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The adverse effect of dilution on the infectivity of *Fusobacterium necrophorum* culture

By G. R. SMITH and ANN TURNER

Nuffield Laboratories of Comparative Medicine, Institute of Zoology, The Zoological Society of London, Regent's Park, London NW1 4RY, UK

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SUMMARY

Dilution had an adverse effect on the infectivity of 24 h cultures of a strain of *Fusobacterium necrophorum*, which became apparent at or near the minimum lethal dose. Thus in mice inoculated subcutaneously the mortality produced by 0·01 ml of undiluted culture was almost invariably greater than that produced by 0·1 ml of a 1 in 10 dilution. The explanation appeared to lie in the increased physical separation of bacterial cells that was the inevitable consequence of dilution.

INTRODUCTION

In titrating bacterial suspensions for virulence microbiologists usually inoculate groups of animals with a standard volume of a series of dilutions, often decimal. Numerous titrations of *Fusobacterium necrophorum* cultures in mice, made at this Institute in the course of a study of necrobacillosis in wallabies, led to a suspicion that dilution had an adverse effect on infectivity. The effect became apparent at or near the minimum lethal dose.

This report describes experiments that define and partly explain the phenomenon.

MATERIALS AND METHODS

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

Organism

The strain (A42) of *F. necrophorum* used throughout was isolated from a necrobacillosis lesion in a Red-necked wallaby (*Macropus rufogriseus*) in the Zoological Society's Collection at Whipsnade Park (Smith, Oliphant & Parsons, 1984). It was freeze-dried after fewer than 10 laboratory subcultures.

Cultures

Liquid cultures for inoculation into mice were grown for 24 h at 37 °C in BM medium (Deacon, Duerden & Holbrook, 1978). In handling them care was taken to minimize accidental aeration and, whenever appropriate, laboratory manipula-

tions were accompanied by 'gassing' of tubes and bottles with CO_2. During the course of each experiment in which repeated inoculations and viable counts were made, the culture and any dilution thereof was kept at 4 °C.

Culture diluent

Either BM medium or a sterile filtrate of 24 h BM culture of strain A42 was used to make 1 in 10 dilutions of culture for inoculation. Unless just prepared, the basal BM medium was boiled and cooled immediately before use. Sterile culture filtrate was prepared with a hypodermic syringe and 0·45 μm Filter Unit (Millex-HA; Millipore S.A., Molsheim, France).

Viable counts

The method was that described by Smith, Oliphant & Parsons (1984). A single count only was made on each occasion. Because the purpose was usually merely to demonstrate or exclude any dramatic loss of viability during an experiment, the greater accuracy that would have been afforded by replicate counts was considered unnecessary. This should be borne in mind in considering apparent slight discrepancies between counts of the same culture made at different times.

Aeration of cultures

In some experiments cultures were deliberately aerated. This was done by filling a hypodermic syringe to as near the full mark as possible with culture and expressing it forcibly back into its container through a 23-gauge needle. Culture in 15 ml volumes was thus-treated twice with a 5 ml syringe ('+' aeration), three times with a 10 ml syringe ('++' aeration), or three times with a 20 ml syringe ('+++' aeration).

Mouse inoculation

Inoculations were made subcutaneously in the right flank. One-millilitre syringes were used to deliver volumes of 0·1 ml and microlitre syringes (Hamilton Bonaduz AG, PO Box 26, CH-7402, Bonaduz, Switzerland) to deliver smaller volumes. Thus volumes of 0·01 and 0·001 ml were delivered with a 50 μl syringe and those of 0·0001 ml with a 10 μl syringe. Delivery of 0·0001 ml required the use of a hand lens.

The course of the fatal disease resulting from subcutaneous inoculation with strain A42 was described by Smith *et al.* (1985).

RESULTS

Preliminary observations

In the course of numerous experiments in mice inoculated subcutaneously with 24 h BM cultures of *F. necrophorum* (strain A42) it was observed that the infectivity of 0·1 ml of a 1 in 10 dilution (in BM diluent) was usually much less than that of 0·01 ml of undiluted culture. Thus in 13 experiments made on different occasions the fatal infections produced in groups of mice by 0·1 ml of 1 in 10 dilution were as follows (the dose of viable organisms in millions being given in parentheses): 1/8 (2·9), 6/25 (6·1), 7/10 (6·5), 0/12 (7·0), 4/10 (7·2), 3/10 (7·8), 4/10

Table 1. *Effect of dilution on the infectivity of* Fusobacterium necrophorum *culture in mice*

Experiment no. (and diluent used)	Period between preparation of a 1 in 10 dilution of culture and its injection into mice	Fatal infection in mice inoculated subcutaneously with	
		0·1 ml culture diluted 1 in 10	0·01 ml undiluted culture
1	20 min	*3/10 (7·8)	8/10 (5·6)
(unseeded BM	2 h	*3/10 (6·7)	8/10 (7·8)
medium)	4 h	*0/10 (7·6)	6/10 (7·8)
2	< 3 min	3/10 (10·8)	7/10 (12·1)
(unseeded BM	1 h	*2/10 (8·8)	9/10 (11·3)
medium)	5 h	*0/9 (5·0)	10/10 (8·0)
3	< 3 min	*6/25 (6·2)	18/25 (8·3)
(sterile filtrate	1 h	2/10 (6·8)	5/10 (8·0)
of BM culture of *F. necrophorum*)	5 h	*1/10 (4·8)	7/10 (4·0)

Viable counts (10^6) of the doses administered are given in parentheses.

* Result differed ($P < 0{\cdot}025$ to $0{\cdot}001$; Wilson & Miles, 1975) from that produced by 0·01 ml of undiluted culture.

(9·7), 3/10 (10·8), 2/10 (11·5), 0/30 (15·2), 6/10 (16·2), 2/6 (unknown), and 3/12 (unknown). In 19 further experiments the corresponding results for 0·01 ml doses of undiluted culture were: 10/10 (3·9), 8/10 (5·6), 9/12 (7·0), 9/10 (8·2), 10/10 (8·3), 18/25 (8·3), 9/10 (10·3), 10/10 (11·2), 11/12 (11·5), 25/26 (11·5), 10/10 (11·6), 10/10 (11·8), 7/10 (12·1), 10/10 (14·5), 10/10 (16·2), 16/16 (18·5), 13/14 (31·2), 6/6 (unknown), and 9/12 (unknown).

Effect of dilution on the infectivity of F. necrophorum *culture*

Table 1 gives details of three representative experiments. In each the infectivity of 0·1 ml of a 1 in 10 dilution of culture was compared at three different times with that of 0·01 ml of the original undiluted culture. In the first two experiments the diluent was BM medium, fully capable of supporting robust growth of the organism at 37 °C. In the third experiment the diluent was a freshly prepared sterile filtrate of *F. necrophorum* culture.

In each experiment the infectivity of the dilution was less than that of undiluted culture. The effect of dilution on infectivity was rapid in onset, probably immediate. It persisted for the duration (several hours) of each experiment and did not depend on any loss of viability.

Occasionally, in similar experiments, cultures diluted with BM medium or culture filtrate showed not only an early loss of infectivity but also, usually after a few hours, a striking loss of viability as compared with undiluted culture.

Effect of deliberate aeration on F. necrophorum *culture*

One of several possible explanations of the phenomenon described above was that the inevitable slight aeration associated with the preparation of a 1 in 10 dilution of culture caused a loss of infectivity.

Table 2 gives details of three representative experiments in which 24 h BM cultures were subjected to three different degrees of aeration. This treatment had

Table 2. *Failure of deliberate aeration to reduce rapidly the infectivity of* F. necrophorum *culture for mice*

Experiment no. (and degree of aeration)*	Period between aeration of culture and its injection into mice	Fatal infections in mice inoculated subcutaneously with undiluted culture (0·01 ml)
1 (+)	< 5 min	10/10 (5·8)
	1 h	10/10 (6·3)
	2 h	10/10 (6·3)
	3 h	9/10 (5·0)
2 (++)	< 5 min	9/10 (9·7)
	1 h	9/10 (14·0)
	2 h	7/10 (11·4)
	3 h	10/10 (1·2)
3 (+++)	< 5 min	9/10 (3·9)
	1 h	10/10 (4·3)
	3 h	8/10 (3·6)
	5 h	8/10 (4·1)

* See Materials and Methods.

Viable counts (10^6) of the doses administered are given in parentheses.

Viable counts (10^6) of the cultures immediately before aeration were 8·3 (Expt 1), 11·7 (Expt 2), and 3·9 (Expt 3).

no observable effect on the infectivity of 0·01 ml of undiluted culture, either immediately or within a period of a few hours. In one of the experiments (no. 2) a sharp fall in the viability of the culture occurred between 2 and 3 h after aeration.

Virulence titration of strain A42 in mice

Undiluted culture was inoculated subcutaneously in volumes of 0·1 ml (115×10^6 viable organisms), 0·01 ml, 0·001 ml, and 0·0001 ml. The fatal infections produced in groups of 12 mice were 12, 11, 2 and 0 respectively.

DISCUSSION

This report shows that the infectivity of 0·01 ml of a 24 h culture of *F. necrophorum* in BM medium was almost invariably greater than that of 0·1 ml of a 1 in 10 dilution. Explanation of this phenomenon necessitates consideration of three main possibilities.

Toxicity of the diluent could be ruled out because the dilutions were made in either (1) BM medium capable of supporting profuse growth of *F. necrophorum*, or (2) sterile filtrate of a well grown BM culture of *F. necrophorum*.

Despite the precautions taken to minimize aeration of culture during the preparation of a 1 in 10 dilution, there was little doubt that slight aeration must nonetheless have occurred. It certainly did not lead to any immediate loss of viability (Table 1), but could it have produced an immediate reduction of infectivity? This seemed unlikely in view of the experiments described in Table 2, in which aeration of a severity far greater than that brought about by the diluting procedure had no noticeable effect on infectivity, either immediately or for hours afterwards.

The most likely explanation of the adverse of dilution on infectivity lay in increased physical separation of bacterial cells, the inevitable consequence of dilution. This led in turn to increased separation of bacterial cells in the tissues of the inoculated host. How this brought about a loss of infectivity is at present uncertain.

The phenomenon described came to light only because the minimum lethal dose of undiluted strain A42 culture fell within the range of volumes easily administered with a micro-syringe. In assessing the true virulence of this strain the use of a micro-syringe and avoidance of culture-dilution are essential.

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