

The weak immunogenicity of *Fusobacterium necrophorum*

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SUMMARY

Three of four extreme methods of immunization completely failed to protect mice against challenge with the homologous strain of *Fusobacterium necrophorum*. Unsuccessful vaccines included (1) broth culture killed by mild heat and emulsified with Freund's complete adjuvant, and (2) a homogenate of heavily infected mouse brains, inactivated by mild heat and given in two doses. Also unsuccessful as a method of immunization was the production of a severe subcutaneous infection with *F. necrophorum*, followed by curative treatment with metronidazole. Slight but significant protection against subcutaneous challenge resulted, however, from two such infections given in rapid succession. It would appear that the main virulence factors of *F. necrophorum* are only weakly immunogenic, and the experiments give little encouragement to the prospect of an effective necrobacillosis vaccine.

INTRODUCTION

Fusobacterium necrophorum causes necrobacillosis in many animal species and man (Beveridge, 1959; Prévot, Turpin & Kaiser, 1967; Simon & Stovell, 1969). Infection may be pure or mixed, and substantial economic loss can result from certain forms of the disease in animals. Hepatic necrosis and abscess formation in cattle, bovine foot-rot, calf diphtheria, and sheep foot-rot – a disease in which *F. necrophorum*, *Bacteroides nodosus* and other organisms play a role – are among the more serious manifestations. Losses are not confined to agricultural animals, and Ashton (1982) reported more than 200 deaths from gastric or facial necrobacillosis in Red-necked wallabies (*Macropus rufogriseus*) in the Zoological Society's collection at Whipsnade Park during an explosive outbreak that occurred during the first four months of 1979. Oliphant, Parsons & Smith (1984) showed that in such cases *F. necrophorum* was the main agent, but other organisms were often present also.

There is no doubt that in animals an effective vaccine would be useful. With this in mind numerous workers have, since the beginning of the century, studied immunization against necrobacillosis. The results have been conflicting, as was well illustrated by a review of the literature from 1891 to 1960 (Simon & Stovell, 1969).

The authors of the review commented that the frequent presence of *F. necrophorum* abscesses of different ages within a single bovine liver indicated that natural infections did not confer immunity. Cameron & Fuls (1977) cited more recent studies that suggested at most a meagre protective response to immunization, a suggestion borne out by their own experiments. Other reports have been more encouraging. Thus Garcia *et al.* (1974) claim to have reduced the incidence of *F. necrophorum* liver abscesses in cattle from 35% to 10% by immunization with a toxoid prepared from the cytoplasmic fraction of the organism. Others (Katitch, 1974, 1979; Banting, Bellenger & Turpin, 1977; Katitch & Matitch, 1977; Bernard *et al.* 1978) believe – unlike Egerton & Roberts (1971) – that vaccines containing *F. necrophorum* have a protective effect against sheep foot-rot; and Beveridge (1983) refers to the apparently successful use of *F. necrophorum* vaccine against bovine foot-rot. Recent studies with inactivated vaccine in mice suggested that hyperimmunization by repeated intraperitoneal injections partly protected against challenge by the same route (Abe, Holland & Stauffer, 1978); but a single large intravenous dose failed to protect against small numbers of viable organisms given intracerebrally (Smith, Oliphant & Parsons, 1984).

Against this background of uncertainty the following experiments were designed to give clearer answers to the questions (1) will *F. necrophorum* immunize against itself, and (2) if so, with what difficulty?

MATERIALS AND METHODS

The mice, culture media, anaerobic methods, and viable count technique were as described by Smith, Oliphant & Parsons (1984).

Organisms

The strain (A42) of *F. necrophorum* used throughout was isolated from a wallaby (*Macropus rufogriseus*) with necrobacillosis of the leg and face (Smith, Oliphant & Parsons, 1984). In an experiment to test the immuno-suppressive or immuno-destructive effect of *F. necrophorum*, the Blenheim strain of *Mycoplasma mycoides* subsp. *mycoides* (Smith, 1968) was used.

Killed vaccine containing Freund's complete adjuvant

A 48 h culture of strain A42 in BM medium (Deacon, Duerden & Holbrook, 1978), with a viable count of 3580×10^6 /ml, was killed by heating in a water bath at 56 °C for 30 min and emulsified by means of a high speed homogenizer and 1-inch tubular mixing unit (Silverston Machines Ltd, Chesham, Bucks) with an equal volume of Freund's complete adjuvant (Difco). This stable emulsion was used several hours later in a dose of 0.2 ml per mouse, 0.1 ml being injected subcutaneously into two separate sites on the right side of each animal. The immunized mice, together with untreated controls, were subsequently challenged.

Killed vaccine prepared from infected mouse brain

A number of anaesthetized mice each received intracerebrally 220 000 viable organisms of strain A42 suspended in 0.05 ml of BM medium. Two days later 33 moribund animals were killed, and their brains were removed aseptically and

homogenized with 33 ml phosphate-buffered saline in a Griffith tube. The homogenate, which had a viable count of 306×10^6 /ml and contained a profusion of fusobacteria as shown by a stained smear, was heat-killed at 56 °C for 30 min, dispensed in convenient volumes, and stored at -20 °C until used. Mice were immunized subcutaneously with two doses (0.25 ml, left side) separated by an interval of 10 days. Control mice were treated similarly but with a homogenate prepared from the brains of mice inoculated intracerebrally with sterile BM medium.

Immunization by means of cured infections

Mice were inoculated subcutaneously with a potentially lethal dose of *F. necrophorum* (0.1 ml of undiluted 24 h culture in BM medium) and three days later, when severely ill, were treated with the first of eight daily doses of metronidazole 0.5 % w/v. This drug (Flagyl Injection; May and Baker Ltd), given intraperitoneally (0.8 ml per mouse) at a dose rate of about 200 mg/kg, effected a complete cure. Uninfected control mice were also given metronidazole.

Challenge

Groups of mice were challenged subcutaneously with either (1) 0.1 ml volumes of undiluted 24-h BM culture, or with decimal dilutions thereof in BM medium, or (2) smaller volumes (0.01 or 0.001 ml) of undiluted culture delivered by means of a Microliter syringe (Hamilton Bonaduz AG, PO Box 26, CH-7402 Bonaduz, Switzerland). The challenge injections were given on the side opposite that on which the mice had been immunized. Dilutions for injection were made up and used immediately. Intracerebral challenge was carried out as described by Smith, Oliphant & Parsons (1984) with 0.05 ml volumes of appropriate dilutions in BM medium. Deaths from subcutaneous challenge occurred in 6–16 days and from intracerebral challenge in 1–5 days.

Passive haemagglutination test

Individual mouse sera were tested in parallel with a known positive serum obtained from a rabbit 10 days after the last of six bi-weekly intravenous injections (0.2 ml, increasing to 1.0 ml) of a 48 h culture of *F. necrophorum* strain A42, killed by heating at 56 °C for 30 min. The culture was made in BM medium lacking horse serum.

The test was made in plastic agglutination plates, 0.05 ml of each doubling dilution of serum being mixed with an equal volume of a 1 % suspension of rabbit erythrocytes sensitized with antigenic polysaccharide from strain A42, as described by Warner, Fales & Teresa (1974). The final dilution of serum in the first well of each series was 1 in 20, and the test was read after incubation at 37 °C for 1 h.

Test of the immuno-suppressive or immuno-destructive ability of F. necrophorum

The test was made in four groups of 10 or 9 mice with a mycoplasma immunization system (Smith, 1968, 1969) currently in use in our laboratory. Mice in one group (unimmunized controls) received 0.25 ml of unseeded BVF-OS broth (Turner, Campbell & Dick, 1935) intravenously. Three further groups were immunized intravenously with 0.25 ml doses of heat-killed (56 °C, 30 min) 3-day

culture of *M. mycoides* subsp. *mycoides* in BVF-OS. Challenge of all mice with the homologous strain was carried out 21 days later by the intravenous injection of 17×10^6 viable mycoplasmas suspended in 0.5 ml of BVF-HS (BVF-OS in which ox serum was replaced by horse serum). This represented a 1 in 100 dilution of 3-day culture. Immunity was assessed by the presence or absence of mycoplasmaemia 24 h after challenge. This was shown by culturing one drop of tail-blood in 5 ml of ONB-OS medium (Hooker, Smith & Milligan, 1979) containing penicillin 100 units/ml and thallos acetate 0.05% to inhibit bacterial and fungal growth. After incubation at 37 °C for 7 days the liquid blood cultures were subcultured on blood agar, also containing penicillin and thallos acetate.

Mice in the unimmunized group and in one of the immunized groups remained untreated with *F. necrophorum*. Mice in a third group received, 2 days before immunization, a subcutaneous injection of *F. necrophorum* (0.1 ml of 24 h BM culture), which produced severe lesions and illness; immediately after the mycoplasma immunization these and all other mice in the experiment were given the first of eight daily doses of metronidazole (see above). This treatment completely cured the fusobacterial infection of the group 3 animals. Mice in a fourth group were similarly infected with *F. necrophorum*, but in this instance 2 days before mycoplasma challenge instead of before immunization. The experimental procedures are summarized in Table 5.

RESULTS

Use of killed vaccine with Freund's complete adjuvant

See Table 1. Mice were challenged either subcutaneously or intracerebrally with graded doses of *F. necrophorum*. The mortality in immunized and control mice was similar. Moreover, immunization failed to delay the onset of symptoms or reduce noticeably the interval between the first clinical signs and death.

Use of killed vaccine prepared from infected mouse brain

See Table 2. Two doses of vaccine failed to protect mice against subcutaneous or intracerebral challenge with moderate doses.

Immunization by means of a single cured infection

See Table 3. Mice were treated subcutaneously in the flank with 397×10^6 viable organisms. In some animals this produced lameness within as little as 24 h in the hind leg on the side of inoculation. Within 48 h almost all mice showed lameness, or ulceration, or both near the injection site. At 72 h the mice were severely ill and becoming emaciated. They had extensive necrotic lesions around the injection site (Figs. 1 and 2), impression smears from the diseased tissues showing many fusobacteria (Fig. 3). At this stage the animals received the first of eight daily doses of metronidazole. Considerable clinical improvement was noted within 48 h, and at the cessation of treatment recovery was invariably complete except for hair loss at the site of ulceration and healing. The mice, together with unimmunized controls, were challenged subcutaneously with graded doses 19 days after the final dose of metronidazole.

The cured infection, severe though it had been, failed completely to protect

Table 1. Failure of killed *Fusobacterium necrophorum* vaccine containing Freund's complete adjuvant to protect mice against challenge with the homologous strain

Challenge dose (10^6)	Challenge method	Fatal infections in	
		immunized mice	control mice
82	Subcutaneous	10/10	10/10
8.2	Subcutaneous	7/10	9/10
0.8	Subcutaneous	4/10	2/10
0.4	Intracerebral	7/7	11/11
0.04*	Intracerebral	6/6	9/10

The mice were immunized subcutaneously on day 1 and challenged on day 46. Details of the vaccine are given in Materials and Methods.

* About $5 \times \text{LD}_{50}$ (Smith, Oliphant & Parsons, 1984).

Table 2. Failure of two doses of killed *F. necrophorum* grown in vivo to protect mice against challenge with the homologous strain

Challenge dose (10^6)	Challenge method	Fatal infections in	
		immunized mice	control mice
30	Subcutaneous	15/18	16/18
0.15*	Intracerebral	19/19	18/20

The mice were immunized subcutaneously on days 1 and 11 and challenged on day 24. Details of the vaccine are given in Materials and Methods.

* About $19 \times \text{LD}_{50}$ (Smith, Oliphant & Parsons, 1984).

against subsequent challenge. The course of the disease, as well as the mortality, was similar in the immunized and unimmunized mice.

Immunization by means of two cured infections

See Table 4. Mice were treated subcutaneously on day 1 with 147×10^6 viable organisms in the flank and on day 22 with 102×10^6 in the shoulder region on the same side. Control mice received unseeded BM medium. Both infections resulted in severe illness and necrotic lesions within 3 days, but were completely cured with metronidazole (as above). Subcutaneous challenge (day 43) on the opposite side with three graded doses revealed slight but significant resistance in the immunized mice. The smallest dose showed protection from death ($P < 0.0025$; Wilson & Miles, 1975) but the two larger doses merely an increase in survival time ($P < 0.03$).

Eight immunized mice and an equal number of controls were killed instead of being challenged, and bled by the method of Evans & Perkins (1954) to provide individual serum samples. None gave a positive reaction at a final dilution of 1 in 20 in the passive haemagglutination test, whereas a hyperimmune rabbit serum had a titre of 2560.

*Test of the immuno-suppressive or immuno-destructive ability of *F. necrophorum**

See Table 5. In mice (group 1) that remained untreated with *F. necrophorum* and did not receive mycoplasma vaccine, challenge with *M. mycoides* subsp.

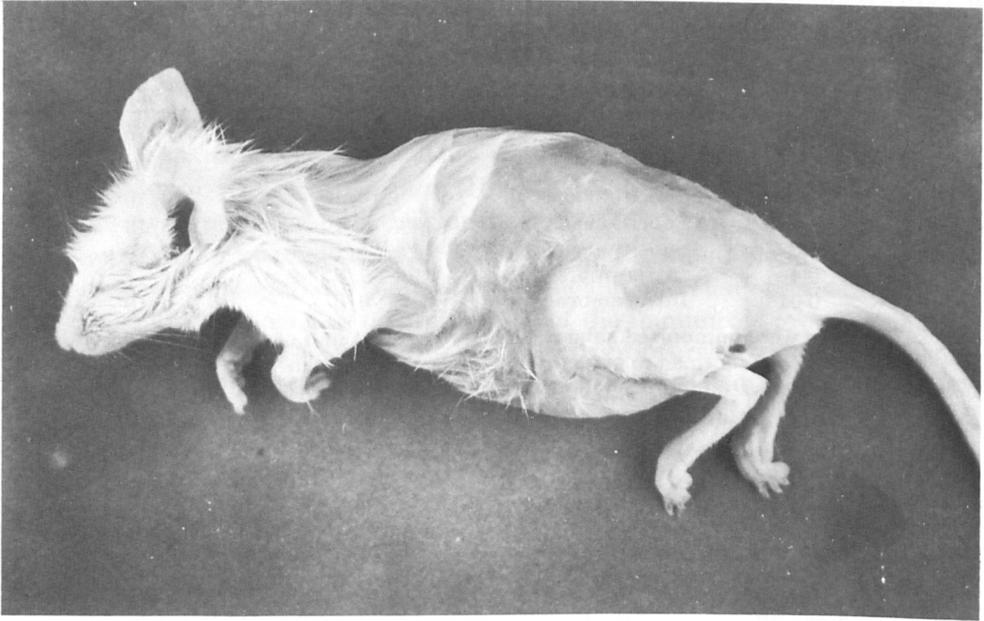


Fig. 1. Necrotic lesion seen through the shaved skin of a mouse 3 days after the subcutaneous injection of 0.1 ml of a 24 h BM culture of *F. necrophorum* strain A42.



Fig. 2. Necrotic lesion (arrows) exposed by dissection in a mouse similar to that shown in Fig. 1.

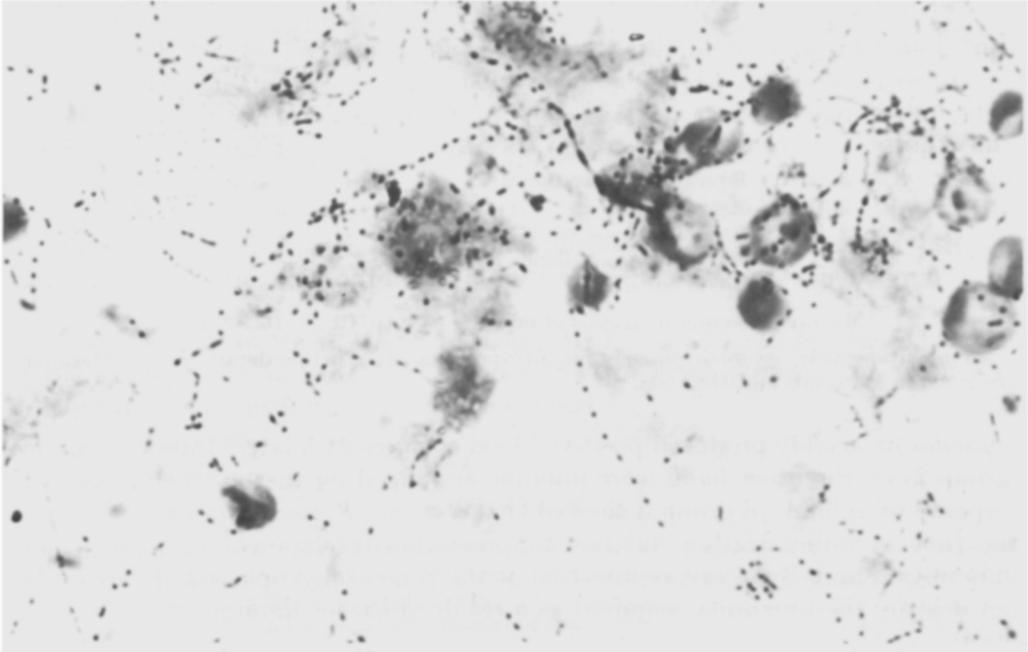


Fig. 3. Impression smear from a necrotic lesion similar to that shown in Fig. 2. Giemsa. $\times 1500$.

Table 3. *Failure of a F. necrophorum infection cured with metronidazole to protect mice against challenge with the homologous strain*

Subcutaneous challenge dose (10^6)	Fatal infections in	
	previously infected mice	control mice
312	14/14	14/14
31.2	14/14	13/14
3.1	3/6	1/6

The mice were infected on day 1, treated (see Materials and Methods) on days 4–11, and challenged on day 30.

Table 4. *Slight protection of mice against challenge with F. necrophorum by two earlier homologous infections cured with metronidazole*

Subcutaneous challenge dose (10^6)	Final mortality in groups of 16	
	previously infected mice	control mice
185	16 (7)*	16 (13)
18.5	16 (3)	16 (10)
1.8	9 (0)	16 (9)

The mice were infected on days 1 and 22, treated (see Materials and Methods) on days 4–11, and 25–32, and challenged on day 43.

* Numbers in parenthesis give the deaths 9 days after challenge, 7 days before the experiment was terminated.

Table 5. *Failure of necrobacillosis to suppress or destroy mycoplasma immunity*

Day	Treatment	Treatments given to mouse group			
		1	2	3	4
1	<i>F. necrophorum</i> sc	—	—	+	—
3	<i>Mycoplasma vaccine</i> iv	—	+	+	+
3–10	Metronidazole ip	+	+	+	+
22	<i>F. necrophorum</i> sc	—	—	—	+
24	<i>Mycoplasma challenge</i> iv	+	+	+	+
25	Blood culture	+	+	+	+
Mycoplasmaemia in groups of mice		10/10	1/10	2/10	2/9

sc, subcutaneously; iv, intravenously; ip, intraperitoneally. Further details of the experiment are given in Materials and Methods.

mycoides invariably produced positive blood cultures 24 h later. Immunized mice (group 2) on the other hand were immune as judged by the blood cultures. The response of animals in-group 3 showed that a severe *F. necrophorum* infection at the time of immunization did not suppress the development of mycoplasma immunity. Similarly, a severe infection at the time of mycoplasma challenge did not destroy the immunity acquired as a result of earlier immunization (group 4 mice).

DISCUSSION

Three of four extreme methods of immunization used in this study failed to produce any trace of resistance against subcutaneous challenge with moderate or graded doses of the homologous strain of *F. necrophorum*. One unsuccessful method, which consisted in the use of heat-killed culture emulsified with a powerful experimental adjuvant (Freund's complete), was open to the possible criticisms that (1) the culture medium, although supporting profuse growth, may not have allowed the organism to produce its full complement of antigens, and (2) the heat-inactivation process, although mild (56 °C, 30 min), might conceivably have destroyed essential antigens. A second unsuccessful method consisted in the use of two spaced doses of organisms grown *in vivo* and therefore presumably equipped with all the necessary protective antigens; once again however the organisms were killed by heat. The third method consisted in producing a severe subcutaneous infection, which was subsequently cured with metronidazole. This procedure, although open to neither of the objections mentioned above, proved no more successful than the earlier methods. A final method, in which mice were infected and cured not once but twice, succeeded in producing increased resistance. This resistance was slight, however, despite the favourable timing of the two immunizing infections and the challenge inoculation.

The great difficulty in producing resistance, and the apparent lack of serological response to a double infection – a point that requires further study – led to an examination of *F. necrophorum* for possible immuno-suppressive or immuno-destructive properties. In mice severe experimental necrobacillosis failed, however, to prevent the development of, or to break down, active immunity against a

mycoplasma infection. The possible effect of necrobacillosis on other immune systems would be of interest.

This clear example of a pathogenic bacterium that immunizes against itself only with extreme difficulty is something of a rarity, but certain parallels can be drawn with the staphylococcus and gonococcus, both of which cause infections that have so far defied immunization. *F. necrophorum* produces an endotoxin, a cytoplasmic exotoxin, a leucocidin, a haemolysin, a lipase and a DNAase, which may be related to pathogenicity (Duerden, 1983). Our experiments in mice offer little encouragement to the prospect of immunization against necrobacillosis in the field, and suggest that the main virulence factors are of weak immunogenicity.

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