

ANTIBODY RESPONSE TO AND IMMUNITY INDUCED BY *CORYNEBACTERIUM PYOGENES* VACCINE

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ABSTRACT

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A procedure is described for producing a high quality *C. pyogenes* vaccine.

A satisfactory antibody response was obtained by administering 3 subcutaneous injections of vaccine at 10-day intervals, using doses of 2,0 ml for rabbits, 5,0 ml for sheep and 10,0 ml for cattle. The titres resulting from this procedure compared favourably with those obtained from following the more intensive schedule and higher doses of vaccine recommended by the British Veterinary Codex.

The antibody response was at its best 1-2 months after immunization but dropped to a plateau 6 months after immunization.

Mice were also successfully immunized against challenge with $1,5 \times 10^8$ organisms by the application of 3 doses of vaccine administered at 10-day intervals.

Résumé

LA PRODUCTION D'ANTICORPS ET L'IMMUNITÉ SUSCITÉE PAR UN VACCIN À *CORYNEBACTÉRIUM PYOGENES*

Les auteurs exposent une méthode pour la production d'un vaccin efficace contre *C. pyogenes*.

En effectuant 3 injections du vaccin par voie sous-cutanée à intervalle de 10 jours aux doses de 2,0 ml chez le lapin, 5,0 ml chez le mouton et 10,0 ml chez le bovin, ils ont obtenu une production satisfaisante d'anticorps. Les taux d'anticorps ainsi produits sont comparable avec ceux suscités par les vaccins aux protocoles plus intensifiés et aux doses plus fortes préconisées par le British Veterinary Codex.

La production d'anticorps atteint son apogée à 1-2 mois après l'immunisation et puis s'abaisse à un niveau constant à 6 mois après l'immunisation.

Des souris immunisées avec 3 doses du vaccin à intervalle de 10 jours se sont révélées résistantes à une infection provoquée par l'inoculation de $1,5 \times 10^8$ bactéries.

INTRODUCTION

Apart from its significance as a cause of abscesses, *Corynebacterium pyogenes* is frequently responsible for mastitis in cattle (Saes, 1970), and it has been incriminated as a cause of abortion (Hinton, 1974). It is also an important pathogen in sheep, and may be responsible for perinatal lamb mortality (Dennis & Bamford, 1966), as well as acute lameness (Gardner, 1961).

Infections due to *C. pyogenes* in both sheep and cattle are widespread in South Africa and, although normally only individual animals are affected, infection may assume epidemic proportions on certain farms. Usually these are in areas where there is severe thornbush encroachment and heavy tick infestation (Cameron, unpublished observations).

Although control of the infection should primarily be based on sound hygienic and epizootiological principles, the application of a vaccine is a most useful adjuvant in the effort.

Apart from the reports by Weitz (1947), Weitz & Longridge (1947), Lovell, Foggie & Pearson (1950), Derbyshire & Matthews (1963) and Cameron (1966), there is very little information on the immune response to and efficacy of *C. pyogenes* vaccines. Nevertheless, Capper (1953) had laid down potency standards as well as an immunization schedule for cattle. This regimen comprises the intramuscular injection of 10, 15 and 20 ml of vaccine at 3-day intervals. The same regimen and doses are used for potency assays in rabbits.

Since these recommendations prescribe excessively large doses of vaccine and presuppose an impracticable schedule, studies were undertaken to determine

whether comparable results could be obtained with smaller doses and a more practicable immunization procedure.

MATERIALS AND METHODS

Bacterial strain

The strain of *C. pyogenes* used in these studies was originally isolated from a case of bovine mastitis, and designated Strain 23402. It was stored in the lyophilized state, and was used for toxin production, antigen production, vaccine production, as well as for challenge of mice.

Vaccine production

The procedure used for producing adjuvant cell toxoid was based on the findings of Lovell (1937). Modified Wright's broth containing meat particles (Jansen, 1961) was distributed in tubes (25 × 200 mm) and 40 litre flasks. The flasks contained 20 litres medium, and were fitted with a glass sparger for aeration.

Two-day-old cultures of *C. pyogenes* 23402 on blood tryptose agar (BTA)* were used to inoculate the tubes, which were then incubated at 37 °C for 5 days. Each production flask containing 20 litres medium was inoculated with the contents of 1 litre tube. If the growth is poor, 2 tubes/flask or alternatively 1 litre seed flask containing 500 ml medium may be used. The production flasks were incubated at 37 °C for 48 h and aerated by means of the sparger from an air cylinder.

At the termination of the growth period, the supernatant fluid was carefully siphoned off into holding flasks and samples were collected for toxin assay and determination of the packed cell volume. Toxin values of 160-320 haemolytic units/ml and 0,4% packed cells were routinely obtained by this method. The residual meat particles were discarded.

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Inactivation and toxoiding were accomplished by adding 0.5% formalin to the supernatant culture and maintaining the holding flasks at 37 °C for 4 days. Samples were again taken for toxin titration, and sterility of the culture was verified by preparing BTA cultures. Thereafter, the inactivated culture was precipitated by the addition of 100 ml/litre of an 11% solution of potassium-alum. Final sterility tests were done in thioglycollate and liquid Sabouraud's agar.

The final vaccine was stored in 100 ml volumes in sealed bottles at 4 °C.

For comparative purposes vaccine was also produced according to the method advocated by Levy (1961).

Toxin production

Haemolytic toxin for antitoxin titration was prepared by growing *C. pyogenes* Strain 23402 in modified Wright's broth tubes for 48 h at 37 °C. The culture fluid from 20 tubes was pooled and centrifuged to remove the bacteria. The supernatant fluid was then lyophilized in 5.0 ml aliquots *in vacuo* and stored at 4 °C. The toxin was titrated by the method described by Capper (1953).

Antigen production

Antigen for agglutination tests was produced in a liver meat broth (Mason & Scheuber, 1936) as follows: *C. pyogenes* was grown for 48 h in tubes of modified Roux flasks containing 200 ml of liver meat broth and then aerated by shaking at 37 °C for 18 h. The purity of the cultures was determined by examination of Gram's stained smears and the cells collected by centrifugation. After they had been washed once in saline, the cells were resuspended in 0.5% phenol-saline and the density adjusted by means of a nephelometer to correspond with that of standard *Brucella abortus* antigen. Only those batches of antigen which gave a good yield and did not agglutinate spontaneously were used for agglutination tests.

Experimental animals

Rabbits: The New Zealand type rabbits used in this study were 4-6 months old. They were reared at the Institute, housed in wire cages and fed a balanced pelleted ration.

Sheep: Cross-bred Dorper-Merino ewes, approximately 12 months old, were used to study the antibody response in sheep. They were obtained from the farm of the Institute and maintained on natural veld.

Cattle: Afrikaner-Simmenthaler cross-bred heifers, 4-6 months old, were used for the experiments in cattle. They were maintained extensively on the farm of the Institute.

Mice: Conventionally-reared male albino mice from the colony maintained at the Institute were used. They were fed a balanced pelleted ration and housed in plastic cages. Four-week-old males were used for the immunity experiments, and males, 9-10 weeks old, for the titration of the challenge material.

Immunization of animals

Rabbits: The first experiment was designed to compare the antibody response following the administration of *C. pyogenes* vaccine at various doses and regimens. Four groups of 8 rabbits were given

C. pyogenes vaccine as follows: Group A, 3 subcutaneous injections, comprising 5, 10 and 15 ml of vaccine, respectively, according to the schedule recommended by Capper (1953); Group B, 2 subcutaneous injections of 5 ml each with an interval of 21 days; Group C, 2 subcutaneous injections of 2 ml each with an interval of 21 days, while Group D served as non-immunized controls. All the rabbits were bled 14 days after the last injection, and the agglutination and antitoxin titres of the sera assayed.

The 2nd experiment was designed to compare the antibody response following the administration of 2 or 3 injections of either 2 or 5 ml of vaccine. Groups of 8 rabbits were given vaccine as follows: Group A, 3 injections of 5 ml each at 10-day intervals; Group B, 2 injections of 5 ml each at 10-day intervals; Group C, 3 injections of 2 ml each at 10-day intervals; Group D, 2 injections of 2 ml each with an interval of 21 days, while group E served as non-immunized controls.

All the immunized rabbits, as well as the controls, were bled 14 days after the last injection of vaccine and the agglutination and antitoxin titres of their sera determined.

Sheep: Four groups of 8 sheep were used. Group A received 3 subcutaneous injections of 5, 10 and 15 ml of vaccine, according to the schedule recommended by the British Veterinary Codex (Capper, 1953); Group B was given 2 injections of 5 ml each with an interval of 21 days; Group C was given 3 injections of 5 ml each at 10-day intervals and Group D served as non-immunized controls.

All the sheep were bled 1 month and 2 months after the last injection. Thereafter, only Group C was bled at monthly intervals for a total period of 12 months.

The sera were stored at -20 °C until the agglutination and antitoxin titres could be determined.

All the sera collected during the first 2 months of the experiment were tested simultaneously, but, since this was not possible with all the other sera, they were divided into 3 lots. The sera of either 2 or 3 individual animals collected over the total period of 12 months were therefore tested simultaneously. This precaution was taken in order to preclude any inaccuracies which might occur owing to the variation in technique or in the sensitivity of different batches of antigen.

Cattle: The same procedure for immunization and testing which was used for sheep was applied to cattle, except that the dosages of the vaccines were different. Group A received 3 injections of 10, 15 and 20 ml, respectively; Group B received 2 injections of 10 ml and Group C received 3 injections of 10 ml.

Mice: In order to assay the protective value of *C. pyogenes* vaccine, 2 groups of 20 mice were immunized with 2 different batches of vaccine. Each mouse was given 3 subcutaneous injections of 0.2 ml at 10-day intervals. Ten mice of each group were challenged 14 days after the last injection of vaccine by the intraperitoneal injection of approximately 1.5×10^8 viable organisms (see below). The remaining 10 mice of each group were similarly challenged 1 week later with approximately 2.0×10^8 organisms. In both instances, 10 non-immunized control mice were challenged simultaneously and deaths were recorded daily for 14 and 7 days, respectively.

Serological procedures

Agglutination tests: Twofold serial dilutions in saline of the sera were made in 1.0 ml volumes from 1:5 to 1:5 120. One ml of antigen was added to each tube and

the tubes incubated overnight at 37 °C. The end point of the titration was taken as the highest serum dilution, showing approximately 75% agglutination. The titres were expressed as the geometric mean of the reciprocal of the dilution.

Antitoxin assays: The antitoxin content of the rabbit, sheep and cattle sera was assayed by the method described by Capper (1953), and the titres of the sera were expressed as the geometric mean in units/ml.

Preparation and standardization of challenge material

Live bacteria for challenge purposes were produced by the method described for the preparation of antigen. The yield of bacteria obtained from 1 200 ml of culture was resuspended in 600 ml of a buffered lactose-peptone solution (BLP) and lyophilized in 1,0 ml aliquots *in vacuo*. Triplicate plate counts were made in order to determine the number of viable organisms per vial. Appropriate dilutions were prepared and groups of 10 9-10-week-old mice were infected by intraperitoneal injection of 0,5 ml volumes to give exposure levels of 6×10^8 ; 3×10^8 ; $1,5 \times 10^8$; 6×10^7 and $1,2 \times 10^7$ per mouse. Based on the outcome of these titrations, challenge doses of approximately $2,0 \times 10^8$ and $1,5 \times 10^8$ per mouse were used for infecting immunized mice (see Results).

RESULTS

Vaccine production

The procedure which we employed gave a consistent toxin value of 160-320 haemolytic units/ml and 0,4% packed cells. In our hands the toxin titres obtained were superior to those obtained by Levy (1961) in a milk medium. Using her procedure, an average titre

of only 23,6 units/ml could be obtained in a series comprising 14 batches of vaccine (Smit, unpublished data).

Titration of challenge material

All the mice given $\pm 6 \times 10^8$ organisms died within 2 days while none of those given $\pm 1,2 \times 10^7$ organisms died. The cumulative deaths of the other groups are shown in Fig. 1.

The mice which received 6×10^8 and 3×10^8 bacteria died too rapidly, while dosages of 6×10^7 and $1,2 \times 10^7$ did not establish an infection. A dosage of $1,5 \times 10^8$ on the other hand gave rise to a chronic infection. Based on these findings, dosages of approximately $2,0 \times 10^8$ and $1,5 \times 10^8$ were used to challenge immunized mice (see below).

Antibody response in rabbits

Table 2 demonstrates that lower doses and fewer injections of vaccine did not give as high an antibody response as the regimen recommended by the British Veterinary Codex (Capper, 1953).

TABLE 1 Antibody titres in rabbits given *C. pyogenes* vaccine by different schedules

Group	Immunization schedule	Mean agglutination titre	Mean antitoxin titre (Units/ml)
A.....	5, 10 and 15 ml	4 063	3 609
B.....	2 x 5 ml	2 826	2 320
C.....	2 x 2 ml	987	2 347
D.....	Controls	20	0

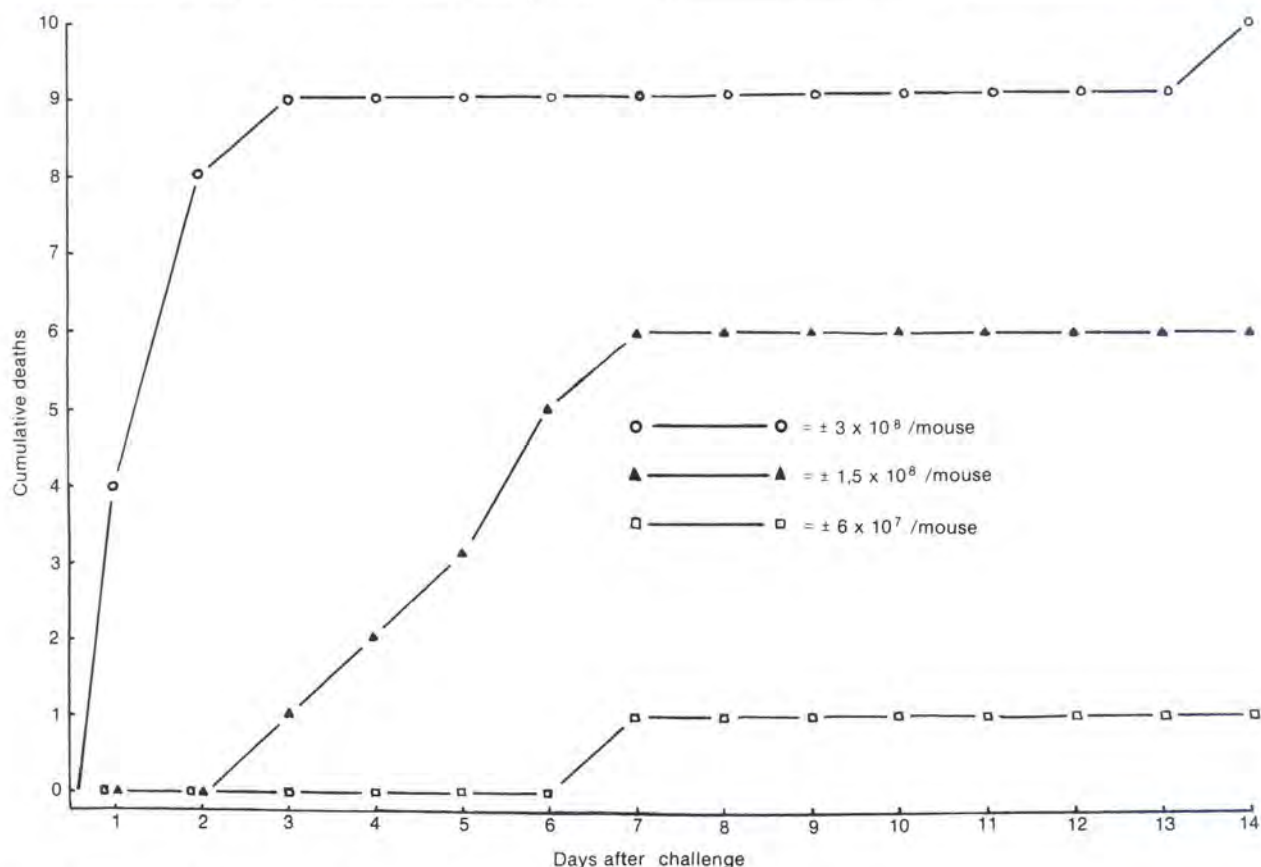


FIG. 1 Titration of *C. pyogenes* challenge material in mice

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Further investigations were designed particularly to determine whether 3 injections would give a better antibody response than 2 injections. From the results shown in Table 3, it is evident that, when 5.0 ml doses were used, the difference in antibody titres is negligible. Three injections each of 2 ml produced higher antibody levels than 2 such injections, but the difference was insignificant. Despite the fact that these dosages are appreciably lower than those recommended by the British Veterinary Codex, the titres compare favourably with those obtained by the latter procedure.

TABLE 2 Effect of dosage and number of injections on the antibody response in rabbits to *C. pyogenes* vaccine

Group	Immunization schedule	Mean agglutination titre	Mean antitoxin titre (Units/ml)
A.....	3 × 5 ml	1 902	3 804
B.....	2 × 5 ml	1 810	5 121
C.....	3 × 2 ml	1 660	3 320
D.....	2 × 2 ml	951	2 320
E.....	Controls	20	20

Antibody response in sheep and cattle

A comparison of the antibody levels obtained using 3 different immunization regimens is shown in Tables 3 and 4.

TABLE 3 Antibody response in sheep given *C. pyogenes* vaccine by different schedules 1 and 2 months after the last injection

Group	Schedule	Agglutination titre		Antitoxin titre (Units/ml)	
		1 month	2 months	1 month	2 months
A....	5, 10 and 15 ml	2 046	1 501	2 421	3 100
B....	2 × 5 ml	1 023	750	2 834	1 607
C....	3 × 5 ml	713	1 227	14 480	3 893
D....	Controls	90	nt*	448	nt*

nt*=not tested

TABLE 4 Antibody response in cattle given *C. pyogenes* vaccine according to different schedules 1 and 2 months after the last injection

Group	Schedule	Agglutination titres		Antitoxin titre (Units/ml)	
		1 month	2 months	1 month	2 months
A....	10, 15 and 20 ml	1 131	2 164	1 544	1 790
B....	2 × 10 ml	485	2 947	1 510	1 607
C....	3 × 10 ml	1 174	3 698	2 393	2 555
D....	Controls	386	nt*	399	nt*

nt*=not tested

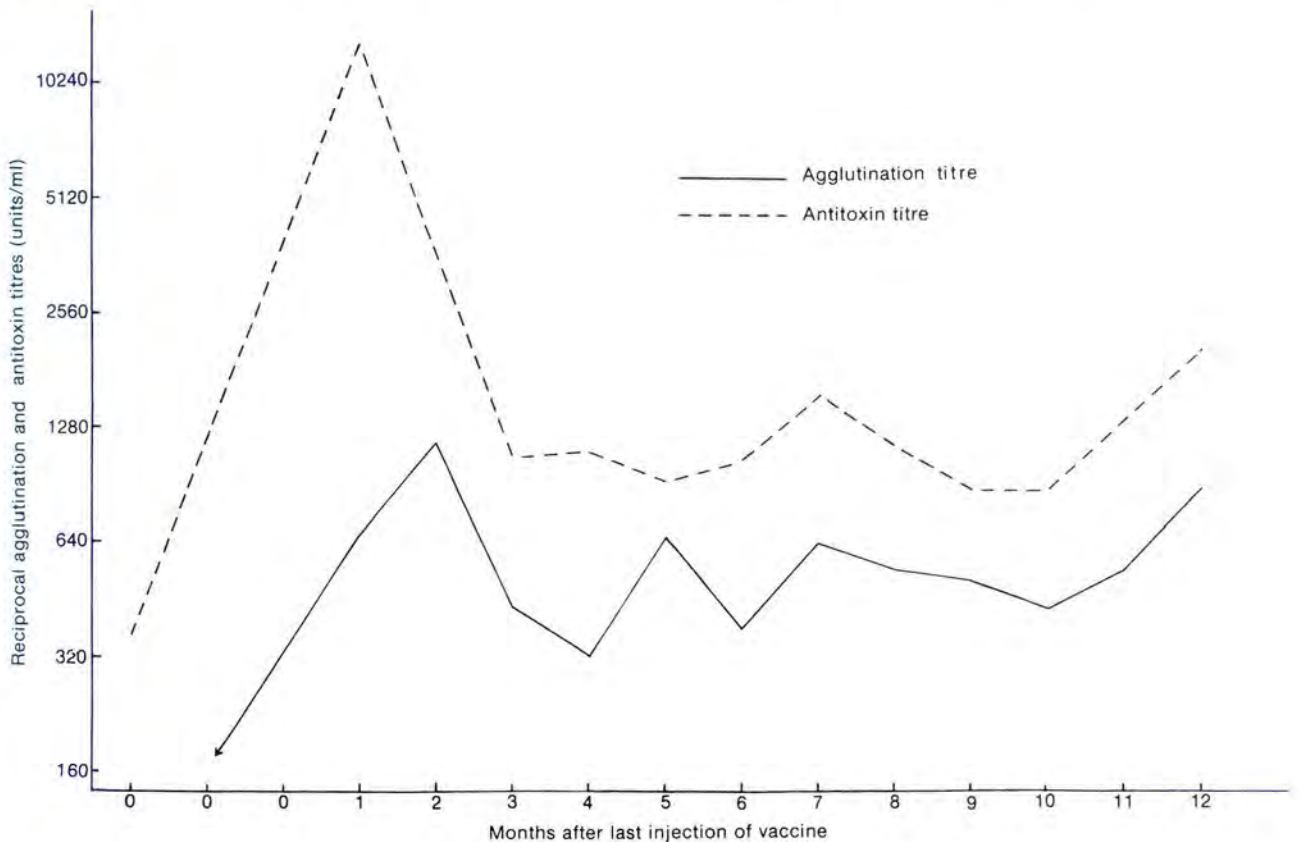


FIG. 2 Duration of antibody response in sheep

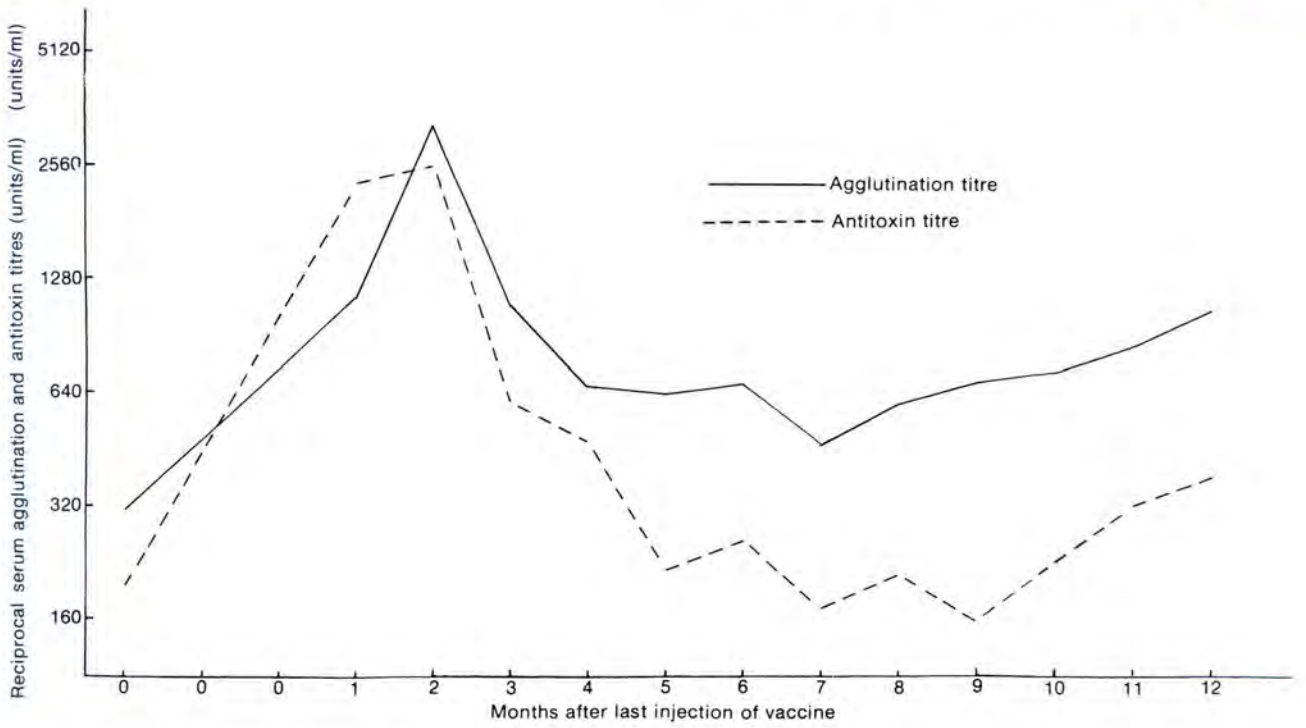


FIG. 3 Duration of antibody response in cattle

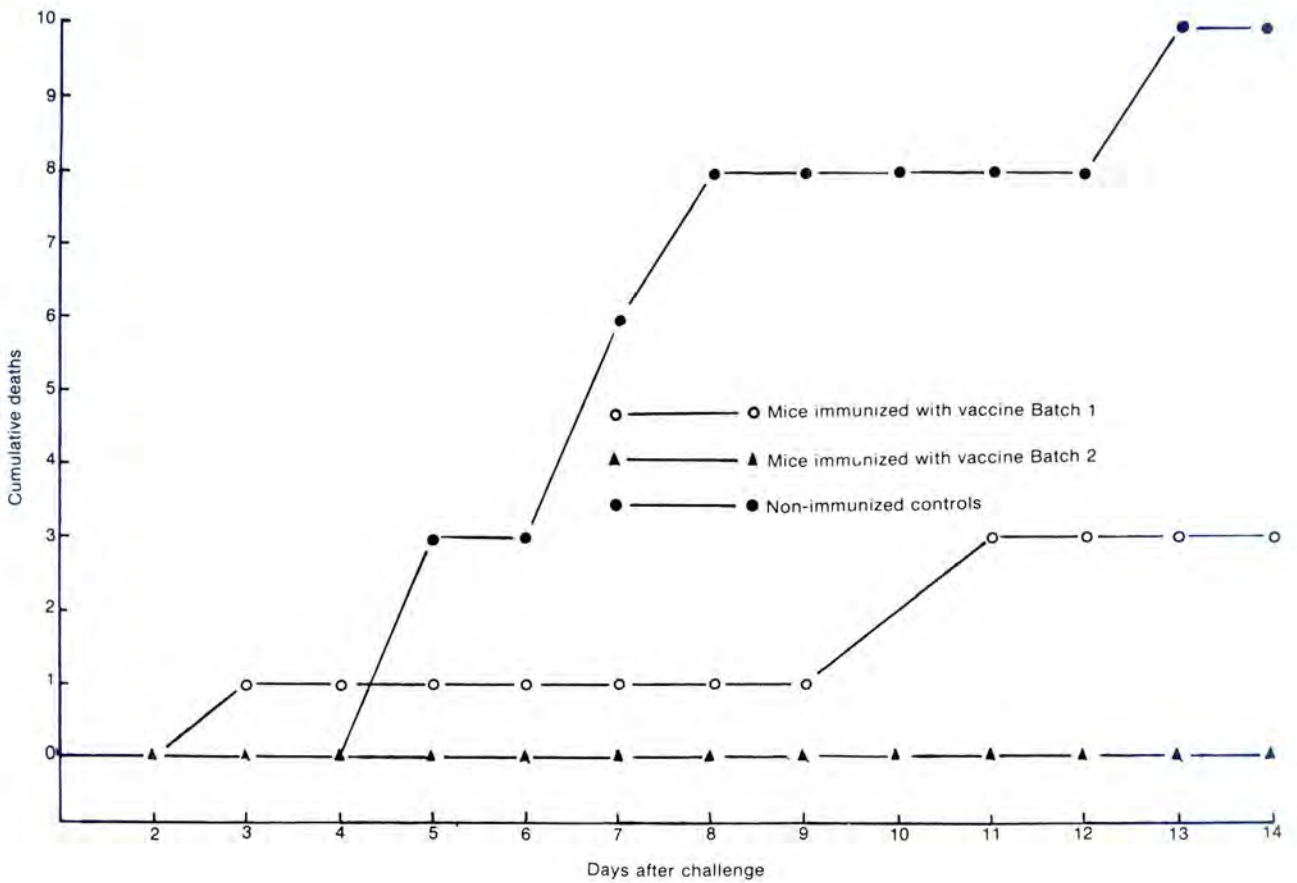


FIG. 4 Immunity in mice challenged with $\pm 1,5 \times 10^8$ bacteria

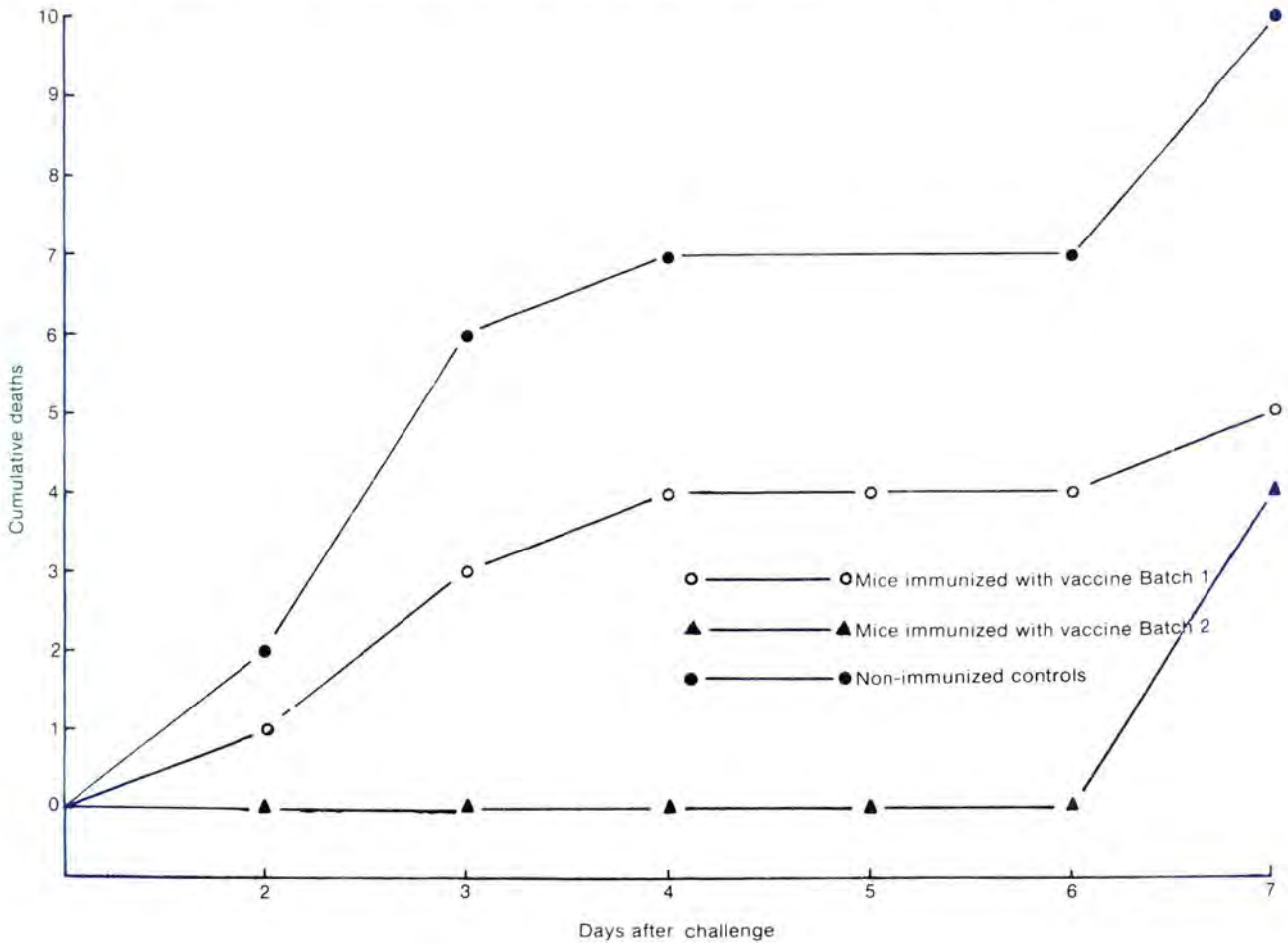


FIG. 5 Immunity in mice challenged with $\pm 2.0 \times 10^8$ bacteria

As in the case of rabbits, there was little difference in the antibody levels among the sheep and cattle groups. In cattle, however, a 3 injection regimen of 10 ml doses of vaccine gave the best results; the results with only 2 injections were distinctly poorer.

Since the application of 3 injections of vaccine of the same volume (5 ml for sheep and 10 ml for cattle) is far more practicable than graded doses (5, 10 and 15 ml for sheep and 10, 15 and 20 ml for cattle), the antibody titres of these groups were followed for a year to determine the duration of the response.

As shown in Fig. 1 and 2, the highest titres in both sheep and cattle were recorded 1-2 months after the commencement of immunization. Thereafter, the titres dropped rapidly, reaching a plateau after 6 months, but they did not drop as low as at pre-immunization levels. The titres fluctuated somewhat from month to month, with a tendency to rise towards the end of the experimental period.

Immunity of mice

The results of the immunity experiments in mice are shown in Fig. 3 and 4. A very definite immunity was demonstrated for both batches of vaccine tested when the mice were challenged with 1.5×10^8 organisms, and 1 batch in fact produced 100% protection. The vaccine also gave distinct protection to challenge with approximately 2.0×10^8 organisms, but was less effective than with the 1st challenge dosage.

DISCUSSION

Although antibody levels are not necessarily a direct measure of protection against infection, their presence is at least a reflection of the ability of a vaccine to elicit an immune response. The response elicited in the experiments reported here was very good indeed.

In rabbits, a higher antibody response was recorded when the dosages and regimen of The British Veterinary Codex (Capper, 1953) were followed than when lower dosages and fewer injections were used. The British Veterinary Codex recommendations, however, prescribe excessively high doses of vaccine and are impracticable because of the variation in dosages, while the response obtained by a modified programme of dosages and regimen was still far above the minimum requirements of the British Veterinary Codex (Capper, 1953).

Similarly, in both sheep and cattle, 3 injections of 5 ml and 10 ml, respectively, gave as good results as were obtained with large and varied dosages. This procedure not only requires less vaccine but is far more practicable under field conditions. Although the antibody response did not remain at a high level for long, it did not drop to pre-immunization levels during the 12 months investigation. It is not known what antibody level is required to afford adequate protection, but it is obvious that the animals remain well primed and should readily respond to antigen stimulation through active infection.

That immunization will indeed provide protection against infection was convincingly demonstrated by the experiments in mice. These findings do not support the conclusion of Derbyshire & Matthews (1963) that the possibility of successfully immunizing animals against *C. pyogenes* is remote. It should be pointed out that the exposure level is extremely critical when assessing protection. Whereas Derbyshire & Matthews (1963) used a challenge dose of 10^9 organisms, we found that, when doses of more than 1.5×10^8 organisms were used, the degree of demonstrable immunity rapidly decreased. A further possible explanation for our positive results may be that we employed 3 injections of a high potency vaccine in the place of their 2.

Although it is clear that immunity is readily overcome by a large challenge dose, the exposure level under field conditions is undoubtedly lower than that required for laboratory experiments. We believe, therefore, that immunization is a valuable aid in controlling *C. pyogenes* infections in sheep and cattle, provided a vaccine of high potency is employed. This conclusion is, in fact, borne out by reports from the field and by the continual demand for vaccine by veterinarians and stock owners over the last 3 years (Cameron, unpublished information).

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