Experimental observations on the pathogenesis of necrobacillosis

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SUMMARY

Earlier studies showed that the minimum infective dose ($> 10^6$ organisms) of a virulent strain of *Fusobacterium necrophorum* could be greatly reduced by suspending the fusobacteria in sub-lethal doses of cultures of other bacteria such as *Escherichia coli* before inoculating mice subcutaneously.

In the present study the infective dose of the same strain of *F. necrophorum* was reduced by a factor of $> 10^3$ by suspending the fusobacteria in sub-lethal doses of 5% homogenate of gaur or wallaby faeces. Sterile faecal filtrate had no such effect. The sites of low grade infection produced by the prior subcutaneous injection of *E. coli* culture or gaur faecal suspension were susceptible to superinfection by doses of *F. necrophorum* far below those required to infect normal tissue.

This work helps to explain the production of necrobacillosis by the faecal contamination of small wounds. It proved impossible, however, to produce necrobacillosis in mice by the subcutaneous injection of faecal suspensions from 33 farm cattle. This suggests that the proportion of cattle with virulent *F. necrophorum* in their faeces is low.

INTRODUCTION

Necrobacillosis, caused by *Fusobacterium necrophorum*, occurs in a wide range of domesticated and wild animals and occasionally in man. In zoological collections it is the commonest affliction of macropods (1). So called biotype A (phase A) strains of *F. necrophorum* (2, 3) are haemolytic, haemagglutinating, highly leucocidinogenic, and pathogenic for mice; biotype B strains produce less leucocidin, are haemolytic but not haemagglutinating, and have no more than slight pathogenicity for mice. *F. necrophorum* is a normal inhabitant of the alimentary tract of herbivores and other species including pigs and dogs. In this situation most but not all strains belong to biotype B, whereas necrobacillosis lesions more often contain biotype A (4, 5). It would appear that infection is usually endogenous and that the source of organisms is often the faeces. *F. necrophorum* has little ability to invade intact epithelium, and its entry into the tissues depends on epithelial damage brought about by trauma, maceration or infection (bacterial or viral). Almost any part of the body may be affected, but

necrobacillosis occurs notoriously in the mouth, foot and liver, the latter usually being infected haematogenously from a primary lesion elsewhere.

The causative agent, *F. necrophorum*, is sometimes isolated in pure culture from the lesions, but other organisms often present include many that originate in the gut and others that inhabit the skin and mucous membranes. An investigation of the possible role of such organisms in necrobacillosis (6) revealed that the subcutaneous infectivity of *F. necrophorum* for mice was greatly enhanced by suspending the organisms in sub-lethal doses of cultures of other bacteria before injection. Thus, for example, suspension of *F. necrophorum* in *Escherichia coli* broth culture reduced the minimum infective dose of fusobacteria from $> 10^6$ to < 10. *Citrobacter freundii* and *Actinomyces* (*Corynebacterium*) *pyogenes* produced effects similar to that of *E. coli*. An α-haemolytic streptococcus, *Pseudomonas aeruginosa*, *Bacteroides fragilis* and *Fusobacterium nucleatum* also enhanced the infectivity of *F. necrophorum*, though less strikingly than *E. coli*.

An extension of this work forms the subject of the present report, which includes observations on the effect of (*a*) pre-existing subcutaneous infections on a superimposed fusobacterial infection, and (*b*) sub-lethal doses of faecal suspension on the subcutaneous infectivity of *F. necrophorum*. It also reports an attempt to produce necrobacillosis in mice by the subcutaneous injection of suspensions of cattle faeces.

MATERIALS AND METHODS

The bacterial strains (*F. necrophorum* A42 and *E. coli* NCTC 10418), culture media, anaerobic methods, viable count technique, and mice were as already described (6, 7).

Suspensions (homogenates) of faeces from a gaur, a wallaby, and 33 farm cattle

Suspensions were prepared from gaur (*Bos gaurus*) and wallaby (*Macropus rufogriseus*) faeces as follows. Within 1 h of being passed, each faecal sample was homogenized in BM broth (8) by means of an Atomix Emulsifier (MSE Scientific Instruments, Crawley, West Sussex) to form a 5% w/v suspension. This was immediately subjected to a viable count, anaerobically and aerobically, and injected into mice without delay. When necessary for control purposes, faecal suspension was sterilized by centrifugation and membrane filtration.

Faecal samples from 33 farm cattle were taken directly from the rectum and within 3 h were homogenized (5% w/v) individually in BM medium by means of mortar and pestle. These homogenates were injected into mice immediately.

Inoculation with F. necrophorum suspended in gaur or wallaby faecal homogenate

Mice were inoculated subcutaneously on the outer aspect of the thigh, in dose volumes of 0·1 ml, with decimal dilutions of *F. necrophorum* culture prepared in a diluent consisting of a 5% w/v suspension of gaur faeces containing $< 2·5 \times 10^6$ intestinal bacteria/ml, or of wallaby faeces containing 67×10^6/ml.

Superimposition of F. necrophorum infection on a pre-existing E. coli infection

Mice were inoculated subcutaneously on the outer aspect of the thigh with 0·1 ml of an 18 h culture of *E. coli* in BM broth (227×10^6 viable organisms). This

treatment was found previously (6) to produce a subcutaneous infection lasting more than 7 days, with a comparatively mild local lesion that usually ulcerated before healing. At intervals (1, 3 and 7 days) after infection, sub-groups of the mice were inoculated with 0·01 ml volumes of an 18 h BM culture of *F. necrophorum*, diluted 1 in 10^5. The inocula, which contained 13–49 fusobacteria, were injected subcutaneously into the pre-existing *E. coli* lesions, which had not yet ulcerated.

Superimposition of F. necrophorum *infection on a pre-existing infection produced by injecting gaur faecal suspension*

Mice were inoculated subcutaneously on the outer aspect of the thigh with 0·5 ml of a 5 % w/v suspension of gaur faeces containing *c*. 110000 intestinal bacteria/ml. During the next week 17 % of the mice died. On the 8th day after infection the majority of the survivors – all of which appeared healthy – had small spherical lesions which could be palpated through the skin. Animals with such lesions, which on investigation proved to be abscesses 2–3 mm in diameter, were used on the 8th day for the experiment. With a microsyringe and 26 gauge needle the *F. necrophorum* inocula (dilutions in BM broth of an 18 h BM culture; dose volume 0·01 ml) were injected into the lesions, held between thumb and forefinger.

Assessment of experiments in which mice were inoculated with F. necrophorum *culture*

Mice were observed for the occurrence of severe and progressive necrobacillosis, the first sign of which was lameness. Because the disease was known to be inevitably fatal, the mice were killed as soon as diagnosis was certain. Anaerobic and aerobic cultures on blood agar, made from a representative selection of affected animals, invariably yielded large numbers of *F. necrophorum*, either alone or more often with smaller numbers of the other bacteria injected. Surviving mice were kept for at least 2 weeks before being discarded.

Attempt to produce necrobacillosis by inoculating mice with faecal suspensions (5 % w/v) from 33 farm cattle

The faecal suspensions were derived from 18 cows on 'farm A', which was apparently free from necrobacillosis, and from 15 young male cattle on 'farm B', on which necrobacillosis of the foot ('foul-in-the-foot') had necessitated the treatment of several animals within the preceding month. The male cattle belonged to a group of *c*. 50 animals, penned in a large barn with manure underfoot.

Each faecal suspension was injected subcutaneously on the outer aspect of the right thigh of three mice that had been pretreated subcutaneously in the left thigh with 0·1 ml of Mixed Gas Gangrene Antitoxin (Wellcome) containing 12·5 units of Tetanus Antitoxin (Wellcome). Faecal suspensions from farms A and B were injected in doses of 0·5 ml and 0·25 ml respectively.

Mice that died or were killed when severely ill were examined (*a*) by macroscopic inspection, for lesions resembling those of necrobacillosis, (*b*) by smears stained by Giemsa's method, for organisms resembling *F. necrophorum*, and (*c*) by culture, for *F. necrophorum*. Apart from healing ulcerations, the survivors were all apparently normal 2 weeks after inoculation.

Table 1. *Enhancement of the infectivity of* F. necrophorum *by suspending the organisms in faecal homogenates*

Experiment no.	Suspending medium	Dose of F. necrophorum	Cases of necrobacillosis in groups of mice
1	Homogenized faeces from a gaur (HFG)	152000	6/6
		15200	6/6
		1520	4/6
		152	3/6
		15	0/6
		0*	0/12
	Sterile filtrate of HFG	152000*	0/12
	Sterile BM medium	152000*	0/12
2	Homogenized faeces from a wallaby	1375000	6/6
		137500	3/6
		13750	3/6
		1375	1/6
		137	0/6
		13	0/6
		0*	0/12
	Sterile BM medium	1375000*	0/12

*Controls.

Table 2. *Enhancement of the infectivity of* F. necrophorum *by superimposing infection on a pre-existing lesion produced by the injection of* E. coli

Dose of F. necrophorum*	Cases of necrobacillosis in groups of six mice that received F. necrophorum* at the site of an E. coli lesion aged (days)		
	1	3	7
13	6	—	—
42	—	4	—
49	—	—	1

Controls. Eight normal mice given 130000 *F. necrophorum* (dose volume 0·01 ml) remained healthy. Of 12 mice given *E. coli* alone, 1 died in < 24 h; the remaining 11 (of which 6 were inoculated on day 3 at the site of the *E. coli* lesion with 0·01 ml sterile BM broth) survived.
* Dose volume 0·01 ml in BM broth.

RESULTS

Enhancement of the infectivity of F. necrophorum *by suspending the organism in faecal homogenates*

Suspension of *F. necrophorum* in a 5% w/v homogenate of gaur faeces greatly enhanced the organism's infectivity, reducing the infective dose by a factor of > 10^3 (Table 1). The faecal homogenate lost its effect when sterilized by membrane

Table 3. *Enhancement of the infectivity of* F. necrophorum *by superimposing infection on a pre-existing lesion produced by the injection of gaur faecal suspension 8 days earlier*

Dose of F. necrophorum*	Cases of necrobacillosis in groups of mice inoculated with *F. necrophorum* at the site of a lesion caused by prior injection of faecal suspension
225000	4/6
22500	4/6
2250	4/6
225	1/6
22	1/6
2	0/6

Controls. Ten normal mice that received 225000 *F. necrophorum* organisms remained healthy; 14 mice inoculated with 0·01 ml of sterile BM broth at the site of a lesion caused by prior injection of faecal suspension remained unaffected.

* Suspended in 0·01 ml of BM broth.

filtration. Table 1 also shows that a homogenate of wallaby faeces exerted a similar effect. By themselves, the faecal homogenates were not lethal.

F. necrophorum *infection superimposed on a pre-existing* E. coli *infection*

Minute doses of *F. necrophorum* were injected into subcutaneous *E. coli* lesions aged 1, 3 and 7 days. Table 2 shows that the *E. coli* infection enabled the fusobacterium to produce necrobacillosis in doses at least 10^3 times smaller than those that would otherwise have been required.

F. necrophorum *infection superimposed on a pre-existing lesion caused by injecting faecal suspension*

Table 3 shows that the infectivity of *F. necrophorum* was strikingly enhanced when the organism was injected into the small abscesses produced by subcutaneous inocula of gaur faecal suspension given 8 days earlier.

Unsuccessful attempt to produce necrobacillosis by inoculating mice with suspensions of faeces from 33 cattle

Each suspension was injected subcutaneously into a group of three mice. The numbers of animals that died or were killed when severely ill are given below. (The number of affected mice in a group of three is followed, in brackets, by the number of faecal suspensions giving that result.) Farm A, 3/3 (1); 2/3 (1); 1/3 (5); 0/3 (11). Farm B, 3/3 (1); 2/3 (3); 1/3 (6); 0/3 (5).

Examination of the dead mice revealed that none of the 33 faecal suspensions produced necrobacillosis.

DISCUSSION

An earlier study (6) showed that the presence of sub-lethal doses of certain bacteria reduced the minimum infective dose of *F. necrophorum* by $10–10^6$ times, depending on species. The main aim of the experiments reported here was to

extend this observation by methods that bore a closer resemblance to the events that probably influence the pathogenesis of necrobacillosis in the field.

The experiments suggested that the sites of low grade subcutaneous infection by organisms such as *E. coli* or by the mixtures of bacterial species to be found in faeces are susceptible to superinfection by minute doses of *F. necrophorum* – doses far smaller than those required to infect normal tissue. Moreover, the infectivity of *F. necrophorum* was greatly enhanced by suspending the organisms in faecal homogenate.

These observations would seem to provide a convincing explanation of the mechanism by means of which the faecal contamination of small wounds leads to necrobacillosis. It was found, however, that the subcutaneous inoculation of mice with faecal suspensions from a considerable number of cattle failed to produce necrobacillosis. It seems justified to conclude that none of the 33 faecal suspensions examined contained – even in small numbers – a strain of *F. necrophorum* with virulence resembling that of the experimental strain A42.

A more complete understanding of the pathogenesis of necrobacillosis requires further study of faecal carriage of virulent strains of *F. necrophorum*, both in normal herds and in those in which the disease is enzootic. The present study suggests that the proportion of animals in which such carriage occurs is low.

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