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FAILURE TO INDUCE IN RABBITS EFFECTIVE IMMUNITY TO A MIXED INFECTION OF *FUSOBACTERIUM NECROPHORUM* AND *CORYNEBACTERIUM PYOGENES* WITH A COMBINED BACTERIN

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ABSTRACT

CAMERON, C. M. & FULS, W. J. P., 1977. Failure to induce in rabbits effective immunity to a mixed infection of *Fusobacterium necrophorum* and *Corynebacterium pyogenes* with a combined bacterin. *Onderstepoort Journal of Veterinary Research,* 44 (4), 253–256 (1977).

Rabbits were immunized with alum-precipitated, oil adjuvant and an untreated bacterin composed of *F. necrophorum* and *C. pyogenes*. Immunized rabbits were challenged intradermally with a mixture of *F. necrophorum* and *C. pyogenes*. Initially a low level of initial transient resistance could be demonstrated but a solid immunity could not be established.

Résumé

ÉCHEC D'UN ESSAI D'IMMUNISATION EFFICACE DE LAPINS CONTRE FUSOBACTERIUM NECROPHORUM *ET* CORYNEBACTERIUM PYOGENES *AU MOYEN D'UNE BACTÉRINE COMBINÉE*

On a immunisé des lapins au moyen d'une bactérine non traitée composée de F. necrophorum *et* C. pyogenes, *avec un adjuvant huileux précipité à l'alun. Les lapins ainsi préparés ont reçu une injection intradermique d'un mélange de* F. necrophorum *et* C. pyogenes. *On a bien pu observer au début un niveau modéré de résistance passagère, mais il n'a pas été possible d'établir une immunité durable.*

INTRODUCTION

By and large, attempts to immunize animals against infection by *Fusobacterium necrophorum* have been disappointing (Simon & Stovell, 1969). Although serum antibodies are detectable after immunization with various vaccines, the protection afforded against infection is meagre (Alexander, Garcia & McKay, 1973; Warner, Fales & Teresa, 1974; Garcia, Dorward, Alexander, Magwood & McKay, 1974; Roberts, 1970). Immunization is further complicated by toxic factors possessed by *F. necrophorum* (Garcia, Alexander & McKay, 1975; Garcia, Charlton & McKay, 1975).

In sheep, *F. necrophorum* is normally associated with a concomitant infection with *Corynebacterium pyogenes* (Roberts, 1967a & 1967b; Roberts, Graham, Egerton & Parsonson, 1968) and, in the light of this knowledge, it was proposed to establish whether a conjoint immunity to both *F. necrophorum* and *C. pyogenes* would perhaps afford effective protection against these organisms.

MATERIALS AND METHODS

Experimental animals

Groups of six 6-month-old conventionally-reared albino rabbits were used in these studies. They were kept in wire cages and fed a commercial pelleted ration.

Preparation of combined vaccines

C. pyogenes bacterin-toxoid was prepared as described previously except that the final product was not precipitated by alum (Cameron, Botha & Smit, 1976).

F. necrophorum strain 150D* was used to prepare the *F. necrophorum* component of the combined bacterin. Seed material was prepared by growing the organisms for 48 h at 37 °C in tubes of Brewer's thioglycolate medium prepared according to the Oxoid† formula (Simon, 1974). These tubes were used to inoculate a flask of MC medium (Garcia & McKay, 1973) which was filled with a gas mixture composed of 90% H_2 and 10% N_2. The flask was incubated at 37 °C for 48 h and the whole culture inactivated by the addition of 0,5% formalin. This procedure yielded a packed cell volume of 1,0%.

Equal volumes of the *C. pyogenes* bacterin-toxoid and *F. necrophorum* bacterin were mixed and divided into 3 portions. The 1st portion was precipitated with potassium alum (10 ml of an 11% solution per 100 ml) and the 2nd was used to prepare an oil emulsion vaccine as follows:

Solution A:	Bayol 72 (1)	72 ml
	Cirrasol(2)	8 ml
Solution B:	Combined bacterin	19 ml
	Tween 80(3)	1 ml

Solution B (20 ml) was gradually added to Solution A (80 ml) with continuous vigorous shaking.

The 3rd portion was not treated any further.

Immunization of rabbits

All injections were given subcutaneously at the dosages, routes and schedules shown in Tables 1 and 2. The immunized rabbits as well as non-immunized controls were challenged 14 days after the last injection of vaccine.

Since the immune response to either bacterin could possibly be jeopardized when they are mixed together, a 3rd experiment was done in which separately prepared alum-precipitated *F. necrophorum* bacterin and *C. pyogenes* bacterin-toxoid were administered at different sites. Six rabbits were each given 3 subcutaneous injections of 2,5 ml of both preparations at 10-day intervals. Subsequently they were bled and challenged as outlined below.

Infection of rabbits and assay of lesions

The experimental animals were challenged intradermally with a mixture of *F. necrophorum* and *C. pyogenes*. This procedure was used in order to ensure the consistent development of lesions in control animals since neither organisms when injected alone gave satisfactory results.

Freeze-dried cultures of *C. pyogenes* were prepared and the number of organisms per vial determined as described previously (Cameron *et al.,* 1976).

† Obtained from Dr D. F. Stewart, CSIRO, McMaster Laboratory, Glebe, N.S.W. 2037, Australia

* The Oxoid Manual, 3rd Ed. (reprint), 1967

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F. necrophorum was grown in MC medium as for the preparation of the bacterin. After 48 h incubation at 37 °C, the bacteria were collected by centrifugation and resuspended in a solution consisting of equal parts of medium 156 (Simon, 1974) and BLP (1% peptone, 5% lactose in 0,1 M phosphate buffer pH 7,3) and 1,0 ml aliquots lyophilized *in vacuo*. In order to determine the number of viable organisms per vial, ten-fold serial dilutions of the reconstituted dry material were made in medium 156 and counts done on blood tryptose agar plates under hydrogen.

For infecting the rabbits, the dried preparations of both organisms were reconstituted to give a concentration of 10^9 bacteria/ml. The *C. pyogenes* was reconstituted in tryptone water and *F. necrophorum* in medium 156.

Equal portions of the 2 suspensions were mixed and 0,2 ml of undiluted, $\frac{1}{2}$ and $\frac{1}{5}$ dilutions injected intradermally at different sites into each rabbit. Each rabbit was therefore infected with mixtures consisting of 10^8, 5×10^7 and 2×10^7 organism of each bacterium. In one experiment $\frac{1}{10}$ and $\frac{1}{25}$ dilutions were also used.

The diameters of the lesions were measured by means of calipers 4 and 10 days after infection and the surface area of the lesions calculated mathematically. The results are expressed as the average surface area of the lesions in each group.

Serology

In the 2nd and 3rd immunity experiments the rabbits were bled on the day before challenge and the serum stored at −20 °C until it was tested.

C. pyogenes antitoxin titres were determined as described previously (Cameron *et al.*, 1976) while the agglutinin and haemagglutination titres to *F. necrophorum* were determined according to the procedures of Roberts (1970) and Warner *et al.* (1974) respectively. The antigen for the *F. necrophorum* tests was prepared in MC broth and fresh sheep erythrocytes were used in the haemagglutination tests.

Results

Development of lesions

As shown in Fig. 1, in non-immunized control animals, distinct lesions had developed by the 4th day after infection. These lesions spread progressively and by the 10th day (Fig. 2) large necrotic areas had developed.

Immunity experiments

Table 1 shows the results obtained in rabbits that were immunized with either 3 injections of alum-precipitated vaccine or 2 injections of oil adjuvant vaccine. Neither vaccine afforded protection of any consequence but, as there was a very slight indication that the alum-precipitated vaccine might have some effect, larger doses were tried in the 2nd experiment

TABLE 1 Comparison of alum-precipitated and oil adjuvant bacterins

Vaccine	Dosage (ml)	Regimen	Average size of lesions after 4 days (cm^2)			Average size of lesions after 10 days (cm^2)		
			Challenge dose			Challenge dose		
			10^8	5×10^7	2×10^7	10^8	5×10^7	2×10^7
Alum-precipitated.....	1,0	3 injections at 10-day intervals	3,3	3,0	1,6	7,0	5,7	3 9
Oil adjuvant.....	1,0	2 injections at 20-day intervals	4,3	3 3	2,0	10,0	8,6	6,2
Non-immunized controls.....	—	—	4,8	3,3	2,6	12,0	6,2	8,3

TABLE 2 Comparison of different dosages and routes with alum-precipitated and unprecipitated combined bacterins

Vaccine	Dosage (ml)	Regimen and route	Average size of lesions after 4 days (cm^2)			Average size of lesions after 10 days (cm^2)			Average *C. pyogenes* antitoxin units/ml
			Challenge dose			Challenge dose			
			10^8	5×10^7	2×10^7	10^8	5×10^7	2×10^7	
Alum-precipitated.....	5	3 subcutaneous injections at 10-day intervals	3,1	1,7	1,5	7,7	6,2	5,2	366
Alum-precipitated.....	2	3 subcutaneous injections at 10-day intervals	2,5	1,5	1,3	5,7	3,8	3,3	150
Untreated bacterin.....	2	3 subcutaneous injections at 10-day intervals	2,6	2,0	1,4	5,6	6,2	4,5	92
Untreated bacterin.....	2	3 intravenous* injections at 10-day intervals	—	—	—	—	—	—	—
Non-immunized controls	—	—	7,1	3,2	3,1	9,5	6,6	5,5	0

* All 6 rabbits died after the first injection

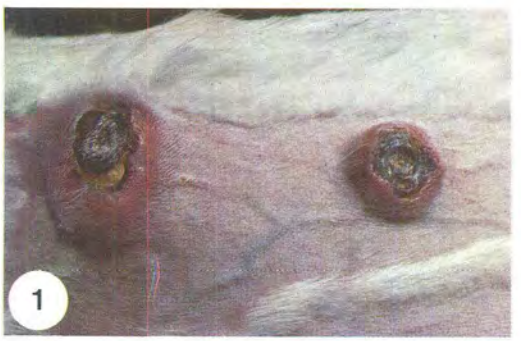

FIG. 1 Skin lesions in a rabbit 4 days after intradermal injection of 10^8 (left) and 5×10^7 (right) *F. necrophorum* and *C. pyogenes*
FIG. 2 Skin lesions in a rabbit 10 days after intradermal injection of 10^8 (left) and 5×10^7 (right) *F. necrophorum* and *C. pyogenes*

TABLE 3 Immunity and antibody response following injection of *F. necrophorum* and *C. pyogenes* bacterin-toxoid at separate sites

Group	Average size of lesions after 4 days (cm^2)					Average *C. pyogenes* antitoxin units/ml
	Challenge dosage					
	10^8	5×10^7	2×10^7	10^7	4×10^6	
Immunized	3,0	2,0	1,1	0,9	0,8	1 707
Non-immunized controls	4,5	4,2	2,5	1,9	1,4	0

However, Table 2 shows that the results were equally disappointing. After 4 days the lesions in the immunized groups were generally smaller than in the control group, but after 10 days there was only a marginal difference.

The *C. pyogenes* antitoxin response was not as good as that produced by a monovalent bacterin-toxoid (Cameron *et al.*, 1976), and no antibodies to *F. necrophorum* could be detected.

The results of an experiment in which *F. necrophorum* bacterin and *C. pyogenes* bacterin-toxoid were given at separate sites are shown in Table 3.

The antitoxin response to *C. pyogenes* was appreciably better than in the previous experiment but again no antibodies to *F. necrophorum* could be demonstrated. Measurement of the lesions 4 days after infection showed a detectable difference between the immunized and control groups but a measurement could not be made 10 days after infection because by this time 5 of the 6 control and 2 of the 6 immunized rabbits had died. The lesions in the surviving animals were very extensive and they were destroyed for humane reasons.

Discussion

Roberts (1970) showed that rabbits immunized with a formalin-killed broth culture of *F. necrophorum* developed humoral bactericidal antibodies which destroy only $\frac{1}{2}$–$\frac{2}{3}$ of a given inoculum either *in vitro* or in the tissues of a vaccinated rabbit. These antibodies had no influence on growth once the organism was established and he concluded that effective protection would probably require a form of vaccination which could induce antibodies able to neutralize the toxin. He also demonstrated that the development of an immediate dermal hypersensitivity jeopardized the establishment of effective immunity.

By using a preparation consisting of a protein protoplasmic toxoid, Garcia *et al.* (1974) were able to reduce the incidence of liver abscess of feedlot cattle from 35%–10%. Recently, Abe, Lennard & Holland (1976) successfully immunized mice with a formalinized bacterin but this could only be accomplished by the administration of 9 or 10 intraperitoneal injections of bacterin over a period of several weeks.

From the literature as well as from our own results it seems possible to induce a low level of resistance to *F. necrophorum* infections by conventional immunization. However, no solid protection is established and there seems to be little prospect of formulating a vaccine which would make a significant impact on the control of *F. necrophorum* infections under natural conditions.

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