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Pathogenicity of *Fusobacterium necrophorum* strains from man and animals

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SUMMARY

Necrobacillosis occurs in man and animals. The typical forms of the disease in animals are caused by *Fusobacterium necrophorum* biovar A; biovar B strains are much less pathogenic. In this study the pathogenicity for mice of eight human isolates of *F. necrophorum* was compared with that of animal biovar A and B strains.

By subcutaneous inoculation seven of the human strains differed from biovar A but resembled biovar B in (1) producing, at the most, mild local lesions that rapidly healed, and (2) showing no enhancement of infectivity when suspended in sub-lethal doses of *Staphylococcus aureus* broth culture. The eighth human strain (A2433) resembled biovar A but differed from biovar B in (1) producing severe lesions, and (2) showing greatly enhanced infectivity in the presence of *S. aureus*. Nonetheless, strain A2433 differed from biovar A, both in the nature of the lesions produced and in its failure to cause severe general signs of illness and rapidly fatal infection.

By intravenous inoculation one of two biovar B strains and all except one of the eight human strains produced purulent lesions, often severe, in the liver and elsewhere, but infection was not usually associated with general signs of illness. In contrast, intravenous injection of a biovar A strain gave rise to a rapidly fatal infection, with severe lesions in the liver or elsewhere.

The results suggest that the term 'necrobacillosis' as used in human and veterinary medicine refers to diseases that differ in important respects.

INTRODUCTION

Necrobacillosis is caused by the Gram-negative anaerobe, *Fusobacterium necrophorum*, first described in 1884 by Loeffler, who recognized it as the cause of 'calf diphtheria'. The organism has two biovars (phases, biotypes), A and B [1–4]. Biovar A strains are haemolytic, haemagglutinating and highly leucocidinogenic, and produce lethal necrotizing infections in mice; biovar B strains produce less leucocidin, are haemolytic but not haemagglutinating, and are much less pathogenic for mice.

Necrobacillosis in man is a severe disease which particularly affects children and adolescents [5, 6]. It often takes the form of throat infection followed by spread via the blood stream to other sites, and abscesses may occur in lung, liver, brain, bone

and elsewhere. The work of Beerens [7] and Fievez [1] suggested that human strains frequently belonged to biovar B.

Necrobacillosis in animals is characterized by necrotic lesions, often severe, which may contain *F. necrophorum* alone but more commonly contain in addition a variable population of other bacteria (anaerobes, facultative anaerobes, aerobes). Agricultural and wild animals, especially macropods, deer and antelope [8], are susceptible. Lesions can occur in almost any part of the body but are found most commonly in the mouth (e.g. calf diphtheria), foot (e.g. bovine foot rot) and liver. The disease in animals is produced by biovar A strains, but biovar B may sometimes also be found in the lesions, probably as an occasional member of the additional microflora that often accompanies the causative organism. Biovar A strains are commonly present as part of the normal commensal microflora in the bovine rumen [9] but are outnumbered by biovar B in both the rumen and elsewhere in the alimentary tract [10]. Necrobacillosis of the liver of ruminants is believed to arise haematogenously from lesions of the ruminal mucosa [11]. Necrobacillosis of the body surface, however, is caused by the contamination of small wounds and abrasions with infected faeces, though under normal circumstances faecal excretion of biovar A organisms occurs in only a minority of animals [9].

It was shown by Smith and colleagues [12, 13] that the minimum infective (lethal) dose of biovar A strains for mice by subcutaneous inoculation could be reduced from $> 10^6$ to < 10 organisms by suspending the fusobacteria in sub-lethal doses of broth cultures of certain other bacteria, for example *Staphylococcus aureus*. Biovar B strains were not susceptible to such enhancement of infectivity [14].

The present report describes experiments in which eight human strains of *F. necrophorum* were tested for pathogenicity in mice by (1) subcutaneous inoculation, with and without *S. aureus*, and (2) intravenous inoculation. The behaviour of these human strains was compared with that of animal strains examined in extensive earlier studies in mice [12–15]. However, limited tests with animal strains were needed to supplement existing knowledge. The main purpose of the work was to discover whether the term 'necrobacillosis' as used in human and veterinary medicine refers to diseases that are essentially similar or different.

MATERIALS AND METHODS

Culture media, anaerobic methods and viable count technique were essentially as already described [16].

Mice

Female Swiss white mice weighing *c.* 20 g were obtained from an outbred closed colony.

Organisms

Eight human and three animal strains of *F. necrophorum* were used. The collection of human strains, supplied by Dr J. S. Brazier and Professor B. I. Duerden (PHLS Anaerobe Reference Unit, University Hospital of Wales,

Cardiff), comprised: A2433, R3949, R4014, R4240, R4267 (all from blood cultures), B49 (cerebrospinal fluid), M3141 (ear infection), and R4065 (neck abscess). Strain A42, a biovar A isolate from a wallaby (*Macropus rufogriseus*) with necrobacillosis has been described previously and used extensively in laboratory experiments [13]. Strains FN2035 and FN2079, both biovar B isolates from bovine liver abscesses, were supplied by Dr J. N. Berg (College of Veterinary Medicine, University of Missouri, Columbia, USA) and have been described previously [14, 17]. All strains produced lipase to a varying degree as shown by growth on Columbia Agar Base (Oxoid, CM331) containing Egg Yolk Emulsion (Oxoid, SR047C) 5%. The conversion of lactate to propionate, a property particularly useful in identifying *F. necrophorum* [18], was confirmed by gas liquid chromatographic analysis kindly carried out by Mr M. J. Hudson (Centre for Applied Microbiology and Research, Porton Down). Except for strain A42, which had undergone no more than 10 subcultures since isolation, the history of laboratory subculture of the strains was unknown.

A strain of *Staphylococcus aureus* was that used in earlier studies to increase the infectivity of *F. necrophorum* [9, 13, 14, 19].

Inocula

The strains of *F. necrophorum* were grown for 24–48 h in BM broth [20]. Mice were inoculated subcutaneously on the outer aspect of the right thigh, or intravenously, with 0·1 ml doses of pure culture, either undiluted or diluted in BM broth. Further mice were inoculated subcutaneously with 0·1 ml volumes of BM cultures diluted in a well-grown 24 h BM broth culture of *S. aureus*. The *S. aureus* culture by itself in a dose of 0·1 ml produced only a mild ulcerating lesion which healed rapidly.

RESULTS

Subcutaneous inoculation of mice with eight human strains of F. necrophorum, *with and without* S. aureus

One strain (A2433) behaved differently from the other seven. Undiluted broth culture of this strain produced severe lesions at the injection site in a majority of mice, and culture diluted tenfold in a minority; infectivity was greatly enhanced when the inoculum contained *S. aureus*, even five A2433 organisms producing severe lesions in 3 of 4 mice (Table 1). The development of severe lesions was accompanied during life by lameness in the right hind leg, swelling around the injection site, and ulceration; however, general signs of illness were slight, though some mice lost their sleekness. The severity of the lesions became clear only after the animals were killed, depilated, and dissected, 25 days after inoculation. A large brown-black scab covered a lesion consisting of pale green caseous material which often involved most of the thickness of the thigh.

Seven further human strains were each used to inoculate mice in 2 groups of 4; one of these groups received undiluted broth culture and the other group culture diluted 1 in 10 in *S. aureus* broth culture. The seven strains with, in parenthesis, the number of viable organisms (millions) in 0·1 ml of undiluted broth culture were as follows: B49 (25), M3141 (50), R3949 (5), R4014 (10), R4065 (40), R4240 (20)

Table 1. *Lesions in mice killed 25 days after subcutaneous inoculation with strain A2433*

Inoculum (0·1 ml) contained		Mice with severe lesions in groups of 4 given A2433 culture diluted in	
A2433 culture diluted 1 in	A2433 organisms in a dose of	Broth	*S. aureus* broth culture
1	57×10^6	3*	—
10	$5{\cdot}7 \times 10^6$	1	4
10^2	570000	0	—
10^3	57000	—	4
10^5	570	—	4
10^7	5	—	3*

* The fourth mouse had a small lesion.

Eight mice given *S. aureus* culture alone (0·1 ml) developed mild lesions which ulcerated and healed.

and R4267 (22). The mice, 56 in all, were observed for 25–27 days. The majority (44) showed either no effects or, at most, mild ulcerating lesions at the injection site, which were healed or healing at the termination of the experiment. The other 12 animals, most of which had received inocula not containing *S. aureus*, showed in addition a swelling at the injection site; the swellings, usually small but in one instance large, had disappeared by the end of the experiment. These seven fusobacterial strains thus differed from strain A2433 in being much less pathogenic by subcutaneous inoculation and in being insusceptible to infectivity-enhancement by *S. aureus*.

Like animal biovar A and B strains, the eight human isolates given subcutaneously produced no lesions at sites other than the site of inoculation.

Intravenous inoculation of mice with eight human and three animal strains of F. necrophorum

The experimental design and results are shown in Table 2. Of the eight human strains, one (M3141) had no apparent effect. The other seven human strains all produced lesions, but except for A2433 the dose required was never less than $1{\cdot}5 \times 10^6$ viable organisms; depending on the strain, doses ranging from $0{\cdot}05 \times 10^6$ to $5{\cdot}2 \times 10^6$ proved ineffective. Of the mice found at necropsy to have lesions, 66% were killed before the termination of the experiment because of swellings, sometimes associated with lameness, or occasionally other clinical signs. The distribution of lesions in affected mice was as follows: liver alone, 24%; liver and other sites, 18%; non-hepatic sites only, 58%.

Lesions in the liver of mice given human strains were circumscribed, cream in colour, single or multiple and coalescing, and 1–9 mm in diameter. They were often present in more than one lobe and sometimes occupied as much as 70% of the entire organ. In consistency they varied from liver-like to near purulent. Severe hepatic lesions were often not associated with obvious clinical signs. Lesions in sites other than the liver consisted of abscesses, single or multiple, containing firm cream coloured pus or caseous material. These lesions, varying in size up to 1·2 cm in diameter, often occurred on the hindquarters, where they were sometimes

Table 2. *Intravenous inoculation of mice with eight human and three animal strains of* F. necrophorum

Strain	Dose (10^6) of organisms	Mice inoculated	Mice with lesions in: Liver only	Liver and other sites	Non-hepatic sites only	Mice without lesions
A2433	67	6§	0	5	1	0
	6·7	6§	0	0	4	2
	5·1	6	1	2	3	0
	0·7	6§	0	0	2	4
	0·5	6	0	0	1	5
	0·05	6	0	0	0	6
B49	62	5	3	1	1	0
	52	6	2	0	2	2
	21	8	3	0	0	5
	6·2	5	1	0	0	4
	5·2	6	0	0	0	6
M3141	31	5	0	0	0	5
	12	8	0	0	0	8
R3949	17	6	0	0	4	2
	1·7	6	0	0	2	4
	0·2	6	0	0	0	6
R4014	9	8	0	2	0	6
	5	6	1	0	2	3
	0·5	6	0	0	0	6
R4065	35	6	0	0	4	2
	4	8	0	0	2	6
	3·5	6	0	0	0	6
R4240	20	6	0	1	3	2
	2	6	0	0	1	5
	1·5	8	3	2	2	1
	0·2	6	0	0	0	6
R4267	15	6	0	1	4	1
	3	8	4	0	2	2
	1·5	6	0	0	0	6
FN2035*	15	6	0	0	4	2
	1·5	6	0	0	0	6
FN2079*	27	6	0	0	0	6
A42†	85	6‖	4‡	0	2	0
	8·5	6	0	0	0	6

* Bovine biovar B strains.
† Biovar A strain from a wallaby.
‡ In three mice infection extended to abdominal wall.
§ Mice killed 16 days after inoculation.
‖ Mice died (or killed because ill) $\leqslant$ 4 days after inoculation.
Of mice other than those marked § or ‖, *c.* 70% were killed 20–22 days after inoculation, but the remainder were killed earlier because of visible swellings or other symptoms.

visible as swellings during life. Other favoured sites were the stifle, the outer aspect of the chest wall, and the dorsal region of the peritoneal cavity. The forelimbs, shoulder region and lung were occasionally affected. Specific lesions were sometimes accompanied by enlargement of the spleen.

One bovine biovar B strain was without apparent effect, but the other (FN2035) gave rise to lesions resembling those produced by the human strains.

In a dose of 85×10^6 organisms the wallaby biovar A strain, A42, produced a gradually developing weight loss and severe illness which in 3–4 days was either fatal or severe enough to necessitate euthanasia. The hepatic lesions, produced in 4 of the 6 inoculated mice, were yellowish, necrotic and non-purulent, affecting 25–50% of the liver tissue and often spreading by extension to the abdominal wall. The other two mice had similar lesions, surrounded by gelatinous oedema, in the region of the stifle. A dose of $8{\cdot}5 \times 10^6$ organisms was without apparent effect.

DISCUSSION

The eight human strains, including one atypical isolate (A2433), differed in their pathogenicity for mice from biovar A strains (as exemplified by the well described [13] strain A42), which cause necrobacillosis in animals.

By subcutaneous injection of doses ranging from 5×10^6 to 50×10^6 (mean 25×10^6) viable organisms, all the human strains except A2433 resembled two animal biovar B isolates [14] in producing, at most, mild ulcerating lesions that rapidly healed. This contrasts with strain A42 which, in a subcutaneous dose of $14{\cdot}3 \times 10^6$ organisms killed 25 of 26 mice in 6–9 days [21]. The same seven human strains, like biovar B but unlike A42 and six further biovar A isolates tested previously [13], showed no enhancement of infectivity in the presence of large but sub-lethal doses of *S. aureus* broth culture.

In contrast to the other seven human strains, the atypical human isolate (A2433) given subcutaneously produced severe lesions in doses of $\geqslant 5{\cdot}7 \times 10^6$ organisms, and in a dose as small as five organisms when the fusobacteria were suspended in *S. aureus* broth culture. In these properties A2433 resembled biovar A strains. However, it differed strikingly from such strains in the appearance of the lesion produced and in the clinical effects of infection. A2433 produced at the inoculation site slowly progressive and eventually very severe lesions containing pale greenish caseous material; however, even 3 weeks or more after infection the lesions, although by this time very extensive, were accompanied by only mild if any general signs of illness. This differs strikingly from the effects of biovar A strains such as A42, which, given subcutaneously, produce a rapidly extending necrotic lesion, surrounded by gelatinous oedema, and severe signs of illness [22] leading to death in *c.* 6–14 days.

In contrast to inoculation by the subcutaneous route, all but one (M3141) of eight human strains injected intravenously gave rise to lesions, usually of considerable size. These were sometimes visible during life as swellings but were usually unaccompanied by general signs of illness. Except for strain A2433, the dose required to produce lesions was never less than $1{\cdot}5 \times 10^6$ viable organisms. The lesions produced by the human strains resembled those resulting from intravenous infection with the animal biovar B strain, FN2035.

Intravenous injection of 85×10^6 organisms of the animal biovar A strain, A42, gave rise to gradually developing weight loss and illness which was fatal or near-fatal in 3–4 days. This, together with the nature of the lesions, clearly distinguished A42 from the human isolates and animal biovar B strains. The complete failure of a smaller dose ($8{\cdot}5 \times 10^6$ organisms) of A42 to produce disease was probably due to the increased physical separation of fusobacterial cells that inevitably results

from injection into the bloodstream. It has been shown in mice inoculated subcutaneously that the mortality produced by 0·01 ml of A42 broth culture is almost invariably greater than that produced by 0·1 ml of a 1 in 10 dilution [23].

In conclusion, the results of pathogenicity tests in mice showed that eight human strains of *F. necrophorum*, although not homogeneous, all differed from biovar A strains, which cause necrobacillosis in animals. Seven resembled biovar B strains. The eighth (A2433) was more pathogenic than the others and its infectivity, like that of biovar A isolates but unlike that of biovar B isolates or of the other seven humans strains, was enhanced by the presence of *S. aureus*. It would seem from this study therefore that the term 'necrobacillosis', as used by medical and veterinary microbiologists, refers to diseases that differ in important respects.

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