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STUDIES IN EXPERIMENTAL TETANUS INFECTION

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CLOSTRIDIUM TETANI is believed to grow in an anaerobic lesion as a result of the development of an area of low oxidation-reduction potential due to necrosis or other factors, and the bacilli are not believed to affect the local lesion significantly (Fildes, 1927, 1929a). There is evidence from both animal experiments (Russell, 1927) and in-vitro culture (Scheibel and Lennert-Petersen, 1958), that the growth cycle of *Cl. tetani* is short—germination, multiplication and resporulation occurring within a period of 3–5 days. In this paper evidence is presented that multiplying tetanus bacilli can influence the extent of the anaerobic lesion, and that growth and toxin production can continue *in vivo* for longer than 4–5 days. These findings are relevant to tetanus prevention in the field, and evidence is given that penicillin can support the prophylactic effect of antitoxin.

MATERIALS AND METHODS

Organisms. Spore suspensions of toxigenic *Cl. tetani* strain T67 and of a non-toxigenic strain (National Collection of Type Cultures 5041) were prepared as described previously (Smith, 1964).

Antitoxin. Pepsin-refined horse serum antitoxin (Wellcome) was used at a concentration of 5000 units per ml and 1500 units per ml, or in appropriate dilutions in normal saline. Antitoxin was injected subcutaneously into the right flank of mice and guinea-pigs.

Antibiotic. The preparation used was a combination of benzathine, procaine and benzyl penicillin G in the proportion 2:1:1 (Penidural All-Purpose). The preparation was injected in an appropriate dilution in distilled water into the left thigh muscle of mice in a volume of 0.05 ml through a finely graduated tuberculin syringe and a needle of 0.45 mm diameter.

Infection of mice. Male albino mice weighing between 16 and 20 g were infected with tetanus by injecting into the muscles of either the right or left thigh 0.2 ml of 2.5 per cent. calcium chloride solution containing 100 LD₅₀ (250 colony-forming units) of tetanus spores. In later experiments 4 per cent. calcium chloride solution was used. Before injection the overlying fur was shaved and the skin treated with tincture of iodine for 2 min. The resulting infection in unprotected mice produced ascending tetanus with signs first noticed as stiffness in the infected leg 16–24 hr after injection. The mice were examined twice daily for 28 days and were killed with chloroform as soon as the tetanus reached a degree judged to be inevitably fatal.

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Mice were bled under ether anaesthesia by exposing and cutting through the axillary vessels and collecting the blood in a Pasteur pipette.

Guinea-pig lesions. Anaerobic lesions were produced in the thigh muscle of albino guinea-pigs weighing between 300 and 500 g by injecting 0.5 ml of 15 per cent. freshly prepared, autoclaved calcium chloride solution to which methylene blue was added to give a concentration of 0.2 per cent. The overlying skin was shaved and treated with tincture of iodine for 2 min. before the injection. At intervals afterwards the guinea-pigs were killed by stunning, and immersed for a minute in 3 per cent. lysol solution; the injected area was immediately exposed with sterile precautions. The extent of the anaerobic area was measured by cutting through the lesion and measuring the dimensions of the region which, white at first, slowly turned blue on exposure to air. Films and cultures were prepared from the cut surfaces; lesions in which a secondary bacterial infection had developed were not considered in the results. In some experiments 1000 colony-forming units of toxigenic tetanus spores were added to the injections, and in others a similar number of non-toxigenic spores.

All guinea-pigs were immunised against tetanus by a single injection of 0.5 ml of a 1 in 10 dilution of adsorbed tetanus toxoid (Wellcome) at least 2 wk before the experiment.

For culture, lesions were excised and ground up in an equal volume of thioglycollate broth with sterile sand and a sterile pestle and mortar. Dilutions were prepared and samples plated on horse blood agar plates prepared with 4 per cent. agar-agar. The plates were incubated anaerobically for 4 days before examination. In order to look for the presence of heat-resistant spores, samples were also cultured after immersion, in a centrifuge tube, in a waterbath at 80°C for 20 min. after which the tube was immediately cooled in running cold water.

Antitoxin titrations. Tetanus antitoxin titrations were made in mice by the method of Glenny and Stevens (1938).

RESULTS

Effect of penicillin in experimental tetanus

Experimental tetanus in mice can be controlled when a large dose of a long-acting penicillin preparation is given within a few hours of infection (Smith, 1964). Table I shows the results obtained when different doses of the penicillin preparation were injected into groups of mice 1 hr after infection. As the dose of penicillin was increased, the time at which signs of tetanus first appeared was progressively delayed until, with a dose containing 1000 units of benzathine penicillin, the mice remained well throughout the 28-day observation period. In mice given the largest non-protective dose of penicillin, containing 750 units of benzathine penicillin, tetanus developed on average 5 days after injection of the spores.

In mice completely protected with penicillin it has previously been found that *Cl. tetani* frequently survives at the infection site, and that it is often possible to reactivate the organisms to produce fatal tetanus by injecting 5 per cent. calcium chloride solution into the area (Smith).

An explanation for these observations is that penicillin prevented the tetanus bacilli from multiplying, provided it remained in the circulation for over 4 days, an explanation that is supported by results previously published (Smith). After 4 days the conditions are presumed to be no longer suitable for multiplication of the bacteria, since tetanus did not develop after 5 days, even though clostridia remained in the lesion. (There is probably an interval of about 1 day between germination of spores and development of tetanus,

because in unprotected mice tetanus appears about 20 hr after the spores are injected.)

Effect of antitoxin in experimental tetanus

Mice were infected with tetanus spores and within 1 hr groups were given an injection of different doses of tetanus antitoxin. The mice were observed for 28 days and their condition was recorded twice daily.

TABLE I

Effect of different doses of penicillin in mice infected with tetanus spores in 2.5 per cent. CaCl₂*

Units benzathine penicillin injected	Proportion of infected mice surviving	Mean number of days after infection when tetanus developed
0	0/12	0.8
100	3/12	2.0
250	4/12	2.8
500	7/12	4.2
750	5/8	5.1
1000	12/12	...

* Penicillin = benzathine 10,000 units, procaine 5000 units and benzyl penicillin G 5000 units.

TABLE II

Effect of different doses of tetanus antitoxin in mice infected with tetanus spores in 2.5 per cent. CaCl₂

Units antitoxin injected	Proportion of infected mice surviving in		Mean number of days after infection when tetanus developed
	expt 1	expt 2	
0	0/4	0/10	0.8
1	0/4	0/10	3.0
5	1/4	1/10	4.8
10	1/4	0/10	6.0
50	1/4	3/10	8.4
100	2/4	7/10	9.6
500	4/4	9/10	...
1000	4/4	10/10	...

Table II, which gives the results of two experiments, shows that as the dose of antitoxin was increased, the time at which signs of tetanus first appeared was progressively delayed until, with a dose of 500 units, 13 out of 14 infected mice remained well throughout the 28-day observation period. Mice given the largest dose of antitoxin failing to give a high level of protection, 100 units, developed tetanus on average at approximately 9–10 days after injection of the spores.

Tetanus organisms could still be demonstrated at the infection site in surviving mice by injecting 5 per cent. calcium chloride solution into the same area. Of 21 surviving mice into which calcium chloride was reinjected on the 28th day after infection, 11 developed fatal tetanus, whereas of 8 surviving mice not so treated all survived for a further 28 days.

An examination was made of the decline of antitoxin in the serum of mice given an injection of different doses of horse tetanus antitoxin. Groups of mice were given a subcutaneous injection of 10, 100 or 1000 units of antitoxin, and at intervals afterwards groups of five were anaesthetised and bled out from

FIGURE.—The serum tetanus antitoxin concentration in mice at intervals after injection of different doses of horse antitoxin.

Each point represents the result on pooled serum from five mice. The arrows indicate the average time at which clinical tetanus appeared in infected mice treated with the same dose of antitoxin. The figures before the first observation give the number of units of antitoxin injected on day 0.

the axillary vessels. The serum from each group of five was pooled and the concentration of tetanus antitoxin in the pools determined by titration. From the results given in the figure it can be seen that at the time when tetanus clinically appeared in mice given 10 and 100 units of antitoxin at the time of infection, the serum tetanus antitoxin concentration (in uninfected mice) was between 0.01 and 0.02 unit per ml. The serum antitoxin in mice given the fully protective dose of antitoxin, 1000 units, had fallen to 0.01 unit per ml at 11 days. It therefore appears that significant toxin release must have ceased by 11 days after injection of the spores.

It is possible to interpret these findings on the assumption that a large dose of antitoxin is needed for protection mainly because it is required to circulate until toxin release ceases. The relation of toxin production to growth of tetanus bacilli *in vivo* does not appear to have been studied, but the cessation of toxin release must imply that growth of the organism has stopped, although the

possibility remains that toxin release continues for some time after growth stops. Since, in mice treated with antitoxin, tetanus did not develop later than 10 days after the start of infection, it is suggested that conditions in the lesion were suitable for growth of *Cl. tetani* for as long as 9 days. This conclusion contrasts with that made from the penicillin experiments where growth could occur only for up to about 4 days after the initiation of infection. According to Fildes (1927, 1929*a* and *b*), the only important requirement for the growth of tetanus bacilli in the tissues is a sufficiently low Eh, and it is therefore possible that multiplication of the bacilli causes the experimental calcium chloride lesion in mice to remain anaerobic for about 9 days instead of the 4-day period observed in the experiments using penicillin to prevent bacterial growth. In

TABLE III

Volume of anaerobic lesions produced in guinea-pigs at various intervals after injection of 0.5 ml of 15 per cent. CaCl₂ solution with and without tetanus spores

Time (days) after injection when lesions examined	Lesions produced in guinea-pigs after injection of					
	15 per cent. CaCl ₂		15 per cent. CaCl ₂ + toxigenic spores		15 per cent. CaCl ₂ + non-toxigenic spores	
	Average volume of lesions (mm ³)	Number of lesions examined	Average volume of lesions (mm ³)	Number of lesions examined	Average volume of lesions (mm ³)	Number of lesions examined
7	308	8	4760	11	5817	4
9	516	11	4034	11	2308	4
14	376	6	4850	11	1081	8
21	402	3	7600	4	...	...

order to study this possibility an examination was made in guinea-pigs of the effect of *Cl. tetani* on the anaerobic lesion produced by calcium chloride.

Effect of Cl. tetani on the anaerobic lesion in guinea-pigs

Calcium chloride solution containing methylene blue, with and without the addition of tetanus spores, was injected intramuscularly into the thigh muscles of guinea-pigs. The resulting lesions were examined at intervals afterwards and the results obtained are shown in table III.

It can be seen that the anaerobic lesions were about ten times larger when toxigenic bacilli were allowed to multiply in them, and that the difference in size of the lesions became even more marked in the 2nd and 3rd wk after injection. When non-toxigenic bacilli were present, the lesions at 7 days were as large as in the case of toxigenic spores, but were appreciably smaller by the 14th day.

Six lesions were examined histologically at 9 days after injection of virulent

spores and calcium chloride, by staining with haematoxylin and eosin and also with Weigert's modification of Gram's stain. Numerous vegetative Gram-positive bacilli could be seen at 9 days together with a proportion—about 10 per cent.—of drumstick forms. On culture of the lesions *Cl. tetani* could readily be recovered, but when the tissue suspension was heated in a waterbath at 80°C for 20 min. (a treatment that had no significant effect on the viable spore count of the original T67 spore suspension) no viable organisms could be recovered. It appears that vegetative tetanus bacilli were numerous in 9-day lesions produced in guinea-pigs and that, although spores could be seen microscopically, fully heat-resistant spores were present, if at all, in only very small numbers.

TABLE IV

Effect of penicillin on the protective dose of antitoxin in mice infected with tetanus spores in 2.5 per cent. CaCl₂*

Dose of antitoxin (units)†	0	25	50	100	500	1000
Proportion of infected mice surviving when given no penicillin	0/10	3/10	2/10	5/10	3/10	8/10
Proportion of infected mice surviving when given penicillin†	0/10	8/10	9/9	10/10	10/10	10/10

* Penicillin = benzathine 10,000 units, procaine 5000 units and benzyl penicillin G 5000 units.

† Both antitoxin and penicillin were given 15 hr after injection of the spores.

Effect of penicillin on the protective dose of antitoxin

It would be expected, if the above findings are correct, that penicillin would be capable of reducing the dose of antitoxin required for protection. By preventing multiplication of *Cl. tetani*, penicillin should prevent the consequent prolongation of the anaerobic lesions so that antitoxin would be required in the circulation for a shorter time. This possibility was tested by measuring the amount of antitoxin required to protect mice given penicillin 15 hr after injection of the spores; at this time after infection penicillin alone has no protective effect (Smith).

A group of 120 mice were infected with tetanus spores in calcium chloride and 15 hr later groups of 10 were given a subcutaneous injection of a range of doses of antitoxin. Half the mice were then given 10,000 units of benzathine penicillin (together with 5000 units both of procaine and benzyl penicillin). The mice were observed for 28 days and the results obtained are given in table IV. It can be seen that in mice given penicillin the protective dose of antitoxin was 50 units, whereas in mice not given penicillin 1000 units failed to give complete protection. These findings support the previous evidence that toxin release can occur for a prolonged period when growth of *Cl. tetani* is allowed to

continue, but they could also be explained on the assumption that the penicillin, by stopping multiplication of the bacteria, merely reduced the amount of toxin formed during a short growth cycle of 4-5 days. An attempt was made to resolve this by testing the effect on the protective dose of antitoxin of giving penicillin on the 4th day after the initiation of infection. If penicillin could still influence the protective dose of antitoxin it would suggest that continued growth of the organism after 4 days was an important factor. From the results shown in table V it can be seen that antibiotic given on the 4th day still reduced the amount of antitoxin required to protect.

TABLE V

Effect of penicillin on the protective dose of antitoxin in mice infected with tetanus spores in 4.0 per cent. CaCl₂*

Dose of antitoxin (units) given 15 hr after infection	0	10	50	100	500	1000
Proportion of infected mice surviving when given no penicillin	1/10	0/10	7/10	8/10	6/10	6/10
Proportion of infected mice surviving when given penicillin* at 15 hr after infection	0/10	9/10	10/10	10/10	10/10	10/10
Proportion of infected mice surviving when given penicillin* at 4 days after infection	0/10	5/10	8/10	10/10	9/9	10/10

* Penicillin = benzathine 10,000 units, procaine 5000 units and benzyl penicillin G 5000 units.

DISCUSSION

The mechanism by which tetanus bacilli are able to enlarge the anaerobic lesion caused by calcium chloride is not known. It is unlikely that neurotoxin is responsible, because it has not been found to have any effect on tissue culture cells (Penso and Vicari, 1957). Furthermore, an appreciable enlargement of the lesion was also caused by non-neurotoxigenic tetanus bacilli, so that if the neurotoxin is involved, it cannot be the only factor. *Cl. tetani* also produces a haemolysin, which has some cell toxicity and has been held responsible for the slight haemorrhage sometimes observed around tetanus lesions (Fildes, 1929*b*). The non-neurotoxigenic strain used in the present experiments did not produce tetanolysin, which suggests that the haemolysin also cannot be the only factor involved.

The extent of the anaerobic lesion might be influenced by the further lowering of the Eh that multiplying tetanus bacilli produce (Fildes, 1929*a*). Thus, it has been suggested that the fatal peritoneal exudation that develops when

Clostridium welchii is allowed to grow in the peritoneal cavity, is a consequence of the reduction in Eh that is produced by the multiplying clostridia (Bullen, Cushnie and Stoner, 1966). However, it might be expected that growth of the non-toxigenic strain would lower the Eh as much as the toxigenic *Cl. tetani*, whereas the non-toxigenic bacilli produced much less effect on the lesion than the toxigenic strain. Moreover, cells in tissue culture are said to be unaffected by anaerobic conditions (Harris, 1956).

The possibility should also be considered that in the immunised guinea-pigs the lesions were made worse as a result of the interaction between circulating antitoxin and locally produced toxin. However, this explanation is unlikely, since the findings in guinea-pigs seem to reflect the observations in mice, which were not immunised against tetanus.

Our findings on the culture and histological examination of 9-day lesions differ from those of Russell (1927), in that no evidence was obtained that re-sporulation was completed in about 4 days. It seems clear that vegetative growth can continue for longer periods and it must be presumed that onset of sporulation is variable—perhaps dependent on the extent of the anaerobic area and the supply of nutrients.

The experimental finding that penicillin reduced the amount of antitoxin required to protect infected mice suggests that this antibiotic can support the prophylactic effect of tetanus antitoxin in man. A combination of antitoxin and antimicrobial substances has also been found to have a better protective effect in experimental gas-gangrene than either alone (see McIntosh and Selbie, 1943). The explanation for the findings reported here may be that, by inhibiting bacterial multiplication, penicillin prevented the prolongation of the anaerobic conditions that multiplying tetanus bacilli might otherwise have caused. They could also be accounted for by a reduction in the amount of toxin produced in the early stages of infection. However, the finding that penicillin started as late as the 4th day after injection of the spores still reduced the amount of antitoxin needed for protection indicates that toxin formation can continue for more than 4 days, and suggests that the indirect effect of penicillin on the lesion may be important. In either case the findings show that an antibiotic can have a valuable effect in animals given antitoxin, and it might be expected that prophylaxis with both antitoxin and antibiotic could be of value in man—particularly in patients with very severe contaminated wounds and in those who have had heterologous antitoxin previously and may therefore eliminate subsequent injections from the body unexpectedly quickly.

SUMMARY

Evidence is given that for penicillin to prevent experimental tetanus in mice a 4-day period of treatment is satisfactory. To prevent tetanus by passive immunisation the antitoxin must remain in the circulation for 9 days. In either case *Cl. tetani* frequently survives at the injection site. To account for these observations it is suggested that when tetanus bacilli grow in an anaerobic lesion, the local conditions are altered so that the time for which the lesion

remains suitable for multiplication of anaerobic bacteria is prolonged. This conclusion is supported by the following findings.

(a) The extent of the anaerobic lesion produced by intramuscular injection of calcium chloride in guinea-pigs is increased about 10-fold when tetanus spores are also injected.

(b) Culture and histological examination of lesions in guinea-pigs at 9 days after injection of spores in calcium chloride showed the presence of numerous vegetative organisms but very few spores.

(c) A long-acting penicillin preparation was able to reduce the dose of anti-toxin required to protect infected mice when the antibiotic was given either at 15 hr or 4 days after infection.

It is suggested that antibiotic may be of value in supporting the effect of antitoxin in prophylaxis in man, particularly in patients who may rapidly eliminate the heterologous horse serum, because it may reduce the time period for which the passive immunity is required.

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