that three of them showed an infection of the lungs with the guinea-pig pneumococcus (Type 19 of man), a consequence of the lowering of resistance brought about by the associated nutritional disturbances.

After the last dose of 0·04 c.c. of endotoxin, the experiment was brought to an end by bleeding the animals to death and collecting and pooling the serum of the immunized group and of the control group.

Heavy guinea-pigs. This group included six guinea-pigs weighing 700–800 g. Owing to the lack of tolerance to the injections only a few doses, most of them containing 0·00125 or 0·00167 c.c. of the endotoxin solution, were given; three of the six animals died after three or four doses.

Immunization of rabbits with meningococcal endotoxin

Two rabbits were immunized by the subcutaneous route and two by the intracerebral route.

Subcutaneous route. Two rabbits, nos. 34 and 35, received an identical course of immunization with subcutaneous doses of endotoxin and toluol-killed suspensions; 78 c.c. of endotoxin solution and 56 c.c. of suspension ($560{,}000 \times 10^6$ cocci) were given in all to each animal. On 21. vi. 34 a sample of blood was taken from rabbit no. 34.

The general health of both animals did not appear to be seriously affected by the treatment but the local swelling persisted, and after death extensive necrosis and abscess formation was found at the various sites of injection; the pus proved to be sterile.

Intracerebral route. Rabbit no. 31 received a series of intracerebral doses of endotoxin in parallel with the group of light guinea-pigs to which reference has already been made (p. 53); the same toxins were used for all. The course lasted from 30. iv. 34 to 23. xi. 34 and a blood sample was taken on 1. xii. 34. Five successive doses of 0·5 c.c. were given and the total dosage was 4·3 c.c. The first and last weighings were 1000 and 2900 g. respectively.

Rabbit no. 32 received identical treatment and it was sampled on 1. xii. 34 and bled to death on 13. xii. 34. The first and last weighings were 1200 and 2600 g. respectively.

Rabbit no. 33 received a succession of intracerebral doses, 3·9 c.c. in all, from 29. vi. 34 to 13. xi. 34, and it was bled to death on 14. xi. 34. The first and last weighings were 3410 and 3060 g. respectively, a decrease of 350 g.

The immunization of these animals made it evident that the rabbit is much less sensitive to intracerebral injections of endotoxin than the heavy guinea-pig, since the lethal intracerebral dose for the latter, when calculated per gram of the body-weight, is about 0·000004 c.c., whereas 0·00025 c.c. per gram is not a lethal dose for the rabbit; a ratio of 1 : 60. This conclusion still holds

https://doi.org/10.1017/S002217240003480X Published online by Cambridge University Press

when the calculation is made on the assumption that the brain tissue alone is susceptible to the endotoxin.

Immunization of horses with meningococcal endotoxin

Two horses, "Endo" and "Endococcus", received successive subcutaneous doses of eight preparations of endotoxin solution, and "Endococcus" received an intravenous dose of a heat-killed meningococcal suspension after each subcutaneous dose of endotoxin solution. Two courses of treatment were given to both horses; in addition a third course consisting of a series of doses of endotoxin solution which contained 0·1 per cent potash alum was given to "Endo"; this method is known to enhance antitoxin production when horses are being immunized against tetanus and diphtheria toxins. The details of the immunization are given in Table II. The horse "Endo" showed some intolerance to the doses and the local swellings were larger and persisted longer than those of "Endococcus", so that on occasion it was necessary to extend the interval between the doses to 7 or even 14 days.

At the same time as these horses were being immunized, two other horses were immunized on behalf of Dr B. Maegraith with filtrates of broth cultures of the meningococcus which, according to Ferry, contain the exotoxin of the meningococcus. Subcutaneous doses of exotoxin were given to both, and in addition intravenous doses of coccal suspensions were administered to one of them.

The general effects as estimated by the temperature and pulse-rate and the local reactions were much the same in all the four horses; the temperature was not raised much in consequence of the doses of exotoxin although the pyrexia sometimes persisted for 48 or even 72 hours. The horse that received subcutaneous doses of the exotoxin alone had an immediate severe allergic attack on two occasions. The horse "Endo" showed allergic symptoms with heavy breathing, general discomfort, and loss of appetite after a subcutaneous dose of 50 c.c. of endotoxin solution, and the horse "Endococcus" had a similar attack after an intravenous dose of six agar slopes of the strain "Pringle".

Immunity test by means of intracutaneous injections of meningococcal endotoxin and of a meningococcal suspension.

Three of the four horses just mentioned, which had been thoroughly immunized with meningococcal products, and a normal horse which served as a control were given four injections intracutaneously in the neck as follows:

(1) 0·25 c.c. meningococcal endotoxin + 0·25 c.c. saline.
(2) „ „ endotoxin + 0·25 c.c. 50 per cent glucose solution.
(3) „ „ suspension ($=5000\times10^6$ cocci) + 0·25 c.c. saline.
(4) „ „ suspension ($=5000\times10^6$ cocci) + 0·25 c.c. 50 per cent glucose solution.

https://doi.org/10.1017/S002217240003480X Published online by Cambridge University Press

Table II. *Giving details of the immunization and bleedings of two horses inoculated with meningococcal products*

Horse	Course	Inclusive dates	Endotoxins used	No. of subcutaneous doses of endotoxin	Total volume of endotoxin injected subcutaneously c.c.	No. of intravenous doses of suspension	No. of slopes given intravenously
"Endo"	1st	14. vi. 34–19. xi. 34 Bleeding 29. xi. 34	4, 5, 6, 7, 8 A, 9 A, 10 A	30	601	—	—
	2nd	10. i. 35–31. i. 35 Bleeding 7. ii. 35	11 A	7	118	—	—
	3rd	28. ii. 35–25. iii. 35 Bleeding 1. iv. 35	11 A + 0·1 per cent alum	8	217	—	—
			Total	45	936	—	—
"Endococcus"	1st	14. vi. 34–19. xi. 34 Bleeding 29. xi. 34	4, 5, 6, 7, 8 A, 9 A, 10 A	31	801	31	109
	2nd	10. i. 35–31. i. 35 Bleeding 7. ii. 35	11 A	7	118	7	22
			Total	38	919	38	131

https://doi.org/10.1017/S002217240003480X Published online by Cambridge University Press

The resulting oedematous reactions were measured after 3, 5, 8 and 22 hours and daily thereafter. After 3 hours they were larger in the meningococcus horses than in the control horse. After 5 hours all the reactions in the immunized group had fused with just a trace of division between them. The total swelling measured 25 × 15 cm., and presented an alarming appearance because it bore some resemblance to an acute pyogenic infection; the temperature and pulse-rate, however, were normal in all the horses. At this time the local reactions in the control horse were separated from each other, and after 8 hours they were still readily distinguishable, whereas those in the meningococcus horses remained fused, but now showed one or more long extensions from the lower edge of the oedematous swelling caused by the action of gravity on the fluid in the tissues. One of these extensions measured 34 cm. and was 10 cm. broad in the middle of its length. After 22 hours the swellings in all the horses were flatter, and thus of larger area than before; those in the meningococcus group were separated from each other and so could easily be measured. On the third day the endotoxin reactions (nos. 1 and 2) in all the animals had virtually disappeared whereas nos. 3 and 4 had taken the form of raised papules. A day or two later either one or both of these papules in all the horses showed a tendency to break down in the centre, but it was not clear whether this was an effect of the endotoxin or due to friction applied by the animal to allay local irritation. There was little evidence that the addition of glucose had increased the size of the local lesion to any significant extent, nor were the reactions caused by the toxic products of the meningococcus modified in the immunized horses, an observation which supports the conclusion that the endotoxin of the meningococcus is not antigenic. The rapid production of an extensive local tissue reaction with an equally rapid subsidence of the oedematous swelling was an allergic effect brought about by an antigen-antibody reaction, the antigen presumably consisting of the specific substance which causes precipitation with an immune serum.

IV. NEUTRALIZATION EXPERIMENTS WITH MIXTURES OF VARIOUS IMMUNE SERA AND MENINGOCOCCAL PRODUCTS

The intracerebral injection of endotoxin together with immune sera derived from the horse, rabbit and guinea-pig

Horse sera. Most of the samples of serum employed were obtained from "Endo" and "Endococcus" (see Table II and p. 56). The intracerebral lethal dose of the endotoxin solution used for the neutralization tests was 0·01 c.c. for light guinea-pigs and 0·001 c.c. for heavy guinea-pigs. The serum-toxin mixtures were incubated at 37° C. for at least 1 hour before injection into the test animal. The serum dose was, as a rule, 0·05 c.c. of the undiluted serum, and the toxin dose was graduated from 0·001 c.c. to 0·004 c.c. (1–4 lethal doses) contained in a volume of 0·05 c.c. The weight of the guinea-pigs ranged between 400 and 550 g. A control set of guinea-pigs were inoculated with the

https://doi.org/10.1017/S002217240003480X Published online by Cambridge University Press

same dose of the corresponding normal serum, that is, a sample taken from the horse before the immunization was begun.

The impression received during this part of the work was that the results lacked consistency, because an experiment which seemed to indicate some degree of protection from the immune serum was not supported in later tests. The individual tests are on too small a scale to permit any definite conclusion, but the summed results are as follows.

Of fifty-four guinea-pigs that received immune sera derived from "Endo" and "Endococcus" twenty died (37 per cent), and of forty-eight control guinea-pigs that received the same test dose at the same time as the others and the same dose of the corresponding normal serum twenty-three died (48 per cent). The difference (11 per cent) is not statistically significant.

A horse, "Minos", furnished a precipitating serum of high titre for Group 1 meningococci after immunization with intravenous doses of meningococcal suspensions. A group of ten guinea-pigs with a mean weight of 190 g. received intracerebrally a mixture of 0·05 c.c. of an endotoxin solution and 0·05 c.c. of the immune serum of "Minos"; seven out of ten died. A control group with a mean weight of 200 g. received the same dose of toxin but this was mixed with 0·05 c.c. of the normal serum of this horse; five out of ten died. The experiment was repeated with a smaller test dose—0·005 c.c.—and an increased dose of serum—0·1 c.c.—with the result that two died out of each group of five animals.

Rabbit and guinea-pig sera. The serum used in this group of experiments was derived from immunized animals to which reference has already been made, namely, rabbits nos. 31, 32, 33, 34 and 35; a pooled serum obtained from guinea-pigs immunized by the subcutaneous route with meningococcal suspensions (groups A and B) and a pooled serum from the control group C; guinea-pig no. 36, which had been immunized subcutaneously with endotoxin; a pooled serum from eight intracerebrally immunized guinea-pigs and also a control pooled serum from guinea-pigs that had received successive doses of saline intracerebrally. The numbers in the individual tests are too small for a strict comparison, but the summed results show that in the immunized group eighteen out of forty animals died and that of the controls twelve out of twenty-four died.

Conclusion to be drawn from the above tests.

The figures relating to all the tests described above have been totalled, with the result that of 109 guinea-pigs which received a dose of immune serum derived from the horse, the rabbit or the guinea-pig forty-seven died: a mortality rate of 43 per cent. There are for comparison eighty-seven control guinea-pigs that received normal serum, and of these forty-two died: a mortality rate of 48 per cent. We may conclude that immune sera, prepared from meningococcal products which contain the endotoxin, are unable to neutralize the lethal action of the endotoxin when serum-toxin mixtures are injected intracerebrally into the guinea-pig.

https://doi.org/10.1017/S002217240003480X Published online by Cambridge University Press

Control experiments showing that, in contrast to meningococcal endotoxin, certain neurotoxins can be specifically neutralized in the cerebral tissues of the guinea-pig

A number of control experiments with three well-known neurotoxins, namely, diphtheria toxin, tetanus toxin and scorpion venom were carried out in order to test the possibility that the cerebral tissues of the adult guinea-pig might be so sensitive to the action of meningococcus toxin that a specific serum could not be expected to neutralize it. The technique of injection of the neurotoxins and the serum-toxin mixtures was the same as that which was used in the work on the endotoxin of the meningococcus. The results were briefly as follows:

In the first place it is noteworthy that the tests for determining the intracerebral lethal dose of the various neurotoxins showed satisfactory regularity. The ease with which many multiples of the intracerebral lethal dose of each of these toxins were neutralized by the corresponding immune serum, even when the serum-toxin mixtures were injected directly into the brain substance, formed a striking contrast to the difficulty of demonstrating by the same technique a specific antibody for the endotoxin of the meningococcus. Thus the pathogenic action of fifty intracerebral lethal doses of a freshly prepared potent diphtheria toxin was so completely neutralized by the addition of two units of diphtheria antitoxin that no toxic symptoms were apparent. The minimum amount of this toxin which produced the typical symptoms of diphtheritic paralysis, when introduced directly into the brain of the guinea-pig, was 0·000005 c.c.; the test dose of toxin that was used in this experiment contained 1250 paralysing doses. Again, a mixture which contained 100 intracerebral lethal doses of a tetanus toxin and 0·4 International unit of tetanus antitoxin was injected into the brain of each of three guinea-pigs. There were no tetanic symptoms and the animals remained healthy.

The results obtained with scorpion venom were even more conclusive, because this venom, like others of its class, has a powerful neurotoxic action with scarcely any latent period when it is injected by the subcutaneous route. The intracerebral injection of the venom causes immediate symptoms that lead to a rapidly fatal issue. And yet guinea-pigs that received a mixture of twenty intracerebral lethal doses and 0·1 c.c. of antiscorpion serum from the horse remained quite well.

These experiments confirm the belief that the failure to obtain evidence of neutralization of meningococcus toxin by immune sera is due to the lack of a specific neutralizing antibody. Apart from this conclusion, the results of the experiments with neurotoxins were striking from the proof they gave of the remarkable efficiency of the neutralizing mechanism. The animals were kept sufficiently long for the foreign horse serum which carried the antibody to be excreted, and yet there was no evidence of dissociation of the toxin-antitoxin complex, since they remained quite well.

https://doi.org/10.1017/S002217240003480X Published online by Cambridge University Press

Intracutaneous tests with mixtures of meningococcal products and immune sera

(1) Doses of 0·5–0·0125 c.c. of an endotoxin solution, the intracerebral lethal dose of which was 0·01 c.c. for light guinea-pigs and 0·001 c.c. for heavy guinea-pigs, were injected into the skin of a guinea-pig weighing 575 g. together with 0·1 c.c. of serum taken from "Endo" on 7. ii. 35; the same doses of the endotoxin were injected into the skin of the other flank of this animal together with 0·1 c.c. of the normal serum of "Endo". Significant differences in the resulting reactions were not detectable.

Here it may be mentioned that there seems to be a difference in the reactivity of the skin of the light guinea-pig as compared with that of the heavy guinea-pig when the endotoxin is injected intracutaneously. Thus amounts of the above-mentioned endotoxin solution ranging from 0·001 to 0·0000019 c.c. prepared in saline and contained in a volume of 0·1 c.c. were injected intracutaneously into a guinea-pig weighing 540 g., and at the same time into a guinea-pig weighing 260 g. Next morning areas of congestion from 10×10 mm. to 5×5 mm. were present on the heavy guinea-pig even at the site of the smallest dose injected, whereas the light guinea-pig was free from reactions. A control injection of 0·1 c.c. saline in the heavy guinea-pig showed some degree of hyperaemia of the blood vessels of the skin but the effect was much less definite than the reaction from the smallest amount of endotoxin. Most of the reactions had disappeared on the second day. Larger amounts up to 0·5 c.c. of the endotoxin were injected into the skin of two guinea-pigs weighing 250 and 270 g.; the reactions were ill-defined and caused little or no hyperaemia of the cutaneous vessels. This observation suggests a parallelism between the susceptibility of the tissues of the skin and of the brain of the guinea-pig in relation to the weight of the test animal.

(2) Doses of a toluol-killed suspension varying from 5 million to 100 million cocci were injected into the skin of a guinea-pig weighing 600 g. together with 0·1 c.c. of an immune serum from the horse "Minos", an immune serum from the rabbit, and a normal rabbit serum. There was no evidence that the immune sera had modified the reactions in comparison with those which followed the doses that contained normal serum.

Attempt to neutralize the toxic action of a toluol-killed suspension of meningococcus (Type 1) in mice by means of an immune serum

The details are given in Table III, and they show no evidence of any neutralizing action of a sample of serum obtained from a horse, "Endococcus", which had been specially immunized against the endotoxin.

https://doi.org/10.1017/S002217240003480X Published online by Cambridge University Press

Table III. *Attempt to neutralize the toxic action of a toluol-killed suspension of meningococcus (Type 1) by means of an immune serum from the horse "Endococcus"—sample 7. ii. 35. In half the mice the cocci were injected intraperitoneally one hour after an intravenous dose of 0·5 c.c. of the serum*

Intravenous dose of serum c.c.	No. of mice inoculated	Intraperitoneal dose of cocci $\times 10^6$	No. of deaths
—	6	20,000	6
0·5	6	20,000	6
—	6	10,000	6
0·5	6	10,000	6
—	5	5,000	2
0·5	5	5,000	3

V. EXPERIMENTS DESIGNED TO CONTROL THE SPECIFICITY OF THE LETHAL ACTION OF MENINGOCOCCAL PRODUCTS WHEN GIVEN BY THE INTRACEREBRAL ROUTE

The failure to obtain evidence of specific neutralization of the toxic and lethal effects produced by the endotoxin led to a critical examination of the question whether other bacterial products can reproduce the pathogenic effects that follow the intracerebral injection of meningococcal products in the guinea-pig. The results of this part of the work are given in summary form as follows.

The toxicity of various physiological and chemical substances

(1) *Normal saline, serum, and blood products.* Seven light guinea-pigs received normal saline or a mixture of normal saline and rabbit serum; no deaths resulted. Each of three guinea-pigs (mean weight, 192 g.) was inoculated with 0·1 c.c. of normal saline on nine separate occasions as a control to an endotoxin experiment; the mean final weight was 522 g.; there were no deaths. Six heavy guinea-pigs received 0·2 c.c. of horse serum, each sample having been obtained from a different horse; there were no deaths. Forty heavy guinea-pigs were inoculated with 0·1 c.c. of various blood products such as horse serum or defibrinated horse blood or peptic digest of human blood or Hiss's serum water or the condensation water of a Loeffler serum slope; there were no deaths.

These results show that sterile normal saline and sterile blood products are not toxic when they are given intracerebrally to heavy guinea-pigs, and that trauma from the technique of inoculation is not a lethal factor to be taken into account in death from the injection of meningococcal endotoxin into the brain tissues.

(2) *Carbohydrates.* Heavy guinea-pigs, nineteen in all, received 2 or 4 mg. of glucose, lactose, galactose or inositol; 2 mg. of the polysaccharides glycogen, starch and gum arabic, and 0·1 mg. of agar. Of these animals three died, namely, one guinea-pig out of four that had received 2 mg. of glucose; a guinea-pig which had received 4 mg. of inositol in 0·2 c.c. of normal saline; and

https://doi.org/10.1017/S002217240003480X Published online by Cambridge University Press

a guinea-pig which had received 2 mg. of starch. Each of the substances mentioned was injected into a light guinea-pig, ten animals in all, with no lethal effect.

(3) *Organic bases.* The observations under this heading were made in an attempt to throw light on the pathogenesis of the peculiar symptoms that have been described as following the intracerebral injection of meningococcal endotoxin in the guinea-pig. It was thought, for example, that the local liberation of histamine might account for some of the symptoms, but no evidence in support of this was forthcoming. The lethal dose of histamine acid phosphate (B.D.H.) when dissolved in normal saline and given in a volume of 0·1 c.c., was 1 mg. for both light and heavy guinea-pigs; 0·2 mg. was without effect. The symptoms appeared rapidly: the animals ran round about and showed a tendency to fall over; the head was turned to one side; defaecation was an obvious feature; dyspnoea and swallowing movements were noted; nystagmus was observed in the heavy guinea-pig and muscular twitchings in the light guinea-pig. This animal died 5–10 min. after the injection, and the heavy guinea-pig in 15–20 min. The post-mortem appearances did not resemble those which were seen in the endotoxin guinea-pigs, for the outstanding feature was congestion of the blood vessels of the stomach and intestines.

Choline in a dose of 2 mg. proved to be innocuous, a result in conformity with the physiological inertness of this substance.

The effect of an intracerebral injection of acetylcholine was difficult to ascertain owing to its instability, except in an acid solution.

A dose of 0·1 mg. of adrenaline was lethal to both light and heavy guinea-pigs; 0·01 mg. was a non-lethal dose for them. The symptoms noted were dyspnoea and twitchings. The light guinea-pig died in 5 min. and there was blood-stained froth on the muzzle; the heavy guinea-pig died in half an hour. The symptoms thus showed some resemblance to those produced by the endotoxin.

The toxicity of sterile nutrient media and their ingredients when injected intracerebrally into the guinea-pig

In the course of the work it was thought advisable to control the toxicity of the media used for the preparation of the endotoxin. The results are as follows:

Samples of seven routine batches of sterile nutrient broth were tested; one was lethal in a dose of 0·01 c.c., two in a dose of 0·1 c.c., and 0·1 c.c. of four samples did not cause death; heavy guinea-pigs were used. A dose of 0·1 c.c. of the condensation water of sterile agar killed a heavy, but not a light guinea-pig. A sterile broth agar medium was diluted with saline; 0·001 c.c. (0·03 mg.) killed a heavy guinea-pig whereas 0·01 c.c. (0·3 mg.) failed to kill a light guinea-pig.

A dose of 2 mg. of eight out of ten samples of various peptone preparations was lethal to heavy guinea-pigs. Freshly prepared meat infusions of various

https://doi.org/10.1017/S002217240003480X Published online by Cambridge University Press

kinds—veal, ox muscle, ox heart, horse muscle and a dried commercial preparation of veal—were tested; one out of five killed a heavy guinea-pig. The same infusions, which were unfiltered, were incubated at 36° C. overnight and then retested, when the same dose of four out of five of the infusions was found to be lethal. Freshly prepared infusions of ox heart, veal, dried veal, beef and horse meat were each divided into three parts: the first was kept in the cold room overnight, the second was filtered through a Seitz disc and kept in the cold room, and the third, an unfiltered portion, was kept at 36° C. overnight. There were no deaths from the injection of the infusions stored in the cold room, whereas four out of five of the incubated samples killed in a dose of 0·1 c.c. The lethal samples were now autoclaved at 130° C. for 10 min., and this treatment was repeated 2 days later but without abolishing their toxicity.

When the endotoxin chiefly used in the neutralization tests was prepared, a number of the flat bottles containing the agar medium were left uninoculated; they were incubated in the hot room for the same length of time as the bottles used for the cultures, and were subsequently treated in exactly the same way as these by the addition of the same amount of saline to each bottle; by further incubation of the contents of the bottles at 36° C. during the period of autolysis of the coccal suspension, and by filtration through a Seitz disc. The control filtrate was then tested in guinea-pigs with satisfactory results, because two heavy guinea-pigs to which 0·2 c.c. was given and two to which 0·1 c.c. was given remained healthy, thus indicating that during the process of preparation toxic substances had not been extracted from the agar by the saline used for making the coccal suspension. The agar used for this batch of endotoxin was diluted in saline and tested in heavy guinea-pigs, with the result that two died from a dose of 0·01 c.c. and one out of two from 0·001 c.c.; the toxicity of an agar medium is evidently due to the broth contained in it.

These results show that nutrient media and their ingredients—meat infusion and peptone—produce lethal effects in the guinea-pig when injected intracerebrally, and they indicate that the toxicity is associated with a thermostable substance derived from contaminating bacteria. Various kinds of peptone as used in bacteriological work are known to contain bacteria, a natural consequence of the method of digestion of meat, fibrin or other substances employed in their manufacture. The greater sensitiveness of the heavy guinea-pig as compared with the younger animal is again apparent. The symptoms and post-mortem appearances which followed the intracerebral injection of these substances were indistinguishable from those produced by meningococcal endotoxin.

The toxicity of Gram-negative cocci of human origin when injected intracerebrally into the guinea-pig

(1) N. gonorrhoeae *and* N. catarrhalis. Toluol-killed suspensions in normal saline were prepared from a meningococcus strain "Robinson" (Type 1); a culture of *N. gonorrhoeae*; and a culture of *N. catarrhalis*. Each suspension

https://doi.org/10.1017/S002217240003480X Published online by Cambridge University Press

contained about 13,000 million cocci per c.c. and the reaction in each case was adjusted to *p*H 6·8. Heavy guinea-pigs were inoculated intracerebrally with doses of 0·1, 0·01, 0·002 and 0·001 c.c. of each suspension. The fatal doses of the meningococcal and the gonococcal preparations were 0·1 and 0·01 c.c.; and the suspension of *N. catarrhalis* proved fatal in doses of 0·1, 0·01 and 0·002 c.c. The usual symptoms and post-mortem signs were observed in every instance.

(2) N. flavescens *and strains isolated from the human throat.* These strains were obtained from the National Collection of Type Cultures and were designated: (*a*) *N. flavescens* 3656, (*b*) *N. flavescens* 3191, (*c*) *N.* "Fleming" 4590, and (*d*) *N.* "Fleming" Sp. 4. *N. flavescens* is a Gram-negative coccus which was isolated by Miss Branham (1930) from the spinal fluid of patients who were suffering from cerebrospinal meningitis during an outbreak of cerebrospinal fever in 1928 in Chicago. Suspensions of (*a*) and (*b*) were prepared in horse-meat infusion broth, and of (*c*) and (*d*) in 1 per cent normal saline. Endotoxins were obtained from each of the suspensions by allowing them to autolyse at 36° C. for 4 days, and then filtering them through a Seitz disc. A light and a heavy guinea-pig were inoculated with each of the preparations and approximately equal doses were given, as estimated by the opacity of the suspensions. The results need not be fully detailed, but again the suspensions and the endotoxins killed all the heavy guinea-pigs. Two of the four light guinea-pigs inoculated with the suspensions and two of the four to which endotoxin was given died. An estimate of the relative toxicity of the suspensions and of the endotoxins was not attempted.

Conclusion to be drawn from the above experiments.

These experiments show that the toxic action of meningococcal strains and extracts derived from them on the cerebral tissues is shared by a variety of Gram-negative cocci of human origin and that, therefore, it is not *specific* in the sense in which this term is applied to diphtheria toxin, tetanus toxin and the toxin of *B. dysenteriae* (Shiga). This conclusion receives support from the work of Belkina *et al.* (1933) on the experimental production of meningitis in the rabbit with meningococcal products, for they found that the reaction of the cerebrospinal nervous system to the suboccipital introduction of meningococci or other pathogenic microbes and their toxic products is essentially the same in its clinical and histological manifestations. In the view of these workers the pathological features of an experimental meningitis caused by the meningococcus are attributable in an important degree to the action of the toxin.

VI. The histological appearances of the lesions caused by the intracerebral injection of meningococcal products and of control materials

A précis of typical results abstracted from the histological findings is given below.

https://doi.org/10.1017/S002217240003480X Published online by Cambridge University Press

Meningococcus endotoxin

Guinea-pig no. 1. Weight 600 g. Dose 0·1 c.c. Death in about 4 hours.

Brain. Marked polymorphonuclear invasion of the meninges, of the perivascular sheaths of vessels penetrating the cortex and of the superficial cortical tissue. Similar appearances in the vessels and nervous tissue beneath the ependyma of the ventricles, in the choroid plexuses, and also, together with some haemorrhage, in the vicinity of the needle track.

Adrenal. Areas of cortical congestion: there is pronounced polymorphonuclear exudate in the cortex, and more particularly in its outer half.

Lung. Areas of congestion and haemorrhage.

Toluol-killed meningococcal suspensions

Guinea-pig no. 23. Male: weight 560 g. Dose of 10×10^6 cocci of strain "Robinson" in 0·1 c.c. Death in 9 hours.

Brain. Intense polymorphonuclear infiltration at the site of injection and in the contiguous brain tissue, the perivascular spaces, the meninges, choroid plexus and ventricular walls. Distant parts of the cortex and meninges are free from change.

Adrenal. Marked excess of polymorphonuclear leucocytes in parts of the cortex.

Control materials

Saline, horse serum and broth. Dr E. Weston Hurst kindly made the following observations on my behalf.

Guinea-pig no. 4. Dose of 0·1 c.c. of saline freshly prepared from glass-distilled water. No symptoms. Killed by etherization after 24 hours. No macroscopic abnormality. A few necrotic nerve cells at site of inoculation. Scarcely any polymorphonuclear infiltration. Sections of other organs normal.

Guinea-pig no. 7. Dose of 0·1 c.c. of horse serum. No symptoms observed. Killed after 24 hours by etherization. No macroscopic abnormality. Changes at site of injection in brain are not more pronounced than with saline. No obvious abnormality in viscera.

Guinea-pig no. 9. Dose of 0·1 c.c. undiluted broth. No symptoms observed. Killed after 24 hours by etherization. No macroscopic abnormality.

Brain. Nerve cell necrosis at site of injection, moderate polymorphonuclear invasion here and in meninges. Lesions are certainly less pronounced than in animal no. 1. Other organs normal.

Guinea-pig no. 10. Received 0·1 c.c. of undiluted broth as prepared for making meningococcus exotoxin. After 4 and 6 hours muscular twitchings were noted. There were no symptoms 24 hours after the injection, when the animal was killed. No congestion of adrenals.

Brain. Similar, but milder changes to those found in guinea-pigs nos. 1 and 2.

Meningococcus exotoxin

The intracerebral lethal dose of this toxin was 0·1 c.c. for a light guinea-pig and 0·0017 c.c. for a heavy guinea-pig.

Guinea-pig no. 2. Weight 260 g. Dose 0·1 c.c. Death in 7 hours. The symptoms and the post-mortem appearances were typical.

Brain. Condition as in guinea-pig no. 1, which received 0·1 c.c. of endotoxin. The lesions due to the exotoxin were more marked in the deeper tissues and less marked in most parts of the meninges than the corresponding appearances in guinea-pig no. 1, but this was probably due to slight differences in the sites of injection or in the amount of the inoculum that regurgitated into the meninges.

Adrenal. Less definitely congested than in guinea-pig no. 1 and with no polymorphonuclear infiltration.

Lung. Areas of congestion, haemorrhage and oedema.

https://doi.org/10.1017/S002217240003480X Published online by Cambridge University Press

Suspensions of Gram-negative cocci

Suspensions containing 100×10^6 cocci per c.c. were prepared from two strains of gonococcus, *N. flavescens*, *N. catarrhalis*, a Gram-negative coccus isolated from the air, and, to provide control material, the meningococcus strain "Robinson"; 0·1 c.c. of each was injected intracerebrally into a guinea-pig (weight 500–550 g.). As soon as possible after death the brain and adrenal were removed and preserved for sections.

The results can be briefly stated because the appearances in the brain were virtually identical in all the animals and are those which have already been described for guinea-pig no. 23, the control to this series. The adrenal showed either some degree of congestion or it was normal, but, as already stated, there was an excess of polymorphonuclear leucocytes in parts of the cortex in guinea-pig no. 23.

Summary of histological findings

The examination of the brain of guinea-pigs that had received intracerebral doses of meningococcal products and of control materials is consistent with the account that has been given of the relative toxicity of these substances for the cerebral tissues. Thus sterile saline and serum are non-toxic, and produce a negligible histological reaction with scarcely any polymorphonuclear exudate at the site of inoculation (guinea-pigs nos. 4 and 7). Sterile broth occasionally contains a toxic element probably of bacterial origin, and produces microscopic changes in the brain that are similar to but less pronounced than those found in animals that receive an injection of meningococcal endotoxin or exotoxin or a suspension of the coccal bodies (guinea-pigs nos. 9 and 10). Meningococcal products are highly toxic and give rise to a severe reaction of the polymorphonuclear type, but this is also seen in animals that receive intracerebrally a suspension of Gram-negative cocci from various sources. These considerations support the view that the toxic action of meningococcal products on the cerebral tissues of the guinea-pig is not specific in the strict sense of the term.

VII. Discussion

The evidence given in the preceding pages indicates that the endotoxin of the meningococcus is an intracellular poison which, if we may judge from the results of its injection intracerebrally into the guinea-pig, represents one of a group of thermostable poisons that are present in various bacterial species. When the guinea-pig, the rabbit and the horse were treated with repeated doses of endotoxin preparations in the form of meningococcal suspensions or extracts, tests for a resultant immunity failed to give any evidence of tolerance to the pathogenic action of the toxin. Moreover, the sera obtained from the treated animals did not confer immunity against the endotoxin when serum-toxin mixtures were injected intracerebrally or intracutaneously into the guinea-pig. In contrast to these results comparable experiments with three neurotoxins, namely, diphtheria toxin, tetanus toxin and scorpion venom, showed that there is no difficulty in neutralizing the pathogenic action of 20–100 intracerebral lethal doses of these neurotoxins when specific serum-toxin

https://doi.org/10.1017/S002217240003480X Published online by Cambridge University Press

mixtures are injected directly into the brain of the guinea-pig. Thus the conclusion may be drawn that the meningococcal endotoxin is not antigenic.

When the meningococcal poison is administered intracerebrally to the guinea-pig, and especially to the adult animal (over 400 g.), it is lethal in smaller amounts than when it is given by the more commonly employed routes of injection. This difference may conceivably be due to the protection from bacterial assaults which is afforded to the central nervous system by its bony casing and the consequent lack of opportunities for its tissues acquiring an immunity. The cerebral tissues of the mouse, rat, rabbit and guinea-pig, differ in their sensitiveness to the poison: the greater susceptibility of the adult guinea-pig as compared with the young animal is not easily explained. The epileptiform symptoms in the guinea-pig are so characteristic as to suggest that this type of experiment might be useful in the study of traumatic epilepsy and particularly in determining the relative importance of trauma and of infection in the pathogenesis of this condition.

The term "endotoxin" cannot be regarded as an apt one when the meningococcus is under discussion, for the reason that it should be applied only to such antigenic substances as, for example, dysentery endotoxin (Shiga). Nevertheless, the importance of the poison as a pathogenic agent is not lessened by the circumstance that it is incapable of functioning as an antigen since the characteristic lesions of cerebrospinal fever are probably attributable to it; these include the widespread inflammation of the meninges, accompanied by an abundant polymorphonuclear exudate, with a tendency to rapid disappearance of the lesions when the coccal invasion is checked. The experimental data given in the previous pages show that the poison contained in meningococcal suspensions and extracts produces pyogenic and necrotizing effects in the guinea-pig, rabbit and horse.

The failure of the poison to take part in specific antibody reactions adds to the difficulty of defining its nature. There is, however, evidence that it lacks the characters of the classical toxins such as diphtheria and tetanus toxin. Gordon *et al.* have found that the meningococcus endotoxin is remarkably heat-stable, and that it is resistant to drastic methods of chemical treatment. In 1932 Dr Douglas McClean and the present writer made observations (unpublished) which indicated that the endotoxin possesses a number of characters that distinguish it from the ordinary bacterial toxins. Thus the treatment of coccal suspensions in various ways showed that the endotoxin is not destroyed when they are submitted to a temperature of 100° C. for half an hour, or to the action of 0·5 per cent formalin at 37° C. for 1 month or to the action of an equal volume of alcohol, ether, chloroform, or acetone for 1 week at 37° C. Moreover, the endotoxin is not readily oxidizable, for hydrogen peroxide has no action on it; and it resists the enzymic action of trypsin and also of diastase. In dialysis experiments the endotoxin does not pass through a cellophane membrane. There is a heavy loss in toxicity apparently owing to an adsorption effect, when a centrifuged supernatant fluid which contains it is passed through the

https://doi.org/10.1017/S002217240003480X Published online by Cambridge University Press

ordinary filtering materials, the loss being roughly proportional to the closeness of the filter; this is one of the chief difficulties in obtaining a concentrated solution of the toxin.

Reference may be made here to the specific polysaccharide of the meningococcus. Some of the properties of the "endotoxin" outlined above are consistent with the theory that it is a polysaccharide substance. It is unfortunate that, in the present work, so little of it was available for examination, because the experiments carried out on light-weight guinea-pigs suggested that it will prove to be more toxic to the adult guinea-pig than the non-bacterial polysaccharides that were tested later as control substances. Comparative experiments with purified polysaccharides derived from other bacteria may throw light upon the chemical nature of the meningococcal poison.

The question whether the endotoxin of the meningococcus is antigenic or not has an important bearing on the mode of action of antimeningococcus serum as a therapeutic agent in the treatment of cerebrospinal fever in man. If it is conceded that an anti-endotoxin is not present in therapeutic sera prepared from the horse, we may suppose that the efficacy of the specific treatment is attributable to an antibacterial mechanism. This view is consistent with the results of work on the virulence of the meningococcus which has been carried out within recent years in the United States. Formerly, in the experience of most investigators, very large intraperitoneal doses of cocci were necessary in order to produce a fatal infection in mice, and consequently it was difficult to dissociate the pathogenic effects due to the endotoxin from those due to the coccal invasion. It now appears that some freshly isolated strains are highly virulent, and that the virulence of other strains is enhanced by incorporating mucin obtained from the gastric mucous membrane of the pig in the dose of the coccal suspension which is injected into the mouse; the lethal dose may contain as few as ten to twenty cocci (Nungester *et al.*, 1936; Miller & Castles, 1936). By this means it has been found possible to arrange protective experiments which demonstrate that immune sera from the horse can neutralize the pathogenic action of many multiples of the lethal dose of living cocci. The function of the mucin is not understood, but it may act by protecting the coccus from the phagocytic cells of the host.

The question arises of the importance of the meningococcal exotoxin as described by Ferry *et al.* (1931).[1] In a communication to the Congress of Microbiology held in London in 1936, Maegraith stated that he had failed to neutralize the poisonous substance in broth filtrates of the meningococcus by "antitoxic" sera prepared at the Serum Department of the Lister Institute, and by sera from other sources. During the course of his work the present writer has had occasion to carry out control tests of this kind, and he has likewise failed to obtain evidence of neutralization of the exotoxin when serum-toxin mixtures are injected intracerebrally into the guinea-pig. Moreover, the symptoms produced by the exotoxin and its lethal action are indistinguishable

[1] *See* also Ferry (1934) and Ferry & Schornack (1934).

https://doi.org/10.1017/S002217240003480X Published online by Cambridge University Press

from those which follow the injection into the brain of the guinea-pig of an endotoxin solution, or indeed any meningococcal preparation. But even if confirmation of the work on the anti-exotoxin should be forthcoming, the present writer would still be disposed to adhere to the opinion that the intracellular poison of the meningococcus plays an important part in the pathology of cerebrospinal fever, and that, therefore, a thorough biochemical investigation should be undertaken of its nature and properties, and of the possibility of its linkage with a protein to form an effective antigen.

SUMMARY

1. The view is advanced that the endotoxin of the meningococcus is one of a group of thermostable intracellular bacterial poisons, and that it is not a specific antigenic toxin.

2. The inability of the "endotoxin" to function as an antigen does not lessen its importance as a pathogenic factor in cerebrospinal fever, since the lesions that are associated with the presence of the meningococcus in the tissues are apparently attributable to the pyogenic action of the intracellular poison.

ACKNOWLEDGEMENT. I am indebted to Dr E. Weston Hurst for valuable help with the histopathological observations.

REFERENCES

ALISSOWA, E. P. & WYGODSCHEKOFF, G. W. (1933). *Z. f. Immunitätsf.* **81**, 143.
BELKINA, A., KRESTOWNIKOWA, W. & LASOWSKY, I. (1933). *Ztrbl. f. Bakt.*, Orig. **128**, 396.
BRANHAM, S. E. (1930). *Pub. Health Rep.* **45**, 845.
BRANHAM, S. E. & LILLIE, R. D. (1932). *Ibid.* **47**, 1683.
—— —— (1933). *J. Bact.* **25**, 90.
ELLIOTT, T. R. & KAYE, H. W. (1917). *Quart. J. Med.* **10**, 361.
FERRY, N. S. (1934). *J. Immunol.* **26**, 133.
FERRY, N. S., NORTON, J. F. & STEELE, A. H. (1931). *J. Immunol.* **21**, 293.
FERRY, N. S. & SCHORNACK, P. J. (1934). *Ibid.* **26**, 143.
FLEXNER, S. (1907). *J. Exp. Med.* **9**, 105.
GORDON, M. H. (1918). *Brit. Med. J.* i, 110.
GORDON, M. H. & collaborators (1920). In "Cerebrospinal Fever", *Med. Research Council. Special Report Series*, No. 50. London.
KRESTOWNIKOWA, W., BELKINA, A., DOSSER, E. & LASOWSKY, I. (1933). *Z. f. Immunitätsf.* **78**, 451.
KRESTOWNIKOWA, W. & RJACHINA, E. M. (1934). *Ibid.* **83**, 164.
MAEGRAITH, B. (1935). *Brit. J. Exp. Path.* **16**, 109.
MALCOLM, W. G. & WHITE, B. (1932). *J. Immunol.* **23**, 291.
MILLER, C. PHILIPP & CASTLES, RUTH (1936). *J. Inf. Dis.* **58**, 263.
MURRAY, E. G. D. (1932). *J. Bact.* **23**, 90.
NUNGESTER, W. J., JOURDONAIS, L. F. & WOLF, A. A. (1936). *J. Inf. Dis.* **59**, 11.
ROBB, GARDNER (1916). *Proc. Roy. Soc. Med.* (Therap. & Pharmacol. Sect.), **9**, 5.
TSCHAIKA, T. (1936). *Ztrbl. f. Bakt.*, Orig. **135**, 492.
ZDRODOWSKI, P. & VORONINE, E. (1932). *Ann. Inst. Pasteur*, **48**, 617.

(*MS. received for publication* 17. x. 1936.—Ed.)

https://doi.org/10.1017/S002217240003480X Published online by Cambridge University Press