THE PREVENTION OF EXPERIMENTAL *CLOSTRIDIUM NOVI* AND *CL. PERFRINGENS* GAS GANGRENE IN HIGH-VELOCITY MISSILE WOUNDS BY ACTIVE IMMUNISATION

N. A. BOYD*, R. O. THOMSON AND P. D. WALKER

*Tidworth Military Hospital, Tidworth, Hampshire, and Wellcome
Research Laboratories, Langley Court, Beckenham, Kent*

SEVERAL vaccines have been developed against the gas-gangrene group of organisms, and their use in animals and man is well documented. In most cases the immune response obtained with these vaccines has been determined in terms of the level of circulating antitoxin, but there are a number of reports in which a challenge by toxin or virulent culture has been used to measure protection. In general, it has not been found possible to correlate the level of circulating antitoxin or the protection against artificial challenge with the degree of protection afforded against natural challenge.

Penfold, Tolkhurst and Wilson (1941) investigated the responses of guinea-pigs and man to injections of *Clostridium perfringens* fluid toxoid and alum-precipitated toxoid; these workers showed that although fluid toxoid in guinea-pigs was a poor antigen, it was very much improved by the addition of alum. A single injection of the alum-precipitated vaccine produced circulating antitoxin in man that was still demonstrable 5 mth later. When immune sera taken from these subjects 2 wk after immunisation were injected into mice, the mice were protected against an intraperitoneal challenge with *Cl. perfringens* culture.

Tytell *et al.* (1947) investigated the antigenicity of alum-precipitated *Cl. perfringens* and *Cl. novyi* culture products in guinea-pigs and man; they used a toxin challenge for the animals and measured antitoxin titres in the human subjects. They showed that the response to three doses of vaccine was greatly improved if the third dose was delayed up to 40 wk. In a study of the value of small doses of concentrated toxoids as vaccines, Robertson and Keppie (1943) used as a method of challenge the injection of *Cl. novyi* or *Cl. septicum* spores or a fresh culture of *Cl. perfringens* with 5 per cent. calcium chloride into the muscle of guinea-pigs. Two injections of toxoid were given 3 wk apart, and the animals were challenged 8–20 wk later. There were four deaths in a group of 61 immunised animals, compared with 48 deaths in 51 controls.

Satisfactory vaccines have also been produced against *Cl. perfringens* type A by Stewart (1942), and Saltykov, Rozepov and Zemskov (1945), and against *Cl. novyi* by Shneerson (1957).

In addition to their association with gas gangrene, the clostridia are responsible for a number of unrelated diseases in sheep, cattle and other animals. Much experimental work has been done in developing satisfactory vaccines against these diseases (Sterne *et al.*, 1962; Thomson and Batty, 1967; Thomson *et al.*, 1969). Since extracellular toxins play a major role in the pathology of these diseases, vaccines containing single and multiple components have been developed which, on injection, produced adequately sustained levels of circulating antitoxin to prevent outbreaks of disease occurring in the field. It is possible that the same type of vaccine may be of value in the prophylaxis of gas gangrene.

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* Present address: Royal Herbert Hospital, Woolwich, London, S.E.18.

In the previous paper (Boyd, Walker and Thomson, 1972), we have considered passive immunisation as a method of protecting sheep against gas gangrene after the contamination of high-velocity missile wounds with spores of *Cl. novyi* type A. In the present paper the efficacy of active immunisation in preventing the disease in the same experimental model is reported, the challenge species being *Cl. novyi* type A and *Cl. perfringens* type A.

MATERIALS AND METHODS

The experimental model. This is described in the previous paper (Boyd *et al.*, 1972).

Experimental vaccine. This was a mixed vaccine consisting of *Cl. novyi* alpha toxoid 3.0 units per ml, *Cl. septicum* alpha toxoid 4.2 units per ml and *Cl. perfringens* type A alpha toxoid 3.0 units per ml. The vaccine contained 2.5 per cent. (w/v) alum as an adjuvant and 0.01 per cent. thiomersalate as preservative.

Immunisation procedure. Sheep were immunised with two 2-ml doses given subcutaneously and spaced 1 mth apart. Blood samples were taken for titration of antitoxin before immunising and at 14 days after the second injection.

Cl. novyi challenge. The sheep were divided into six groups, each comprising animals with high and low titres of *Cl. novyi* alpha antitoxin. The first group was challenged in the left leg 11 wk after vaccination by the standard muscle wound using the spore suspension of *Cl. novyi* type A described previously. Subsequent groups were challenged at 10–12-wk intervals, the last being challenged 1 yr after vaccination. At the time of each challenge, two unprotected sheep were used as controls.

Cl. perfringens challenge. In order to determine the effectiveness of *Cl. perfringens* immunisation, 20 sheep were selected from those that survived the *Cl. novyi* challenge. These were divided into four groups, the first group being challenged with *Cl. perfringens* in the right leg by the standard method 43 wk after vaccination. The remaining groups were challenged at 44, 45 and 56 wk after vaccination. Two unvaccinated sheep were challenged at the same time as each of the four groups and these served as controls. The challenge consisted of a 5-hr growth of *Cl. perfringens* type A strain CN1491 (Wellcome Culture Collection) in Robertson's meat broth containing 0.5 per cent. glucose. These cultures contained no spores and about 3×10^9 organisms per ml (Brown's tube no. 10). One millilitre of culture was placed on a piece of battledress cloth immediately before challenge and formed a damp area of 1–2 cm diameter in the centre of the cloth through which the bullet passed.

Antitoxin determination. Blood samples were taken before and after vaccination, at challenge and 3 wk after challenge. Antitoxin in the serum from these samples was assayed in mice by determining the extent to which it neutralised specific toxin. Titres of less than 0.1 unit per ml for *Cl. novyi* and less than 0.005 unit per ml for *Cl. perfringens* were not determined.

RESULTS

Cl. novyi challenge

The response to the *Cl. novyi* alpha component of the gas-gangrene vaccine is shown in table I. All 37 sheep used had less than 0.1 unit of antitoxin per ml before vaccination and it was therefore likely that they had had no previous experience of the antigen. No sheep had less than 2 units of antitoxin 2 wk after vaccination. In the interval between vaccination and challenge there was a considerable fall in titre, but the concentration rose markedly in those vaccinated sheep that survived the challenge. This indicates the severity of the challenge and the satisfactory state of immunity in the sheep.

There were 12 unvaccinated controls and, of these, 11 died of gas gangrene within 48 hr; one animal died under anaesthesia.

Of the 37 protected sheep, three died of gas gangrene between 3 and 6 days after challenge. The level of protection in these sheep is shown in table II.

TABLE I

Clostridium novyi alpha-antitoxin titres obtained in sheep at various times before and after vaccination with two injections of experimental gas-gangrene vaccine given at an interval of 1 mth and subsequently challenged with *Cl. novyi*

Group	Number of sheep	Interval between last dose of vaccine and challenge* (wk)	Geometric mean of <i>Cl. novyi</i> antitoxin titres in each group (units per ml serum)			
			immediately before vaccination	2 wk after vaccination	at challenge	2 wk after challenge
1	6	11	<0.1	18.8	2.8	119
2	6	22	<0.1	13.1	0.43	708
3	6	28	<0.1	15.3	0.23	383
4	6	38	<0.1	11.3	0.37	134
5	6	46	<0.1	14.7	0.28	585
6	7	55	<0.1	19.3	0.54	468

* See *Methods* section for details.

TABLE II

Antitoxin titres of three vaccinated sheep that died of gas gangrene after being challenged with spores of Cl. novyi type A

Group	Time of death after challenge (days)	<i>Cl. novyi</i> antitoxin titre (units per ml)	
		2 wk after vaccination	at challenge
1	6	10-20	2-5
2	3	2-5	<0.1
6	3	10	0.1-0.2

All three survived longer than the controls and to this extent vaccination gave them some protection. The antitoxin titres of the two from groups 2 and 6 were the lowest in the two groups and well below the geometric means for these groups. On the other hand, the sheep from group 1 that died had a titre comparable with those of vaccinated survivors.

The survival rate within 90 per cent. confidence limits following the *Cl. novyi* challenge was 2.5-8.5 per cent. in the control sheep and 82.5-94.5 per cent. in the vaccinated groups.

Cl. perfringens type A challenge

The *Cl. perfringens* alpha-antitoxin titres of the sheep selected for challenge with this organism are shown in table III. In some sheep there were significant titres before vaccination and this indicates previous experience of the antigen. Antitoxin was not measured immediately after vaccination, but the titres observed in all groups at the time of challenge suggested that vaccination had resulted in a high level of immunity. A marked increase in the level of circulating antitoxin occurred after challenge.

TABLE III

The Cl. perfringens alpha-antitoxin titres of sheep before vaccination, at the time of challenge and 3 wk after challenge

Group	Number of sheep	Interval between last dose of vaccine and challenge (wk)	Geometric mean of <i>Cl. perfringens</i> titres (units per ml serum)		
			immediately before vaccination	at challenge	3 wk after challenge
1	4	43	0.005	0.38	8.7
2	4	44	0.006	0.16	12.6
3	4	45	0.006	0.03	3.7
4	8	56	0.014	0.13	9.2

Two of the unvaccinated controls from groups 2 and 3 survived and the remaining six died of gas gangrene within 4 days. Of the 20 immunised animals challenged in this series, only one died of gas gangrene. This was a group-4 animal with a pre-challenge titre of 0.01–0.02 unit per ml. The survival rate, within 90 per cent. confidence limits, following the *Cl. perfringens* type A challenge was 12–50 per cent. for the control group and 83–94.5 per cent. for the vaccinated group.

Pathology associated with Cl. novyi and Cl. perfringens challenges

Within a week of wounding, the protected animals all developed a pustular abscess within the adductor group of muscles. The pathology exactly mimicked that seen in those sheep treated with antitoxin alone (Boyd *et al.*, 1972). It was not always possible to identify organisms in the pus, because frequently at necropsy performed 3 wk after challenge, complete lysis of the organisms had occurred. In sheep that were alive many months after challenge, the abscesses had pointed and drained and there remained only a fibrous track.

DISCUSSION

The results show that the experimental gas-gangrene vaccine protected sheep against a massive *Cl. novyi* or *Cl. perfringens* challenge for at least a year after

immunisation. At this time the concentrations of circulating antitoxin were quite low, and in some individuals they fell below the minimum level demonstrable with our tests.

Gas gangrene has a relatively short incubation period and, as has been shown in the previous paper, antitoxin is of little benefit if given more than 21 hr after challenge. Such levels of antitoxin as were present in the actively immunised sheep must, therefore, have been sufficient to contain the disease since it is unlikely that an anamnestic response to toxin produced in the wound could have provided sufficient antitoxin in time to contribute to the control of the disease.

It therefore seems that even antitoxin concentrations of less than 0.1 unit per ml of serum were sufficient to prevent infection and that an effective amount of antitoxin may have been present before the animal was challenged. The fact that antitoxin titres were increased to quite high levels after challenge suggested two things: first, that sufficient toxin was produced by growth of the organism in the wound to provoke a secondary response, and second, that the effective immune response of the animal was directed not against the organism but against the toxin that it produced, otherwise growth and toxin production would not have occurred in the wound. This is confirmed by the fact that *Cl. novyi* and *Cl. perfringens* could be cultured from the wound several weeks after challenge and lends weight to the contention that the necessary type of immunity is antitoxic.

While it is unwise to draw conclusions on the efficiency of a vaccine in man from the results obtained in sheep, the fact that protective levels of circulating antitoxin have been established should encourage attempts to establish the conditions necessary for attaining the same levels in man and maintaining them. It is not unreasonable to assume that the protective level required in man may be comparable to that in sheep. It should be noted that, at least in the case of *Cl. novyi*, the severity of the challenge given to the test sheep was much greater than would be encountered in man even in war. With regard to *Cl. perfringens*, one could imagine circumstances in which contamination was very great, i.e., in wounds grossly contaminated with faeces or manured soil or in wounds involving rupture of the large intestine. There seems, therefore, to be a case for an investigation into the antitoxin responses that clostridial vaccines provoke in man, in order to be able to develop satisfactory gas gangrene vaccines. These might find a place not only in the protection of forces engaged in combat but also in the protection of sections of the civil population from gas gangrene as a complication of road accidents, major disasters or surgery, e.g., the surgery of obliterative arterial disease (Parker, 1969).

SUMMARY

Sheep were immunised with a mixed vaccine containing the toxoids of *Clostridium perfringens*, *Cl. septicum* and *Cl. novyi* and challenged at different intervals after immunisation. The sheep were wounded and challenged in the thigh muscle by a high-velocity bullet that passed through a piece of battledress

impregnated with a suspension of either *Cl. novyi* spores or *Cl. perfringens* culture.

Immunisation was almost completely effective in preventing the onset of gas gangrene due to *Cl. novyi* even 1 yr after immunisation, the survival rate being 82.5–94.5 per cent. within 90 per cent. confidence limits (rate for unvaccinated controls: 2.5–8.5 per cent.). Vaccinated sheep that survived challenge with *Cl. novyi* were later challenged with *Cl. perfringens* and survived this, the rate of survival being 83–94.5 per cent. (rate for unvaccinated controls: 12–50 per cent.). The role that such a vaccine might play in the prevention of gas gangrene in man is discussed.

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