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IMMUNE RESPONSE OF MERINO SHEEP TO INACTIVATED *CORYNEBACTERIUM PSEUDOTUBERCULOSIS* VACCINE

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ABSTRACT

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The brand of yeast extract used for medium production is critical for the cultivation of large numbers of *Corynebacterium pseudotuberculosis*.

The agglutination test was used to measure the antibody response which followed the inoculation of various inactivated *C. pseudotuberculosis* vaccines. The best results followed two subcutaneous injections of 5 ml vaccine prepared by inactivation with 0,5% formalin and standardized so that each dose contained a total of 0,025 ml packed cells.

The addition of adjuvants to the vaccine had a negligible effect on the antibody response, but increasing the interval between the primary and secondary injections from 2 or 4 to 6 weeks resulted in a higher level of agglutinating antibodies. The antibody titres, however, returned to pre-immunization levels within 3 to 4 months.

Freshly prepared vaccine was very toxic, apparently due to incomplete toxoiding of the exotoxin, but older vaccine was shown to be quite safe.

A method was devised whereby chronic pulmonary abscesses could be established in experimental animals. Exposure of immunized sheep to artificial infection showed that they were well protected against subacute infection and death but that they were not able to restrict the development of abscesses effectively.

The possible reasons for the disappointing results are discussed in terms of the mechanisms of immunity which may be involved.

INTRODUCTION

Apart from the reports on field trials quoted by Benham, Seaman & Woodbine (1962) there is no information available in the literature on the antibody response of sheep to *Corynebacterium pseudotuberculosis* on or the protective effect of such vaccines against infection by this organism. It was therefore proposed to study various factors influencing the production and immunogenicity of inactivated *C. pseudotuberculosis* vaccine and to follow the immune response of immunized sheep.

It has been shown that the antigen(s) of *C. pseudotuberculosis* responsible for inducing a protective immunity reside(s) in the cell wall (Cameron, Minnaar & Purdom, 1969) although the precise chemical nature of the antigen is unknown (Cameron & Purdom, 1971). It has furthermore been shown that in mice the mechanism of immunity is dependent on serum antibodies (Cameron & Engelbrecht, 1971). An agglutination test using whole bacteria as antigen would therefore seem to be a fair measure of the immune status of an animal and was consequently selected to follow the antibody response of immunized sheep.

It was also necessary to correlate the agglutinin response of immunized sheep with protective immunity. In order to investigate this, a method was devised whereby sheep could be artificially infected and the magnitude of infection estimated with reasonable accuracy.

MATERIALS AND METHODS

Strains

C. pseudotuberculosis strain 137B was used in all the experiments. It was originally obtained from Prof. H. R. Carne of the University of Sydney.

Preparation of vaccine

Medium was prepared and bacteria were grown in static Roux flasks for 48 hours at 37°C as previously described (Cameron & Swart, 1965). The composition of the medium was varied in order to find the most satisfactory formulation (Table 1). The yield was determined by measuring the amount of packed cells by means of Hopkins tubes. Comparative measurements showed that a concentration of 1,5% packed cells was equivalent to approximately 10^9 bacteria per ml or 2,0 mg dry bacteria per ml.

Standard vaccine was prepared in such a way that the final product contained 0,5% packed cells. For this vaccine a dose of 5,0 ml was routinely used in sheep. Each injection thus contained a total of 0,025 ml packed cells, which is equivalent to approximately $1,5 \times 10^9$ bacteria or 3,5 mg dry bacteria.

After diluting the cultures to the desired concentration with sterile 0,85% NaCl they were inactivated for 7 days unless otherwise indicated by the addition of 0,5% formalin, or 0,5% phenol, or 0,02% merthiolate or by autoclaving at 120°C for 15 min according to the requirements of the particular experiment.

In those vaccines which contained adjuvants, the cell density was adjusted to allow for the dilution caused by the adjuvant. All the vaccines contained a final concentration of 0,5% packed cells unless otherwise indicated. The following adjuvants were employed in this study:

(a) *Claassen's oil adjuvant (CO)*: 18 ml of a mixture of 0,8 ml Lissapol NX[1] and 19,2 ml Lubrol MOA[1] was mixed with 120 ml of bacterial suspension (0,9% packed cells). This mixture was then slowly added to 102 ml Ondina 17[2] oil and vigorously shaken.

(b) *Incomplete Freund's adjuvant (IF)*: 15 ml Arlacel A[3] was mixed with 85 ml Bayol 55[4] and emulsified with 100 ml bacterial suspension by vigorous shaking.

1. Imperial Chemical Industries
2. Shell Oil Co.
3. Atlas Chemical Industries
4. Esso Petroleum Co. Ltd

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(c) *Complete Freund's adjuvant (CF)*: The same formulation as for IF was used except that 100 mg dried *Mycobacterium phlei* was added to the oil.

(d) *Alhydrogel:* Alhydrogel[5] was used as supplied by the manufacturers and mixed in a 50:50 ratio with bacterial suspension.

(e) *Aluminium hydroxide gel* was prepared according to a method employed by the Instituto Zooprofilattico Sperimentale, Brescia, Italy, for use with foot-and-mouth disease vaccine, by mixing 150 ml aluminium ammonium sulphate solution (76,7 g/150 ml) with 600 ml ammonium sulphate solution (22,9 g/600 ml) and adding 50 ml liquid ammonia. The gel was washed thoroughly and mixed in a 50:50 ratio with bacterial suspension (1,0% packed cells).

(f) *Potassium alum:* Two vaccines were prepared, the one contained a final concentration of 0,5% packed cells and the other 1,25% packed cells. Doses of 5,0 ml and 2,0 ml were respectively used for sheep. Both vaccines contained a final concentration of 1,0% alum.

(g) *Tricalcium phosphate gel:* Ca_3PO_4 gel was prepared as described by Alexander & Blok (1960). The gel was mixed in a 50:50 ratio with bacterial suspension.

(h) *Aluminium phosphate gel* was prepared as described by Stern & Wentzel (1950). As in the case of the alum-containing vaccines two vaccines were prepared containing either 0,5% packed cells or 1,25% packed cells. Equal volumes of adjuvant and cell suspension were mixed to give the desired concentrations. Doses of 5,0 and 2,0 ml were respectively used for sheep.

(i) *Alugel*[6] was used as supplied by the manufacturers and mixed in a 50:50 ratio with bacterial suspension.

(j) *Wellcome adjuvant (BW)*: For preparation of vaccine containing Wellcome adjuvant, a bacterial suspension containing 6,0% packed cells was used in order to obtain the desired final concentration. The vaccine was prepared according to the formulation described by Thomson, Batty, Thomson, Kerry, Epps & Foster (1969) and the dose for sheep was 2,0 ml.

Immunization of mice

Mice were immunized as described previously (Cameron & Minnaar, 1969). In the experiment in which the effect of different inactivation procedures were compared, the mice were immunized with a total of 4,0 mg dry bacteria and challenged intravenously with 4×10^4 live bacteria.

Immunization of guinea pigs

In the experiment designed to compare the antibody response to whole cell vaccine and vaccine prepared from disrupted bacteria, (Cameron, Minnaar & Purdom, 1969) the guinea pigs were immunized with 2 injections of 2,0 ml each at an interval of 4 weeks. The vaccines contained 0,5% packed cells and the equivalent of 0,5% packed cells respectively.

Immunization of sheep

All vaccines were administered subcutaneously in the thigh. The dosages and interval between injections varied according to the composition of the vaccines and the requirements of each experiment as indicated under 'Results'.

Assay of immune response in sheep

Groups of either 6 or 8 Merino wethers were used in all the experiments. The dosages and concentration of vaccine varied from one experiment to another as indicated under 'Results'. The vaccines were always administered subcutaneously and serum samples were taken at periodic intervals. The sera were stored at —20°C until the agglutination titres were determined.

In the initial experiments the primary and secondary doses of vaccine were given at intervals of 4 weeks until it was experimentally shown that an interval of 6 weeks gave a better antibody response.

C. pseudotuberculosis is notorious for its tendency to aggregate and it is extremely difficult to obtain a stable suspension of dispersed bacteria. The following procedure gave the best results: *C. pseudotuberculosis* 137B was grown in shake cultures for 18 to 24 hours on the same medium as that used for vaccine production and the bacteria harvested by centrifugation at 2000 *g* for 20 min. The deposited bacteria were suspended in 0,85% NaCl to give a creamy suspension. The suspension was homogenized in a teflon blender for 10 min and allowed to settle out for 2 hours. The upper portion was decanted and kept. The sediment was resuspended by the addition of a small quantity of 0,85% NaCl and the homogenization process repeated. The pooled supernatant fluid was then diluted with 0,85% NaCl to give a milky suspension and allowed to settle out in a separatory funnel overnight. All sediment which settled out was drawn off and the supernatant used for antigen. The density of the suspension was adjusted to that of standard *Brucella* antigen by means of a nephelometer and used on the day of preparation.

Twofold serial dilutions of the sera to be tested were made in 0,5 ml volumes in Dreyer tubes starting at a dilution of 1:5. To each tube 0,5 ml antigen was added and the tubes were then incubated at 37°C for 6 hours when the reactions were recorded. The highest dilution of serum showing at least 50% agglutination was taken as the end-point of the titration.

Challenge of mice and sheep

Immunized mice were challenged intravenously as described by Cameron & Minnaar (1969) and sheep by intravenous injection of live bacteria as outlined under 'Results'.

The degree of infection in sheep slaughtered a month after infection was estimated by counting the number of abscesses larger than 1,0 mm which were visible on the surface of both lungs. The number of live bacteria present in 1,0 g of lung tissue taken from the right apical lobe was also determined. The specimen was homogenized in 9,0 ml of saline, tenfold serial dilutions prepared and plate counts done by spreading 0,1 ml volumes on the surface of blood tryptose agar plates. The plates were incubated at 37°C for 48 hours when the number of colonies was counted.

Density estimations were done on three representative samples of tissue from each lung according to the method described by Crowle (1958).

Clinical pathological tests

The haematocrit was determined according to the standard procedure of Wintrobe (1961) and haemoglobin measured as described by Drabkin (1949). Unconjugated bilirubin was assayed by the method of Malloy & Evelyn (1937).

5. Dansk, Svovlsyre, Denmark
6. Noristan Laboratories

Results

Influence of medium on growth

The medium described by Cameron & Swart (1965) has given good results on an experimental scale. However, it was considered necessary to examine various formulations for routine large scale production of vaccine as well as the possibility of using commercial broths for production of smaller quantities of vaccine for experimental purposes. The results of one such experiment are listed in Table 1. From the data it is clear that the nutrient broth prepared from fresh ingredients is far superior to commercial broth bases and it is also apparent that Oxoid yeast extract is not a satisfactory product for enhancing growth of *C. pseudotuberculosis* in static cultures even at double the concentration of Difco yeast extract. Therefore, all vaccines were produced hereafter on medium prepared from Onderstepoort broth (Cameron & Swart, 1965) enriched with 1,0% lactalbumin (Difco) as well as 0,5% yeast extract (Difco).

Effect of inactivation method on immunity in mice

The immunizing ability of vaccines inactivated by either 0,5% formalin, 0,5% phenol, 1:5000 merthiolate or autoclaving at 120°C for 15 minutes was compared in mice. The results of a typical experiment shown in Fig. 1 confirm previous results which Cameron (1964) obtained in guinea pigs when it was shown that formalinized vaccine affords the best protection, while vaccine prepared from autoclaved bacteria is very poor.

Table 1 *Yield of bacteria in fluid media of different compositions*

Composition of medium	Flask No.	Yield per cent packed cells
Lactalbumin (Difco) 20 g Yeast extract (Difco) 10 g Onderstepoort broth to 2 l	1 2 3 4 5 6	2,5 2,5 3,0 2,0 2,5 3,5
Lactalbumin (Difco) 20 g Yeast extract (Oxoid) 10 g Onderstepoort broth to 2 l	1 2 3 4 5 6	0,5 0,5 0,5 0,75 0,5 0,5
Lactalbumin (Difco) 20 g Yeast extract (Oxoid) 20 g Onderstepoort broth to 2 l	1 2 3 4 5 6	0,5 0,4 0,5 0,5 0,6 0,5
Lactalbumin (Difco) 20 g Yeast extract (Difco) 10 g Nutrient broth (Difco) 16 g Dist. water to 2 l	1 2 3 4 5 6	1,0 1,2 1,0 1,0 0,75 0,75
Lactalbumin (Difco) 20 g Yeast extract (Oxoid) 10g Nutrient broth (Oxoid) 26g Dist. water to 2 l	1 2 3 4 5 6	0,2 0,2 0,2 0,2 0,2 0,1

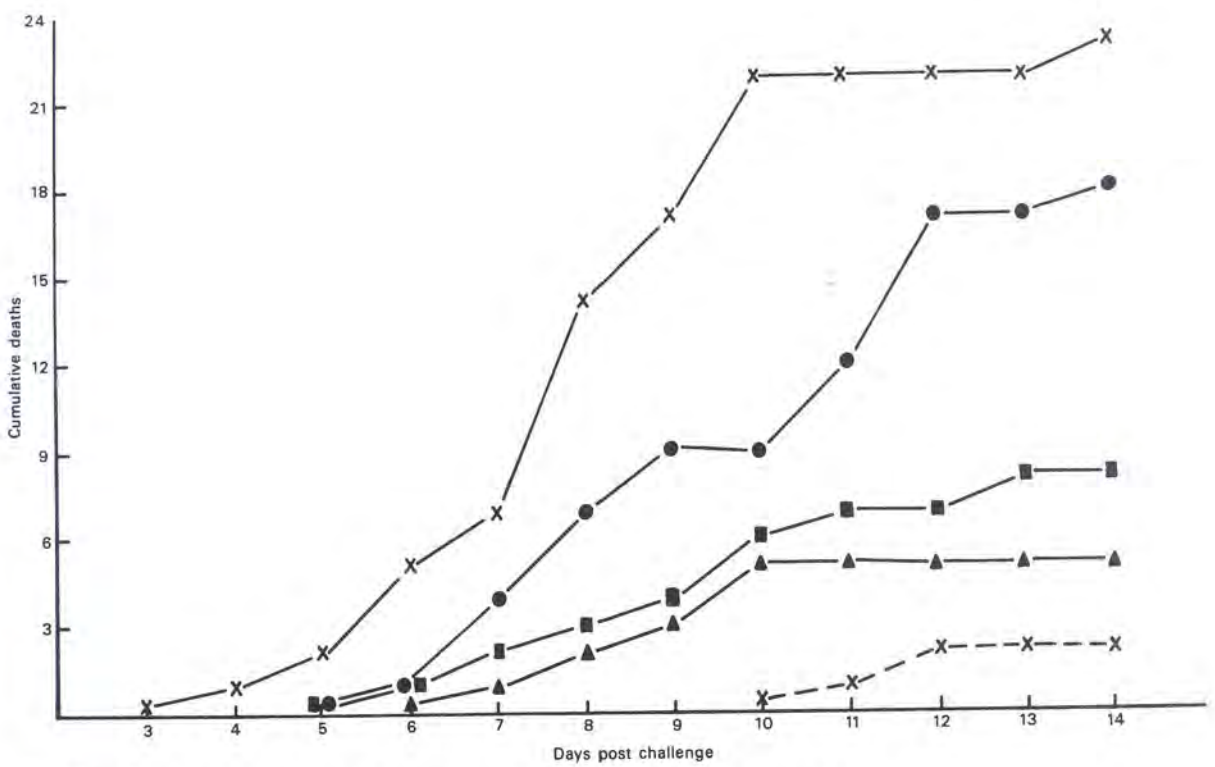

Fig. 1 Effect of inactivation procedure on immunogenicity of *C. pseudotuberculosis* vaccine in mice
×——× = Control mice given complete Freund's adjuvant only
●——● = Vaccine inactivated by autoclaving 120°C for 15 min
■——■ = Vaccine inactivated by 0,02% merthiolate
▲——▲ = Vaccine inactivated by 0,5% phenol
×- - - -× = Vaccine inactivated by 0,5% formalin

Comparison of antibody response in guinea pigs to whole cell vaccine and vaccine containing disrupted cells

It has been claimed by Greenberg & Cooper (1961) that staphylococcal vaccines prepared from lysed staphylococci induce a better immunity than vaccines prepared from whole organisms. It has not been possible to confirm these results with *Pasteurella* vaccines (Cameron & Smit, 1970) but it was nevertheless of interest to investigate this possibility with respect to *C. pseudotuberculosis*. Examination of sera obtained over a period of 12 weeks from groups of guinea pigs that were immunized with either disrupted or intact bacteria failed to show any difference. As shown in Fig. 2 the titres in the two groups were virtually identical throughout the experiment. It may also be noted that the agglutination titres obtained in guinea pigs were higher than those that were subsequently observed in sheep.

Effect of adjuvants on the antibody response in sheep

The agglutination titres that developed over a period of 12 weeks in groups of sheep that were immunized with vaccines containing different adjuvants are shown in Fig. 3. Vaccines containing Freund's incomplete (Group B) or Freund's complete adjuvant (Group C) gave the best antibody response but they were only slightly superior to vaccines containing alhydrogel (Group D), alum (Group F) or aluminium phosphate gel (Group H). None of the adjuvant vaccines were, however, superior to vaccine without adjuvant (Group J). These results are in accordance with previous reports on the ineffectiveness of adjuvants in mice (Cameron & Minnaar, 1969).

Furthermore the oil adjuvant vaccines gave rise to suppurative lesions at the injection site, making their further use impractical.

A further comparison of alum and aluminium phosphate adjuvant vaccines showed the latter to be preferable. It gave a higher peak 2 weeks after the second injection, but after 8 weeks the antibody titres of both groups were approximately the same and had dropped to below pre-immunization levels by the 12th week (Fig. 4).

Effect of dosage and immunization schedule on the immune response in sheep

The agglutination titres obtained in sheep given either the standard dose of aluminium phosphate adjuvant vaccine or twice or four times the standard dose are shown in Fig. 5. From these results it is clear that increasing the dose has no effect on the antibody response.

Similarly, the administration of three doses of vaccine at 4-weekly intervals instead of the customary two, has little influence on the antibody response. The titres persisted for a little longer in the group that received three injections but in both groups the titres were very low by the 12th week and had returned to pre-immunization levels by the 16th week (Fig. 6).
FIG. 6.

An extension of the interval between the primary and secondary injections from 4 to 6 weeks had a definite beneficial effect on the agglutination titres obtained 2, 4 and 8 weeks respectively after the second injection (Fig. 7). But, despite the higher levels obtained, the titres receded to pre-immunization levels by the 16th week.

Toxicity of vaccine

During the course of these studies it was observed that a few sheep lost condition and showed signs of

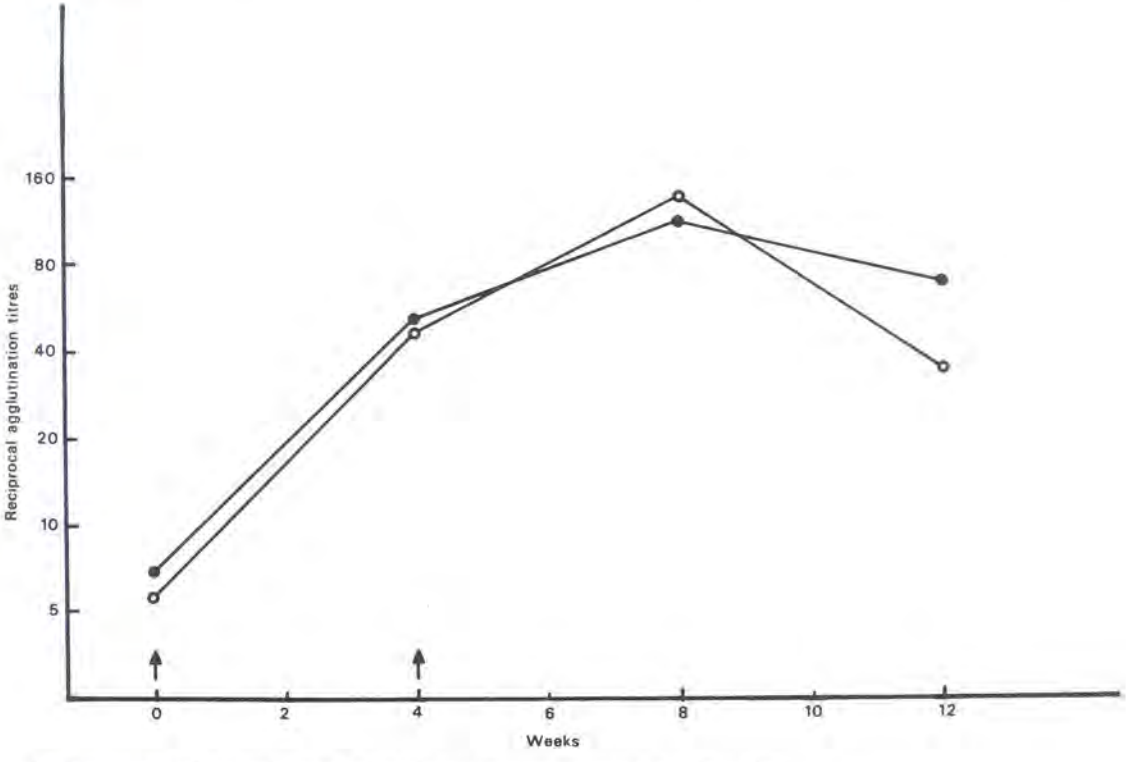

FIG. 2 Comparison of the antigenicity of whole and disrupted bacteria in guinea pigs
●——● = Agglutination titres of guinea pigs immunized with whole cell vaccine
O——O = Agglutination titres of guinea pigs immunized with disrupted bacteria

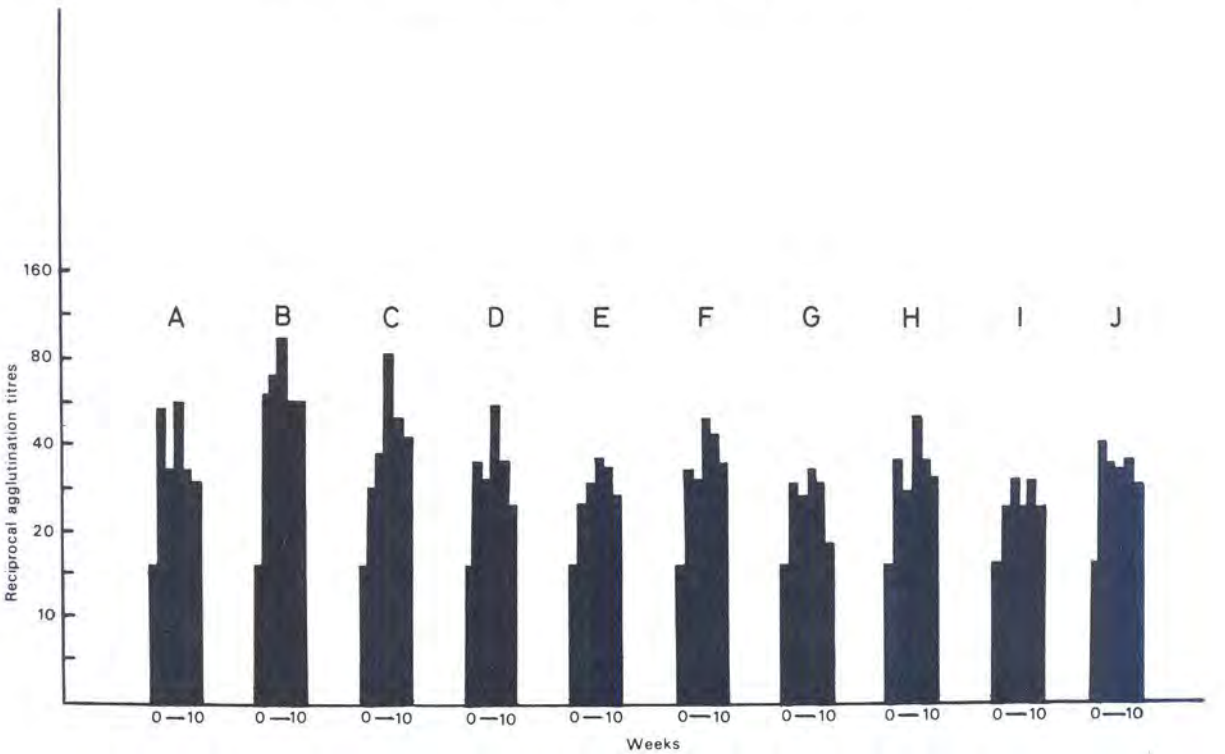

FIG. 3 Antibody response of sheep to vaccines containing various adjuvants
A = Claassen's oil adjuvant
B = Incomplete Freund's adjuvant
C = Complete Freund's adjuvant
D = Alhydrogel
E = Aluminium hydroxide
F = Potassium alum
G = Tricalcium phosphate
H = Aluminium phosphate
I = Alugel
J = None
Each bar indicates the average reciprocal agglutination titre at two weekly intervals from 0 to 10 weeks after the first administration of vaccine.

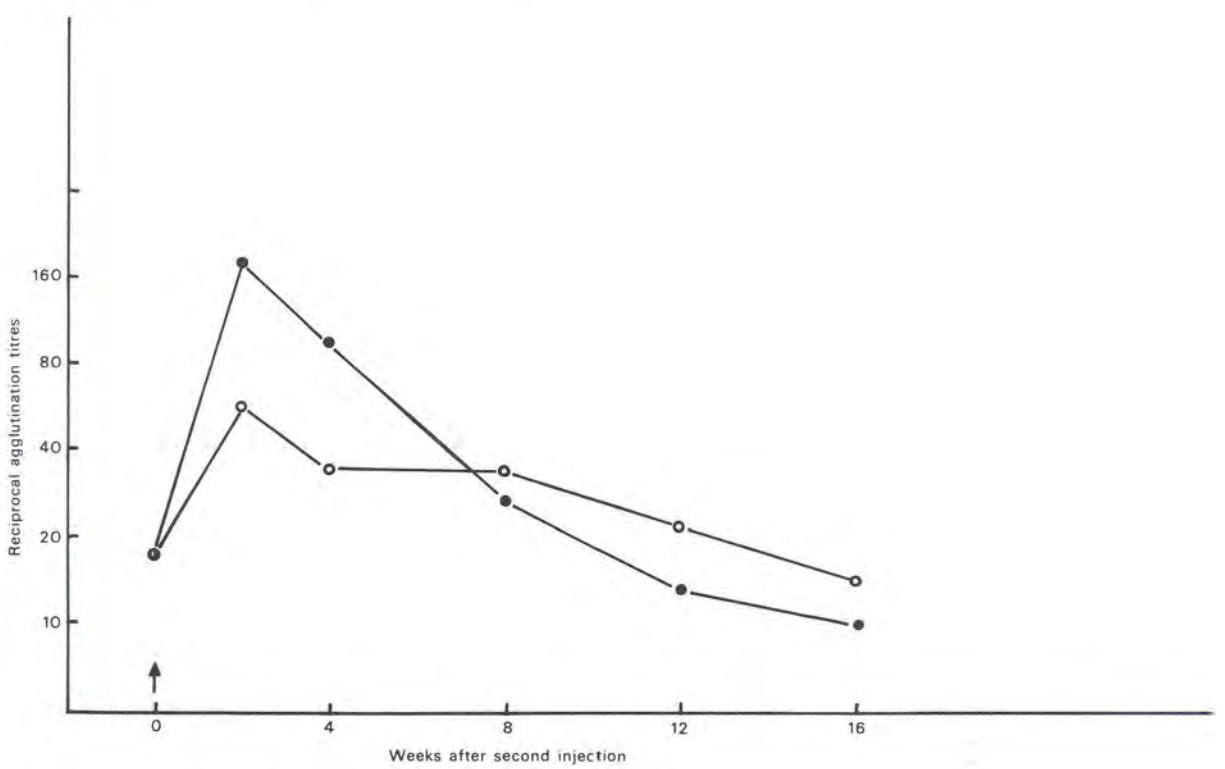

FIG. 4 Comparison of antibody response of sheep to aluminium phosphate adjuvant vaccine and potassium alum precipitated vaccine
●——● = Sheep immunized with two 5,0 ml injections of aluminium phosphate vaccine containing 0,5 per cent packed cells
O——O = Sheep immunized with two 2,0 ml injections of potassium alum precipitated vaccine containing 1,2 per cent packed cells

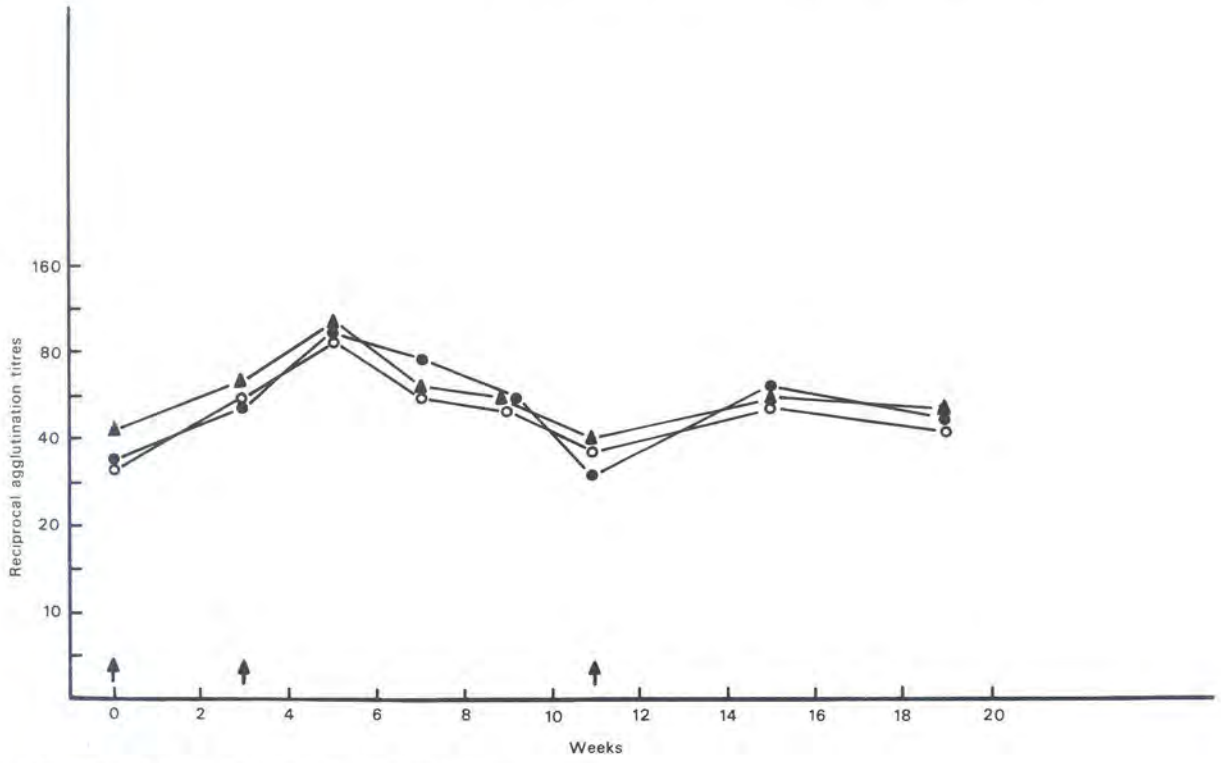

FIG. 5 Effect of dosage of vaccine on antibody response in sheep
●——● = Sheep given 5,0 ml per injection
O——O = Sheep given 2 × 5,0 ml per injection
▲——▲ = Sheep given 4 × 5,0 ml per injection
Sheep were immunized with vaccine containing 0,5 per cent packed cells and aluminium phosphate adjuvant.
Arrows indicate administration of vaccine

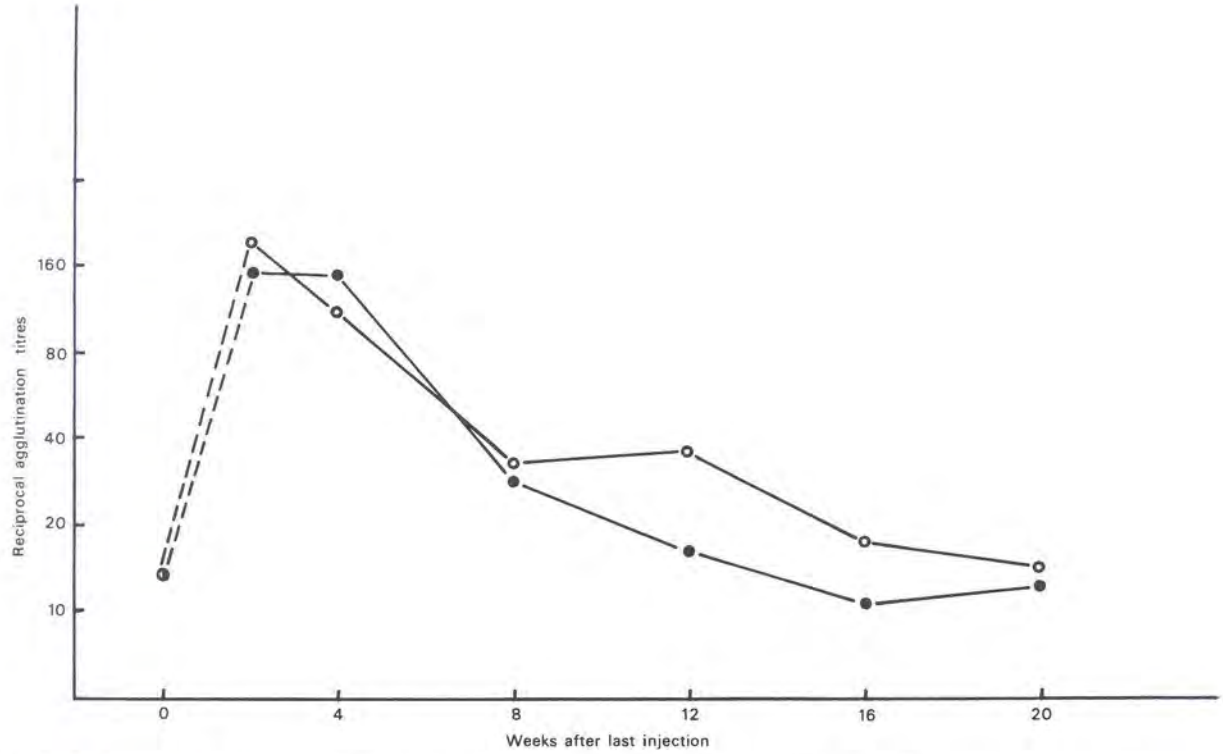

FIG. 6 Antibody response of sheep following administration of 2 or 3 initial injections at 4 weekly intervals
●——● = Sheep given two injections
O——O = Sheep given three injections

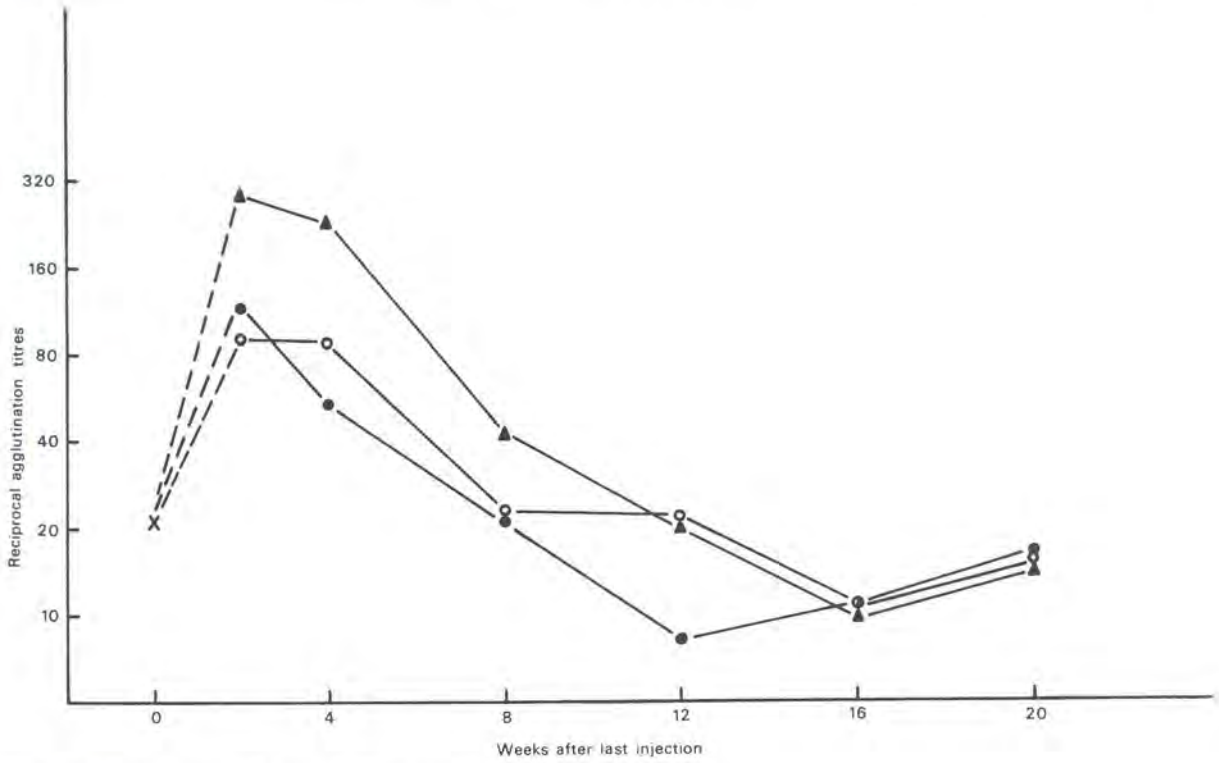

FIG. 7 Antibody response of sheep to vaccine given at different intervals
●——● = Primary and secondary injections given at 2 weeks interval
O——O = Primary and secondary injections given at 4 weeks interval
▲——▲ = Primary and secondary injections given at 6 weeks interval

anaemia, while in one particular instance a large number of sheep died 3 days after the administration of a freshly prepared batch of experimental vaccine. These sheep showed very severe icterus which is clearly illustrated in Fig. 8.

Examination of liver, kidney, spleen and lung sections of these cases revealed the following histopathological changes:

Liver: Fairly extensive centrilobular necrosis manifested by shrinking, eosinophilic and nuclear changes. In parts a mild neutrophile infiltration was associated with the necrotic areas. The rest of the liver showed degenerative changes, some fatty degeneration, swelling of cells and portions of the cytoplasm of some cells were homogenous and eosinophilic.

Kidney: Swelling of the cells of the glomeruli; otherwise mild nephrosis.

Spleen: Congestion.

In view of these observations it was considered necessary to investigate the toxicity of the vaccine more thoroughly.

The results of toxicity experiments in guinea pigs given vaccine without adjuvant are shown in Table 2. Administration of freshly prepared vaccine (24 hours) proved to be lethal despite the absence of any live bacteria and it was shown that this effect was associated with the supernatant fluid and not with the dead bacteria. All the guinea pigs died between 3 and 6 days after a single injection. Neither formalinized nor fresh toxin could be shown to have any *in vitro* haemolytic activity.

Increasing the concentration of formalin from 0,5% to 1,25% did not result in better toxoiding within 24 hours. Vaccine kept for 7 days, however, as well as vaccine prepared from 7 day-old cultures was virtually non-toxic.

TABLE 2 *Effect of formalin concentration, toxoiding time and growth time on toxin activity in guinea pigs*

Growth time	Composition	Formalin concentration	Toxoiding time	Deaths in guinea pigs 4 days
48 hours	Whole culture	0,5	24 h	$^{5}/_{6}$
48 hours	Cells only	0,5	24 h	$^{0}/_{6}$
48 hours	Supernatant only	0,5	24 h	$^{5}/_{6}$
48 hours	Supernatant only	1,25	24 h	$^{6}/_{6}$
48 hours	Supernatant only	0,5	7 d	$^{1}/_{6}$
7 days	Supernatant only	0,5	24 h	$^{1}/_{6}$

The results obtained in guinea pigs were confirmed in sheep. Animals which were given 24 hour old vaccine invariably died after 3 days and showed severe anaemia, icterus and varying degrees of liver degeneration and centrilobular necrosis, while animals given 7 day or older vaccine showed very little adverse reaction.

The haematocrit, haemoglobin and unconjugated bilirubin levels of three groups of sheep given 24 hour, 7 day old and 90 day old vaccine are given in Fig. 9a, 9b and 9c respectively. The sheep given 24 hour old vaccine showed marked aberrations 3 days after administration of vaccine while in the other 2 groups only transient deviations from the normal levels were observed after both the first and second doses of

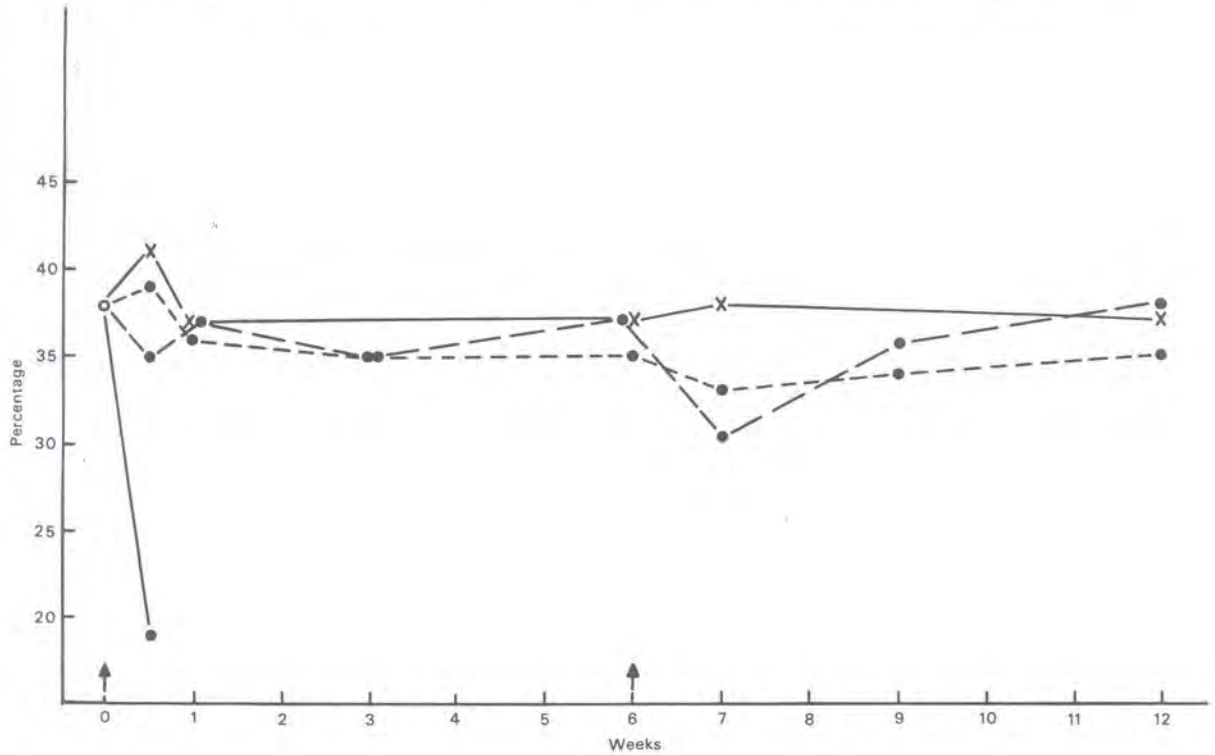

FIG. 9a Haematocrit of sheep after administration of fresh and stored vaccine
×——× = Unimmunized control sheep
●——● = Sheep given 2 day old (fresh) vaccine
●— —● = Sheep given 7 day old vaccine
●- - -● = Sheep given 90 day old vaccine
Arrows indicate administration of vaccine

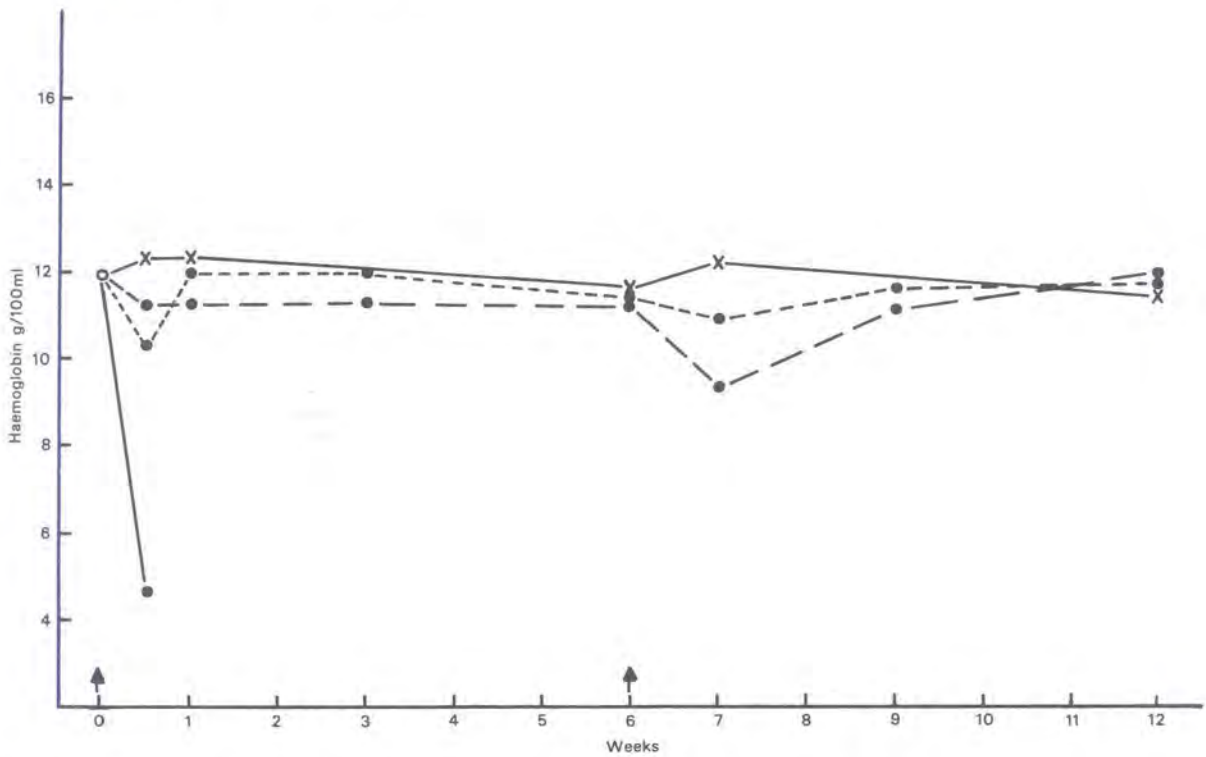

FIG. 9b Haemoglobin values of sheep after administration of fresh and stored vaccine
×——× = Unimmunized control sheep
●——● = Sheep given 2 day old (fresh) vaccine
●— —● = Sheep given 7 day old vaccine
●- - -● = Sheep given 90 day old vaccine
Arrows indicate administration of vaccine

FIG. 8 Sheep showing acute intoxication after administration of fresh vaccine. Note severe icterus.

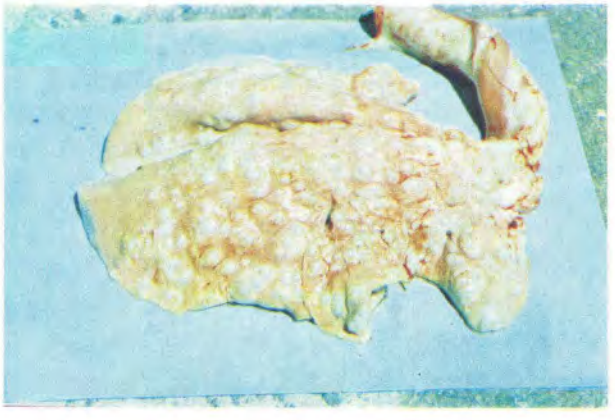

FIG. 10a Macroscopic appearance of abscesses in lung of a sheep artificially infected by intravenous inoculation with bacteria grown on static medium

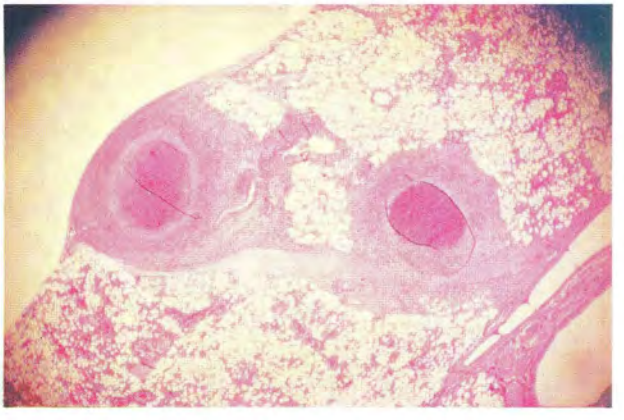

FIG. 10b Histological appearance of small abscess in the lung of a sheep artificially infected with *C. pseudotuberculosis*

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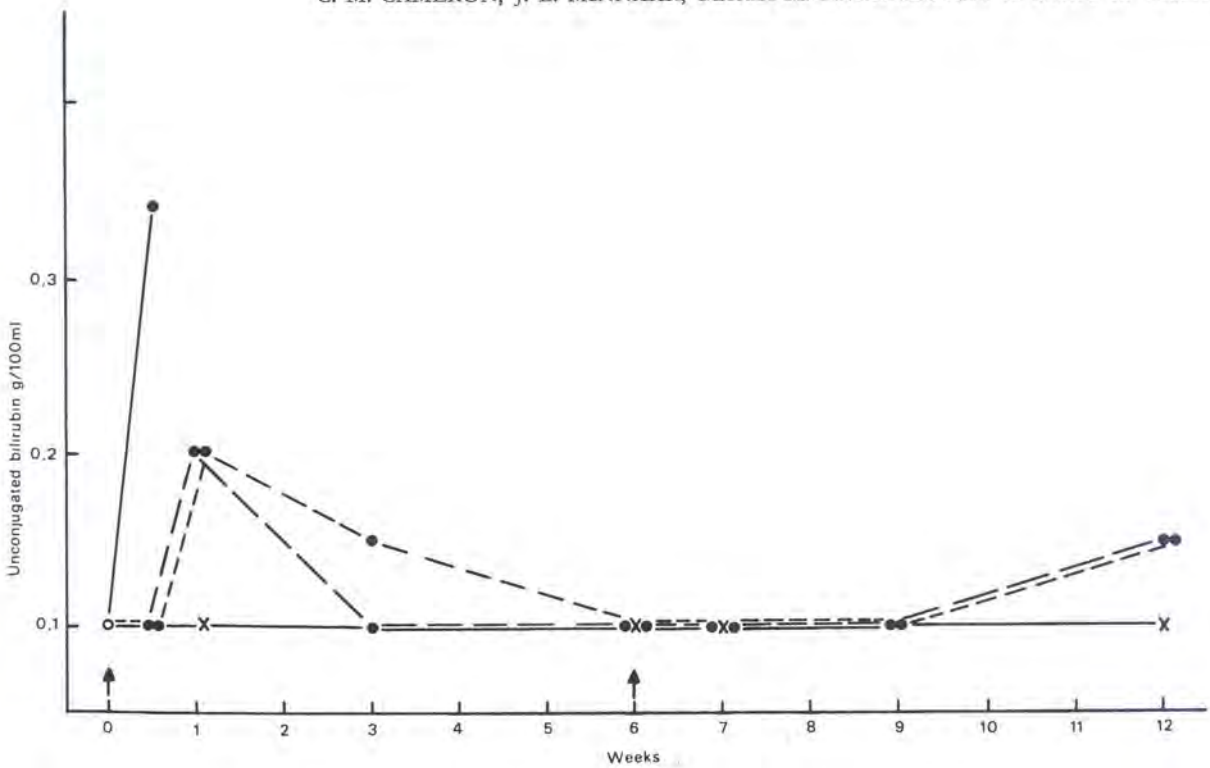

FIG. 9c Unconjugated bilirubin values of sheep given fresh and stored vaccine
× ——— × = Unimmunized control sheep
● ——— ● = Sheep given 2 day old (fresh) vaccine
● — — ● = Sheep given 7 day old vaccine
● - - - ● = Sheep given 90 day old vaccine
Arrows indicate administration of vaccine

vaccine. It therefore appears that even old vaccine is slightly toxic but the sheep are only temporarily affected and the slight changes in the blood composition are rapidly reversed.

Artificial infection of sheep

Preliminary attempts to establish lymph node abscesses by percutaneous injection of living bacteria invariably resulted in failure. Abscesses were seldom seen in the nodes but extensive subcutaneous lesions developed. These lesions were so variable in size that they were not considered to be a satisfactory method of assaying immunity.

Results of experiments with different intravenous doses of dispersed bacteria derived from shake cultures were very disappointing. Doses in the range of 10^9 to 10^{10} usually resulted in acute death due to pulmonary oedema while sheep which received doses between 10^8 and 2×10^7 seldom developed any lesions. When a dose of 5×10^8 was used, abscesses developed in the lungs and kidneys but some sheep still died within as short a period as 3 days after infection with signs of acute infection while others recovered completely.

Far better results were obtained when fresh bacteria derived from pellicles in static cultures were injected intravenously. The small floccules or clumps of bacteria appeared to lodge in the lung and kidney capillaries and subsequently gave rise to discrete abscesses. These abscesses were usually well dispersed throughout the lung tissue but there was a tendency for more pronounced infection to occur in the right apical lobe. Abscess formation was considered to be a true chronic infection. In some instances sheep died 7 to 14 days after infection showing diffuse purulent pneumonia or early abscess formation. Such cases were regarded as subacute infections.

The results of one experiment are summarized in Table 3.

A lung with typical lesions is shown in Fig. 10a and the pathology of a small abscess is illustrated in Fig.

TABLE 3 *Lesions in sheep infected intravenously with different doses of live bacteria*

	Dose	Interval before death	Post mortem lesions or lesions observed 4 weeks after infection
Sheep infected with frozen dispersed culture 24 hours	10^{10}	3 days	Pulmonary oedema; mild liver degeneration
	2×10^9	3 days	Pulmonary oedema; mild liver degeneration
	1×10^9	1 day	Severe pulmonary oedema
	5×10^8	13 days	Multiple pulmonary abscesses and icterus
	5×10^8	17 days	Few pulmonary abscesses and icterus
	10^8	survived	No lesions
	2×10^7	survived	No lesions
Sheep infected with fresh static culture 48 hours . .	10^9	survived	Disseminated focal purulent pneumonia
	5×10^8	survived	Disseminated focal purulent pneumonia

10b. It is clear that the superficial abscesses can be counted with ease. This system therefore proved to be a most satisfactory method for infecting sheep artificially and evaluating immunity.

Counts of the total live bacteria in mass-measured lung samples and density measurements were very erratic and were unsuitable criteria for measuring and expressing the degree of infection.

Assay of immunity in sheep

The results of an experiment in which sheep were given two injections of 5,0 ml vaccine (0,5% packed cells) with an interval of 6 weeks, are summarized in Table 4. The outstanding feature of this experiment was that 9 out of 14 of the control sheep died before termination of the experiment while only one of the immunized sheep died. This was probably due to a somewhat high challenge dose but proved that immunized sheep are clearly immune to a sub-acute infection. When the surviving control sheep were slaughtered a month after infection they were, however, found to have just as many pulmonary abscesses as the immunized sheep.

In a second experiment two groups of sheep were immunized; one group received 2 doses of 2 ml of an aluminium phosphate adjuvant vaccine containing 1,2% packed cells, the other group 2 doses of 2 ml of vaccine with BW oil adjuvant. The average agglutination titres at challenge were 1:35 and 1:59 respectively for the two groups. The results of this experiment are presented graphically in Fig. 11. As in the previous experiment, three of the six control sheep died before termination

TABLE 4 *Abscess development in the lungs of sheep 4 weeks after challenge with 10^9 live bacteria intravenously. Immunized sheep received two 5 ml injections of vaccine containing 0,5% packed cells*

	Agglutination titre at time of challenge	Interval before death	Abscesses in lungs four weeks after challenge
Immunized sheep	1:320	7 days	—
	1:40	—	215
	1:40	—	106
	1:80	—	273
	1:40	—	476
	1:40	—	185
	1:40	—	56
	1:80	—	123
	1:20	—	56
	1:80	—	193
	1:80	—	249
	1:80	—	40
	1:160	—	144
	1:160	—	115
Mean	1:90		172
Unimmunized sheep	1:20	11 days	—
	1:20	4 days	—
	1:10	7 days	—
	1:10	—	173
	1:20	7 days	—
	1:20	—	108
	1:40	—	198
	1:40	—	111
	1:80	6 days	—
	1:20	4 days	—
	1:20	4 days	—
	1:40	—	248
	1:20	4 days	—
	1:40	4 days	—
Mean	1:28		169

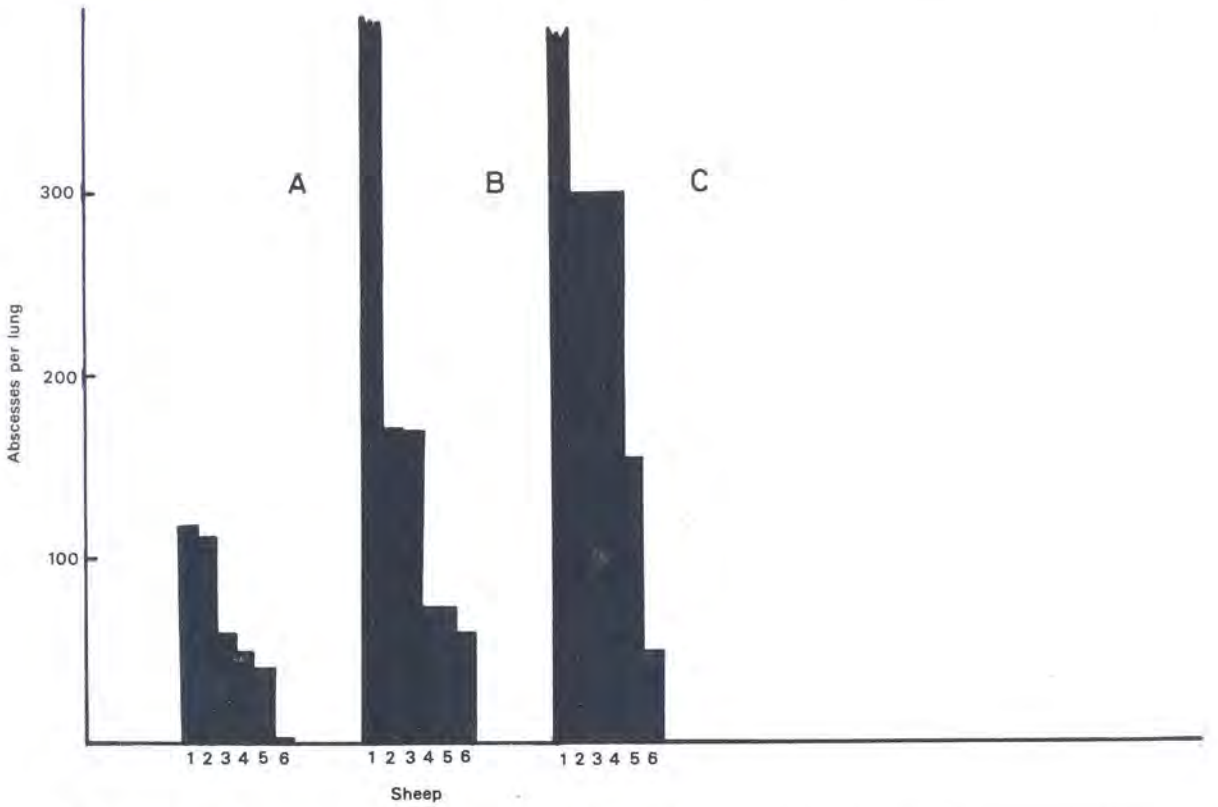

FIG. 11 Comparison of immunizing potency of aluminium phosphate adjuvant vaccine and BW oil adjuvant vaccine in sheep
A = Sheep immunized with aluminium phosphate adjuvant vaccine
B = Sheep immunized with BW oil adjuvant vaccine
C = Unimmunized control sheep

of the experiment (300 abscesses were arbitrarily allocated to each of these three animals).

It is clear that the sheep which received the aluminium phosphate adjuvant vaccine had appreciably fewer abscesses than the control group, while the group which received the BW oil adjuvant vaccine exhibited no detectable degree of resistance.

A certain degree of immunity to a chronic infection was thus established but with the exception of a single sheep it was not absolute and although immunized sheep developed fewer abscesses than control sheep they were not solidly protected.

A curious feature was that sheep which had received aluminium phosphate vaccine developed a mean agglutination titre of only 1:43 weeks after challenge while the group which had received BW adjuvant vaccine had a mean value of 1:183.

Discussion

The antibody response of Merino sheep to *C. pseudotuberculosis* vaccine was poor and the response generally only lasted for a period of 3 or 4 months even after the administration of large or repeated doses.

Nevertheless the poor antibody response was apparently sufficient to protect immunized sheep against the lethal effects of a subacute infection of living bacteria injected intravenously. This may be partially due to the fact that the sheep were able to overcome the toxic effects following establishment and multiplication of some of the invading bacteria. On the other hand it appeared that they were unable to prevent the establishment of foci of infection in the lung which subsequently gave rise to abscesses.

It could be concluded that the immunized sheep probably destroy the greater majority of bacteria, or effectively restrict their multiplication, but that enough survive and eventually give rise to abscesses.

There is a number of possible explanations for the above observations:

(a) Effective immunity may not necessarily be dependent on adequate levels of circulating antibody alone; cellular immune mechanisms may also be necessary in order to obtain solid protection. However, experiments in which hyperimmune rabbit serum was used to protect mice passively, conclusively proved that this is definitely not the case in mice (Cameron & Engelbrecht, 1971). Exclusive humoral immunity may nevertheless not suffice in sheep and the use of live vaccines which could also induce a cellular immunity might prove to be of particular value.

(b) Humoral immunity may be the only mechanism of immunity but the antibody levels obtained by conventional methods of immunization may be inadequate to afford a solid immunity. Manipulation of the antigen in order to render it more immunogenic or the application of alternative methods of immunization may overcome the problem.

(c) Hitherto only Merino sheep have been used and this breed may be exceptionally unresponsive to *C. pseudotuberculosis* antigen. Furthermore, sheep in general may be inherently less responsive to this antigen than other animals.

(d) A fourth possibility is that immunized sheep may be quite capable of destroying single bacteria but may be unable to cope with clusters of organisms, which might be more difficult to phagocytize and destroy.

These possibilities are being studied at present. It should, however, be recalled that the challenge doses used in this investigation were probably vastly in excess of any infection which could conceivably be encountered under natural conditions and therefore, the vaccine may indeed prove to be effective when used under field conditions. This can only be proved by very extensive trials. In the light of this and the briefness of the antibody response it is suggested that vaccine be administered shortly before expected exposure to infection, e.g. shearing, so that the animals will have maximum protection at this particular time.

Summary

A medium consisting of freshly prepared nutrient broth augmented by 1,0% lactalbumin (Difco) and 0,5% yeast extract (Difco) was found to give the highest yield of bacteria.

The method of inactivation had a marked influence on the immunogenicity of the vaccine for mice. Formalin was the best, while heat inactivation destroyed virtually all immunogenicity. The physical state of the organisms had no influence on the antibody response in guinea pigs.

The addition of adjuvants had very little effect on the antibody response in sheep but aluminium phosphate had a slight advantageous effect and was routinely used in subsequent vaccines.

Increasing the dose of vaccine above two injections of 5,0 ml each containing 0,5% packed cells, did not give a superior antibody response. Similarly, the administration of three initial doses of vaccine instead of the conventional two injections did not elicit higher titres. On the other hand a better response was obtained when the primary and secondary injections were given at an interval of 6 weeks rather than 2 and 4 weeks interval. The antibody response was, however, of short duration and agglutination titres dropped to pre-immunization levels after 12 to 16 weeks.

Freshly prepared vaccine (2 days old) was shown to be highly toxic for both guinea pigs and sheep. This was due to the exotoxin and resulted in a marked haemolysis and icterus in sheep which caused the death of the animals. Vaccine which was allowed to undergo toxoiding for 7 days or longer produced only mild temporary chemical pathological changes and proved to be quite safe.

A method was devised whereby disseminated pulmonary abscesses could be artificially produced in sheep. This consisted of injecting sheep intravenously with pellicle suspensions, obtained from 48 h static cultures. A dose of 5×10^8 bacteria gave the most satisfactory results.

Immunized sheep proved to be protected against a subacute infection, but they were not solidly immune to chronic abscess formation.

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