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Further observations on enhancement of the infectivity of *Fusobacterium necrophorum* by other bacteria

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SUMMARY

It had already been shown with a single virulent strain (A42) of *Fusobacterium necrophorum* that suspension of the fusobacteria in sub-lethal doses of broth cultures of other bacteria reduced the minimum infective dose ($> 10^6$ organisms) for mice by subcutaneous inoculation, sometimes to < 10 organisms. The present study extended the known range of bacteria with strong infectivity-enhancing properties to include *Bacillus cereus*, *Klebsiella oxytoca* and *Staphylococcus aureus*; and of those with weaker effect to include *Bacillus subtilis*, '*Bacteroides melaninogenicus*', *Clostridium sporogenes*, *Pasteurella haemolytica*, and *Proteus mirabilis*.

The study also showed that five further virulent strains of *F. necrophorum* closely resembled A42 in respect of striking susceptibility to infectivity enhancement by *Escherichia coli*, *Actinomyces* (*Corynebacterium*) *pyogenes* and *S. aureus*. One further strain (A6) of *F. necrophorum* resembled A42 in respect of strong infectivity enhancement by *A. pyogenes*, *S. aureus*, *B. cereus* and *K. oxytoca* but differed from it and the other five strains in being only slightly affected by *E. coli*.

This work was a necessary prelude to the development of a method, based on infectivity enhancement, for the detection and isolation of *F. necrophorum* present in small numbers in heavily contaminated material such as faeces.

INTRODUCTION

It was recently shown with a virulent strain (A42) of *Fusobacterium necrophorum*, capable of producing fatal necrobacillosis in mice by subcutaneous inoculation, that the minimum infective dose ($> 10^6$ organisms) could be greatly reduced by suspending the fusobacteria in sub-lethal doses of cultures of certain other bacteria [1]. For example, suspension in *Escherichia coli* culture reduced the minimum infective dose to < 10 organisms. *Citrobacter freundii* and *Actinomyces* (*Corynebacterium*) *pyogenes* had a similar effect. An α-haemolytic streptococcus, *Pseudomonas aeruginosa*, *Bacteroides fragilis* and *Fusobacterium nucleatum* also enhanced infectivity, but less strikingly.

The aims of the present study were (*a*) to test the susceptibility to infectivity enhancement of a number of virulent *F. necrophorum* isolates other than strain A42, and (*b*) to test the infectivity-enhancing properties of an extended range of

bacteria. The study was made as a necessary prelude to the development of an efficient *in vivo* method, based on infectivity enhancement, for the detection and isolation of *F. necrophorum* present in small numbers in heavily contaminated materials such as faeces – an objective that has since been attained and reported [2].

MATERIALS AND METHODS

The mice, culture media, anaerobic methods and viable count technique were essentially as already described [3].

Organisms

F. necrophorum strain A42, isolated from a wallaby (*Macropus rufogriseus*) with necrobacillosis, has been used extensively in laboratory experiments [1–8]; strains A10 and A70 were also isolated from wallabies with necrobacillosis and strain A6 [3] originated from a roan antelope (*Hippotragus equinus*) with a subcutaneous abscess; strains L6 and L13 originated from farm cattle litter and strain F1 from the rectal faeces of a calf. All of these *F. necrophorum* strains were capable of producing fatal necrobacillosis in mice inoculated subcutaneously with 0·1 ml of an 18 h culture in BM [9] broth.

Actinomyces (*Corynebacterium*) *pyogenes*, '*Bacteroides melaninogenicus*' (strains 1, 2 and 3), *Klebsiella oxytoca*, *Proteus mirabilis* and *Staphylococcus aureus* were isolated from cases of bovine endometritis, all of which yielded mixtures of bacterial species.

All the organisms mentioned so far had undergone less than 10 subcultures since isolation. The following strains were of unknown laboratory history: *Bacillus cereus* strain F1093/90 (serotype H.2) supplied by Dr J. M. Kramer; *B. cereus* var. *mycoides* strain 5901, *B. subtilis* subsp. *niger* strain 10073, and *Escherichia coli* strain 10418, all supplied by the National Collection of Type Cultures; *Clostridium sporogenes* strain 2154 (University of Surrey); *Pasteurella haemolytica* type A6, supplied by Dr N. J. L. Gilmour.

Dual infection experiments

These were made, by methods already fully described [1], for the purpose of investigating the ability of various organisms to enhance the infectivity of strains of *F. necrophorum*. Briefly, mice were inoculated subcutaneously on the outer aspect of the right thigh, in dose volumes of 0·1 ml, with 18 h cultures grown in BM broth. Test mice received decimal dilutions of *F. necrophorum* culture prepared in a diluent consisting of a neat culture of the organism under examination. Control mice received either an appropriate dilution (in sterile BM) of *F. necrophorum* culture alone, or undiluted pure culture of the organism under examination.

In all experiments, cultural examination of a representative sample of mice with advanced necrobacillosis revealed that *F. necrophorum* was present in the lesions in numbers that greatly exceeded those of the second organism injected.

Virulence tests of strains A42 and A6

Because dilution of pure *F. necrophorum* culture in sterile diluent has an adverse effect on infectivity [5], 18 h culture of strains A42 and A6 in BM medium were

titrated subcutaneously in mice in dose volumes of 0·1, 0·01, 0·001 and 0·0001 ml, delivered with microliter-syringes (Hamilton Bonaduz AG, PO Box 26, CH-7402, Bonaduz, Switzerland).

RESULTS

Dual infection with F. necrophorum *(strain A42) and 10 strains of other bacterial species*

Table 1 shows that the infectivity of *F. necrophorum* A42 was strongly enhanced by *S. aureus*, *B. cereus*, *B. cereus* var. *mycoides* and *K. oxytoca*. Enhancement of intermediate strength was given by *B. subtilis* subsp. *niger* and *Prot. mirabilis*. *Cl. sporogenes* and *Past. haemolytica* gave slight enhancement. *Bact. melaninogenicus* strains 1 and 2 gave none, but in a subsequent experiment in which heavy cultures of strains 2 and 3 were used (527×10^6 and 1389×10^6/0·1 ml respectively) slight but significant enhancement was demonstrated.

Infectivity enhancement of F. necrophorum *strains other than A42*

Because the previous experiment and earlier tests [1] had been made with only one strain (A42) of *F. necrophorum*, six further strains (L6, L13, F1, A6, A10 and A70) were examined. A preliminary experiment in which *E. coli* was used to enhance infectivity suggested that all except A6 resembled A42 in their behaviour. Leaving aside strain A6 for a more detailed examination (see below), this resemblance was confirmed in the following experiment.

BM cultures of *F. necrophorum* strains L6, L13, F1, A10 and A70 were diluted 1 in 10^4 and 1 in 10^6 in broth cultures of *E. coli* (viable count 2025×10^6/ml), *S. aureus* (103×10^6/ml) and *A. pyogenes* (1850×10^6/ml). The doses of fusobacteria in 0·1 ml volumes of the 1 in 10^6 dilutions ranged from 9 to 350 (mean 109). Groups of four mice received the 30 inocula and for each *F. necrophorum* strain a group of 8 control mice received culture diluted 1 in 10^2 in sterile BM medium. The 40 control mice all remained healthy except that necrobacillosis developed in 2 of the animals given strain A10. However, necrobacillosis developed in all except 6 of the 120 test animals. Those remaining unaffected had received dilutions (1 in 10^6) of strains A10 (1 mouse) and A70 (1 mouse) in *S. aureus* culture; and of strains L6 (3 mice) and A70 (1 mouse) in *A. pyogenes* culture.

Detailed examination of F. necrophorum *strain A6*

The results are shown in Table 2. They confirm that strain A6 differed strikingly from the other strains of *F. necrophorum* examined – strain A42 [1] and strains L6, L13, F1, A10 and A70 (see above) – in that its infectivity was enhanced only slightly by *E. coli*. It closely resembled these strains, however, in respect of greatly enhanced infectivity in the presence of *A. pyogenes* and *S. aureus* ([1] and see above). Also, in respect of its greatly enhanced infectivity in the presence of *B. cereus* and *K. oxytoca* it resembled strain A42, the only other *F. necrophorum* isolate tested with these two organisms.

It is worth noting that pure cultures of strains A6 and A42 were of approximately equal virulence for mice by subcutaneous inoculation. Thus, in

Table 1. *Dual infection with* F. necrophorum *strain A42 and 10 strains of other bacterial species*

Dose: 0·1 ml of FN diluted 1 in	Mice with necrobacillosis in groups of four given FN (strain A42) diluted in neat culture of									
	SA	BC	BCM	BSN	CS	KO	PM	PH	BM1	BM2
10^2	4	4	4	4	4	4	3	4	0	0
10^3	4	4	4	4	0	4	3	2	0	0
10^4	4	4	4	2	0	4	3	0	0	0
10^5	4	4	4	1	0	4	1	0	0	0
10^6	4	4	4	0	0	4	1	0	0	0
10^7*	4	4	4	0	0	1†	1	0	0	0

* Dose contained 7–24 (mean 11) fusobacteria, except in the BSN experiment (in which the 10^6 dilution contained only 3).

† Group of six mice.

FN, *F. necrophorum* culture.

Other abbreviations (and viable counts, in millions/0·1 ml of neat culture): SA, *S. aureus* (32); BC, *B. cereus* (6); BCM, *B. cereus* var. mycoides (0·5, but organisms clumped); BSN, *B. subtilis* subsp. *niger* (2·5); CS, *Cl. sporogenes* (70); KO, *K. oxytoca* (44); PM, *Prot. mirabilis* (162); PH, *Past. haemolytica* (82); BM1 and BM2, '*Bact. melaninogenicus*' strains 1 (40) and 2 (77).

Controls: in each of the 10 experiments 12 mice given 0·1 ml of a 1 in 10^2 dilution of FN in sterile diluent remained healthy; and 12 mice given 0·1 ml of the neat culture used as FN diluent showed no more than trivial symptoms, except that BC sometimes caused severe ulceration which nonetheless healed.

Table 2. *Dual infections with* F. necrophorum *strain A6 and* E. coli, A. pyogenes, B. cereus, K. oxytoca *or* S. aureus

Dose of fusobacteria (in 0·1 ml of FN dilution)	Mice with necrobacillosis in groups of 4 inoculated with FN (strain A6) diluted in neat culture of				
	EC	AP	BC	KO	SA
500000	4	4	N	N	N
50000	0	4	4	4	4
5000	0	4	4	4	4
500	0	4	4	4	4
50	0	4	4	3	4
5	0	0	3	0	1
0	N	N	2	0	0

FN, *F. necrophorum* culture; N, not done.

Other abbreviations (and viable counts, in millions/0·1 ml of neat culture): EC, *E. coli* (40); AP, *A. pyogenes* (150); BC, *B. cereus* (2·5); KO, *K. oxytoca* (160); SA, *S. aureus* (352).

Controls: groups of 12 mice given 0·1 ml of neat EC, AP, BC, KO or SA culture all survived, except one animal that received BC; 12 mice given 500000 fusobacteria in sterile diluent survived.

groups of six mice, dose volumes of 0·1, 0·01, 0·001 and 0·0001 ml of a BM culture of strain A6 containing 110×10^6 organisms/ml produced necrobacillosis in 6, 5, 1 and 0 animals respectively; the corresponding figures for strain A42 culture (390×10^6/ml) were 6, 2, 0 and 0.

DISCUSSION

The present and earlier [1] experiments showed that 18 bacterial strains belonging to 15 species and 13 genera fell into 2 groups. Organisms that strongly enhanced the infectivity of *F. necrophorum* included *A. pyogenes*, *B. cereus*, *B. cereus* var. *mycoides*, *Cit. freundii*, *E. coli*, *K. oxytoca* and *S. aureus*. Those giving weaker though variable enhancement included *B. subtilis* subsp. *niger*, *Bact. fragilis*, '*Bact. melaninogenicus*', *Cl. sporogenes*, *F. nucleatum*, *Past. haemolytica*, *Prot. mirabilis*, *Ps. aeruginosa* and an α-haemolytic streptococcus.

Three points deserve comment. (*a*) Although it has been suggested [10] that '*Bact. melaninogenicus*' acts synergically with *F. necrophorum* in the causation of bovine foot rot, it was, of all the organisms tested, the least able to enhance the infectivity of *F. necrophorum* for mice. (*b*) *B. cereus* and *B. subtilis*, although members of the same genus, differed considerably in their effect on *F. necrophorum*. (*c*) *Cl. sporogenes*, which may sometimes enhance the invasiveness of pathogenic clostridia [11], had only a slight effect on *F. necrophorum*.

So-called phase (type, biovar) A strains of *F. necrophorum* [12, 13] are haemolytic, haemagglutinating, highly leucocidinogenic, and pathogenic for mice; phase B strains produce less leucocidin, are haemolytic but not haemagglutinating, and have no more than slight pathogenicity for mice. The seven strains of *F. necrophorum* used in this study were all capable of producing fatal necrobacillosis in mice by the subcutaneous inoculation of 0·1 ml of undiluted BM broth culture; a 1 in 10^2 dilution was without effect. No phase B strains were used.

The striking enhancement of the infectivity of five strains (L6, L13, F1, A10 and A70) of *F. necrophorum* by *E. coli*, *A. pyogenes* and *S. aureus* closely resembled that of *F. necrophorum* strain A42, found in this and an earlier [1] study. One further strain, A6, was exceptional in that although its infectivity – like that of A42 – was greatly increased by the presence of *A. pyogenes*, *S. aureus*, *B. cereus* and *K. oxytoca*, *E. coli* had only a slight effect.

The possible exploitation of infectivity enhancement as an aid to the detection and isolation of *F. necrophorum* present in small numbers in heavily contaminated material such as faeces necessitates the choice of an enhancing organism that (*a*) enables minute numbers (< 10) of *F. necrophorum* organisms to produce progressive and potentially fatal necrobacillosis in mice by subcutaneous inoculation, (*b*) affects all virulent strains of *F. necrophorum* equally, and (*c*) causes by itself no more than a trivial lesion. The observations reported here have already been used in making such a choice [2].

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