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AN IMPROVED *CORYNEBACTERIUM PSEUDOTUBERCULOSIS* VACCINE FOR SHEEP

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ABSTRACT

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Extensive experiments in mice confirmed that the immunogenicity of a *Corynebacterium pseudotuberculosis* vaccine could not be significantly improved with the use of various adjuvants. Immunity against *C. pseudotuberculosis* likewise could not be enhanced by incorporating various immunostimulants into the vaccine or by the use of live vaccines.

However, a combination of aluminium hydroxide gel and saponin as adjuvant did have a beneficial effect. This vaccine was tolerated better, and a smaller dose apparently protected sheep more effectively against intralymph node challenge than the currently available alum-precipitated vaccine.

INTRODUCTION

Cameron & Minnaar (1969) and Cameron, Minnaar, Engelbrecht & Purdom (1972) reported that mice and sheep can be effectively immunized against *Corynebacterium pseudotuberculosis* infection with the use of formalin-inactivated, alum-precipitated whole culture *C. pseudotuberculosis* vaccine. However, the immunity thus obtained was not absolute and could not be enhanced by the use of various adjuvants (Cameron & Fuls, 1973). During these studies it was observed, however, that, in sheep, a capsule, which very quickly formed around the injected vaccine, possibly prevented the effective absorption and processing of antigen. Moreover, the processing of antigen and the production of antibody in the lymph nodes is an important event in the immune response of sheep to *C. pseudotuberculosis* (Husband & Watson, 1977). In the light of the above it was considered advisable to re-examine the possibility of eliciting a better immune response to *C. pseudotuberculosis* than had thus far been possible.

Since it was impracticable to use sheep for all the proposed investigations, mice were used in the initial experiments to determine the effect of live vaccines, to assess the use of immunostimulants and to compare the value of various adjuvants. The best product thus formulated was finally assayed in sheep.

MATERIALS AND METHODS

Bacterial strains

The source and characteristics of the 2 strains of *C. pseudotuberculosis*, namely, Strain 137B (virulent) and 133A (attenuated), have been described (Cameron & Fuls, 1973).

Experimental vaccines

Alum-precipitated vaccine was prepared as described by Cameron & Minnaar, 1969 and Cameron, 1977, except that the sediment was not washed, only sufficient supernatant fluid being removed to give the desired packed cell volume (PCV) of 2,5 %.

Three preparations of aluminium hydroxide gel were used, namely, Rehsorptar[1], O.P. 1 (prepared from aluminium ammonium sulphate and ammonia) and O.P. 2 (prepared from potassium alum and sodium hydroxide). All 3 preparations were adjusted to a 50 % gel density and 12,5 mℓ added per 100 mℓ of vaccine.

When saponin[2] was used, a 3,0 % solution was prepared in distilled water and a volume sufficient to give the desired final concentrations added prior to the addition of $Al(OH)_3$ gel to the vaccines. All the vaccines containing adjuvant had a final PCV of 2,5 % and were stirred at room temperature for 60 min before being stored at 4°C.

Live vaccines were prepared from Strain 133A, as described by Cameron & Fuls (1973), and administered according to the dosages shown in the tables. A commercial vaccine[3] was also used for comparison.

Application of immunostimulants

Hyaluronidase and dimethylsulphoxide (DMSO)[4], well known for their dispersing and absorption enhancing properties, were added to vaccine containing no adjuvant to final concentrations of 300 units/mℓ and 4,0 % respectively.

Levamizole[5] and 2-mercapto-ethanol[4] reportedly have an immunostimulatory effect (Irwin & Knight, 1975; Unanue, 1981). To test their stimulatory effect when incorporated into *C. pseudotuberculosis* vaccine, they were added to the vaccine to give final dilutions of 1/200 (= 5 mg/kg) and 1/1250 (= 2×10^{-5}M) respectively.

Mycobacterium tuberculosis (BCG) reportedly stimulates immunity against *C. pseudotuberculosis* in guineapigs (Bakarat, Saber & Awad, 1974). In an attempt to verify this claim, a group of experimental mice was given a single injection of 10^6 BCG[6] bacteria subcutaneously and challenged 6 weeks later (vide infra).

Immunization and challenge of mice

Immunity experiments in mice were done exactly as were outlined by Cameron & Minnaar, 1969 and Cameron (1977). Briefly, groups of 33 mice were immunized by the routes and dosages as shown in the tables. They received 2 injections of vaccines at a 4-week interval and were challenged 2 weeks after the 2nd injection by the intravenous injection of graded doses of live bacteria. The cumulative deaths of the experimental groups and of the non-immunized, control groups were recorded over 14 days. A challenge dose of 2×10^6 bacteria/mouse generally gave the most consistent and the most realistic results. To avoid the confusion of excessive data, only the results obtained at this challenge level are given in the tables.

Immunization and challenge of sheep

Four groups of 6–12-months-old merino wethers were used to compare vaccines containing either potassium alum or $Al(OH)_3$ and saponin as adjuvants. They were given 2 injections of the respective vaccines as shown in Table 8 at a 4-week interval. All the sheep were challenged 2 weeks after the 2nd injection of vaccine by the injection of live bacteria into their prescapular lumph nodes (Ashfaq & Campbell, 1980). The left node was

[1] Armour Pharmaceutical Company, Kankakee, Illinois 60901, USA

[2] Biolab Chemicals, P.O. Box 14574, Verwoerdburg 0140

[3] CLA Vaccine, Commonwealth Serum Laboratories, Melbourne, Australia

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[4] E. Merck, Darmstadt, West Germany

[5] Ethnor, Halfway House Transvaal

[6] Japan BCG Laboratory, Tokyo, Japan

injected with 10^6 bacteria and the right node with 10^7 bacteria contained in 0,1 mℓ volumes. The sheep were also bled at this time and their sera stored at −20 °C until passive protection tests were done.

Two sheep from each group were slaughtered 6 weeks after challenge and a further 2 sheep from each group were slaughtered at 2 weekly intervals thereafter. Their lymph nodes were removed and all surrounding facia and fat dissected away. The extent of abscessation was assessed (see Table 8), and the lymph nodes were mass-measured.

Passive protection tests on the sera were done in mice as described by Cameron & Engelbrecht (1971).

RESULTS

Live vaccine

In a preliminary experiment, the immonogenicity of alum-precipitated vaccine, of vaccine containing 0,15 % saponin and of live vaccine was compared with a vaccine containing no adjuvant. From the results shown in Table 1, it appears that the live vaccine was superior to the others. The dosage used, however, caused extensive local lesions at the site of administration in the experimental mice.

Further assessment of live vaccines, however, did not substantiate the above finding. Vaccines containing 10^5, 10^6 and 10^7 live bacteria of Strain 133A all afforded a measurable degree of immunity after 1 or 2 injections. However, as Table 2 shows, none of them was better than the 2 injections of alum-precipitated vaccine.

In the following experiment (Table 3), the challenge was unfortunately rather severe, but it is nevertheless apparent that the intravenous or intraperitoneal routes of immunization do not have any particular advantage over the subcutaneous route for either the live or the inactivated vaccines.

Immunostimulants and adjuvants

From the results shown in Table 4 it is clear that the inclusion of DMSO, levamizole, 2-mercapto-ethanol and hyaluronidase had no immuno-stimulatory effect on the antigenicity of the alumprecipitated vaccine. Immunization with BCG alone also had no protective effect.

Further investigations on the effect of various adjuvants showed that, whereas all the adjuvants, when used individually, had little if any effect, the results given in Table 5 suggest that a combination of $Al(OH)_3$ and saponin may offer more promise.

TABLE 1 Comparison of the effect of different adjuvants and live vaccine

Vaccine*	Dosage	Cumulative deaths/10													
		Days post challenge with ± 10^7 bacteria/mouse													
		1	2	3	4	5	6	7	8	9	10	11	12	13	14
A. Inactivated alum precipitated	2 injections of 0,2 mℓ s.c.	0	0	0	0	1	2	4	5	7	7	7	7	7	7
B. Inactivated plus 0,15 % saponin	2 injections of 0,2 mℓ s.c.	0	0	0	0	2	7	8	8	8	8	9	9	9	9
C. Live Strain 133A 5 × 10^7 live bacteria/mℓ	2 injections of 0,2 mℓ s.c.	0	0	0	0	1	1	3	3	3	3	3	5	5	5
D. Inactivated vaccine without adjuvant	2 injections of 0,2 mℓ s.c.	0	0	0	0	1	3	5	6	7	8	8	8	8	8
E. Non-immunized controls	—	0	0	0	4	10	10	10	10	10	10	10	10	10	10

* Inactivated vaccines contained 2,5 % packed cells

s.c. = subcutaneously

TABLE 2 Comparison of live vaccine at different dosage levels with inactivated alum precipitated whole culture (2,5 % packed cells)

Vaccine	Dosage/mouse	Number of injections	Cumulative deaths/10													
			Days post challenge with ± 2 × 10^6 bacteria/mouse													
			1	2	3	4	5	6	7	8	9	10	11	12	13	14
Live Strain 133A	10^7	1	0	0	0	0	0	0	0	0	1	1	1	2	3	3
Live Strain 133A	10^7	2	0	0	0	0	0	0	0	4	4	4	4	4	4	4
Live Strain 133A	10^6	1	0	0	0	0	2	2	3	4	5	5	5	5	5	5
Live Strain 133A	10^6	2	0	0	0	0	0	1	1	2	2	2	2	2	3	3
Live Strain 133A	10^5	1	0	0	0	0	1	2	2	2	2	3	3	4	4	4
Live Strain 133A	10^5	2	0	0	0	0	0	1	1	1	3	3	3	3	3	3
Alum-ppt whole culture	0,2 mℓ	1	0	0	0	0	0	0	0	1	1	2	2	3	3	4
Alum-ppt whole culture	0,2 mℓ	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Non-immunized controls	—	—	0	0	0	0	1	4	5	7	8	8	8	8	9	9

TABLE 3 Effect of route of administration of the immunogenicity of live and inactivated bacteria

Route	Vaccine	Dosage	Cumulative deaths/10													
			Days post challenge with 2 × 10^6 bacteria/mouse													
			1	2	3	4	5	6	7	8	9	10	11	12	13	14
Intravenous	Inactivated	2,5 % pcv.	0	0	1	4	6	10	10	10	10	10	10	10	10	10
	Live	4 × 10^5	0	0	0	0	0	3	4	4	4	7	7	7	9	10
Intraperitoneal	Inactivated	2,5 % pcv.	0	0	0	1	1	2	6	7	8	8	9	9	9	9
	Live	2 × 10^6	0	0	0	3	5	6	7	8	8	8	9	9	10	10
Subcutaneous	Inactivated	2,5 % pcv.	0	0	0	3	4	7	8	8	8	8	9	9	9	9
	Live	10^7	0	0	0	0	0	2	4	4	5	6	6	6	8	8
Controls			0	0	5	10	10	10	10	10	10	10	10	10	10	10

TABLE 4 Failure of immunostimulants to enhance immunity to *C. pseudotuberculosis*

Vaccine	Cumulative deaths/10													
	Days post challenge with 2 × 10^6 bacteria/mouse													
	1	2	3	4	5	6	7	8	9	10	11	12	13	14
Inactivated 2,5 % cells plus DMSO	0	0	0	0	0	2	6	7	7	7	7	7	7	8
Inactivated 2,5 % cells plus levamizole*	0	0	0	0	1	3	5	6	7	8	8	9	9	9
Inactivated 2,5 % cells plus 2–ME	0	0	0	0	1	2	3	6	6	6	6	7	7	8
Inactivated 2,5 % cells plus hyaluronidase	0	0	0	0	3	5	5	7	7	7	8	8	8	8
Inactivated 2,5 % cells no stimulants	0	0	0	0	0	1	2	4	5	5	5	5	5	7
BCG (10^6) only	0	0	0	1	7	10	10	10	10	10	10	10	10	10
Non-immunized controls	0	0	1	6	8	8	8	8	9	10	10	10	10	10

* Ripercol-ℓ 150

TABLE 5 Comparison of various adjuvants on the immunogenicity of inactivated vaccine

Adjuvant	Cumulative deaths/10													
	Days post challenge with 2 × 10^6 bacteria/mouse													
	1	2	3	4	5	6	7	8	9	10	11	12	13	14
$Al(OH)_3$–O.P. 1[1]	0	0	0	0	0	0	1	3	5	5	5	6	7	7
$Al(OH)_3$–RA[2]	0	0	0	0	0	0	1	3	5	5	5	5	5	5
Potassium alum	0	0	0	0	0	0	2	4	4	6	6	6	7	7
Saponin 0,15 %	0	0	0	0	0	0	0	0	2	2	3	4	4	5
$Al(OH)_3$–RA + Saponin 0,15 %	0	0	0	0	0	0	0	2	3	3	3	3	3	3
No adjuvant	0	0	0	1	1	2	2	4	4	5	5	6	6	6
Non-immunized controls	0	0	0	0	0	1	3	7	8	8	9	9	9	9

[1] Prepared at the VRI Onderstepoort

[2] Rehsorptar, Armour

TABLE 6 Determination of optimum concentration of saponin in conjunction with $Al(OH)_3$–RA

Vaccine composition		Cumulative deaths/10													
		Days post challenge with 2 × 10^6 bacteria/mouse													
$Al(OH)_3$–RA %	Saponin %	1	2	3	4	5	6	7	8	9	10	11	12	13	14
10	—	0	0	0	0	0	0	2	4	4	5	5	5	5	6
—	0,3	0	0	0	0	0	0	0	1	2	2	2	2	2	2
—	0,15	0	0	0	0	0	0	0	1	1	1	1	1	1	2
—	0,075	0	0	0	0	0	0	0	2	2	2	2	2	2	2
—	0,04	0	0	0	0	0	0	0	3	5	6	7	7	7	8
10	0,3	0	0	0	0	0	0	0	0	0	0	1	1	1	1
10	0,15	0	0	0	0	0	1	2	2	2	2	2	2	2	2
10	0,075	0	0	0	0	0	0	0	0	1	1	1	1	1	1
10	0,04	0	0	0	0	0	0	1	1	1	1	1	1	1	1
Cells only		0	0	0	0	0	2	3	3	3	3	3	3	3	3
Non-immunized controls		0	0	4	4	6	7	10	10	10	10	10	10	10	10

TABLE 7 Comparison of the immunopotentiation of various aluminium hydroxide preparations in conjunction with 0,075 % saponin

Adjuvant added	Dosage per mouse (mℓ)	Cumulative deaths/10													
		Days post challenge with 2 × 10^6 bacteria/mouse													
		1	2	3	4	5	6	7	8	9	10	11	12	13	14
$Al(OH)_3$–R	0,2	0	0	0	0	0	0	0	0	1	1	1	1	1	1
	0,1	0	0	0	0	1	1	2	2	2	2	2	3	3	3
$Al(OH)_3$–O.P.1	0,2	0	0	0	0	0	0	0	0	0	0	0	0	1	1
	0,1	0	0	0	0	0	0	0	0	0	1	1	2	2	3
$Al(OH)_3$–O.P.2	0,2	0	0	0	0	0	0	1	2	2	2	2	2	4	5
	0,1	0	0	0	0	0	0	0	0	0	0	0	0	0	1
Alum 1 %	0,2	0	0	0	0	0	0	1	1	1	1	1	1	1	2
	0,1	0	0	0	0	0	0	0	0	1	1	5	5	5	5
Saponin only	0,2	0	0	0	0	0	0	0	0	0	0	1	2	2	2
	0,1	0	0	0	0	0	0	1	1	3	4	4	4	5	6
None	0,2	0	0	0	0	0	0	0	0	1	2	4	4	4	5
	0,1	0	0	0	0	0	1	1	3	3	3	3	4	5	5
Commercial	0,1	0	0	0	3	7	8	8	8	8	8	8	8	8	8
Non-immunized controls		0	0	0	1	1	2	4	7	8	9	9	10	10	10

TABLE 8 Protection afforded by various vaccines against lymph node infections in sheep

Vacine group	Adjuvant	Dosage (mℓ)	Sheep No.	Mass of excised nodes		Visual* assessment of tissue disintegration	
				Left Infected with 10^7 bacteria g	Right Infected with 10^6 bacteria g	Left	Right
A	$Al(OH)_3$ plus saponin	2,0	1	10,4	4,7	++	−
			2	5,0	5,5	+	−
			3	11,9	8,6	−	−
			4	18,6	6,4	+++	−
			5	5,4	6,2	−	−
			6	7,8	4,2	+	−
			7	4,5	3,1	−	−
			8	5,5	3,6	−	−
				Mean 8,6	Mean 5,3		
B	$Al(OH)_3$ plus saponin	5,0	1	10,4	8,6	++	−
			2	7,7	8,3	+	+
			3	11,8	8,8	−	−
			4	14,6	6,9	+++	−
			5	9,4	9,1	+++	+++
			6	9,2	6,2	−	−
			7	3,3	3,6	−	−
			8	9,4	6,6	−	−
				Mean 9,4	Mean 7,2		
C	Alum	5,0	1	6,0	5,6	+	+
			2	6,2	4,0	+	−
			3	19,4	15,3	++++	+++
			4	10,6	4,8	+++	−
			5	7,8	8,9	−	+
			6	11,6	7,3	++	+
			7	30,6	6,9	++++	++
			8	8,6	7,5	−	−
				Mean 12,6	Mean 7,5		
D	Non-immunized	—	1	12,0	12,8	++++	++
			2	24,9	14,4	++++	++
			3	16,35	11,5	+++	++
			4	12,6	13,3	+++	+++
			5	17,0	11,4	++++	++
			6	14,1	15,2	++++	++++
			7	7,9	10,1	+	−
			8	10,6	16,8	++	+++
				Mean 15,5	Mean 13,2		

++++ = Diameter of abscesses > 5 cm
+++ = Diameter of abscesses > 3–5 cm
++ = Diameter of abscess 1–3 cm
\+ = Diamter of abscess < 1,0 cm
− = No visible lesion

* *Note:* Exact measurements could not be made because of the varying shapes of the abscesses

This observation was, in fact, confirmed in the following experiment in which vaccines containing $Al(OH)_3$ and different concentrations of saponin were compared with vaccines containing 1 of the adjuvants only (Table 6). It is noteworthy, however, that in this particular experiment vaccine containing no adjuvant also induced good immunity.

Since the cost of using a commercial $Al(OH)_3$ preparation for mass production would be exorbitant, the efficacy of the 2 locally prepared $Al(OH)_3$ gels used in conjunction with saponin was examined. It will be seen from Table 7 that the results obtained with preparation O.P. 1 were as good as those obtained with the commercial product.

A commercially available *C. pseudotuberculosis* vaccine[1] was found to be inferior to all the experimental vaccines.

[1] CLA Vaccine, Commonwealth Serum Laboratories, Melbourne, Australia

Immunization of sheep

Table 8 shows the results of the immunity experiments in sheep.

None of the 3 vaccines provided an absolute immunity in all the animals, but it is evident that the vaccine containing $Al(OH)_3$ plus saponin afforded better protection that the vaccine containing only potassium alum as adjuvant. Moreover, a 2,0 mℓ dose of the former vaccine was as effective as a 5,0 mℓ dose.

Post-immunization sera from the different groups of sheep were ineffective in protecting mice against *C. pseudotuberculosis* infection.

DISCUSSION

The previous finding of Cameron & Fuls (1973) that vaccines prepared from live bacteria are able to induce an effective immunity in mice against *C. pseudotuberculosis* was confirmed in these experiments. Such vaccines, however, are no better than inactivated vaccines,

and the lesions they cause at the injection site are unacceptable. It was also confirmed that the addition of single adjuvants to *C. pseudotuberculosis* vaccine has little effect on the potency of inactivated vaccines (Cameron *et al.,* 1972). Conversely, the advantage of employing vaccines containing a combination of $Al(OH)_3$ and saponin is quite evident from the experiments conducted in both mice and sheep. This formulation has the advantage that a dose of 2,0 mℓ is as effective as a 5,0 mℓ dose and, in addition, the reaction at the injection site is far less severe than that caused by the currently available alum-precipitated product.

Although an absolute immunity was not established in all the sheep, it should be borne in mind that the challenge dose used was probably far in excess of that which would occur under field conditions. Consequently, the vaccine will be expected to be more effective in a situation of natural exposure. This contention should nevertheless be confirmed by extensive field trials.

The mechanism of immunity against *C. pseudotuberculosis* is not yet fully understood. However, in agreement with previous observation (Cameron & Engelbrecht, 1971), it was found that our vaccine, which primarily consists of inactivated bacteria, is superior to a commercial product, which is essentially a toxoid (Nairn, Robertson Middleton & McQuade, 1982). This finding supports the contention that immunity against *C. pseudotuberculosis* is antibacterial rather than antitoxic in nature. The protective role of serum antibodies could not be confirmed in sheep, and it is possible that cellular immune mechanisms may play a prominent role in sheep (Irwin & Knight, 1975). However, the negative results that were obtained could be explained by the fact that in the sheep that were given only 2 injections of vaccine the serum antibody levels were probably far lower than in the hyperimmune sera used previously (Cameron & Engelbrecht, 1971), and therefore could not be detected by passive mouse protection tests. We were unable to show in mice that the route of immunization had any detectable influence on the immune response. However, when the observations of Beh & Lascelles (1981) are taken into account, this finding may not necessarily apply equally to sheep. These questions should be experimentally resolved to obtain a better understanding of the immune response and mechanisms of immunity against *C. pseudotuberculosis* in sheep.

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