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The pathogenic properties of *Fusobacterium* and *Bacteroides* species from wallabies and other sources

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SUMMARY

Intracerebral inoculation was more effective than intraperitoneal, intravenous or subcutaneous inoculation as a means of producing lethal infections with *Fusobacterium necrophorum* in mice. Strains varied in virulence but, of five examined, two had LD50 values as low as *ca.* 8000 and 14000 viable organisms. Profuse bacterial multiplication in the brain was demonstrated. Intravenous vaccination with a single large dose of heat-killed whole culture or washed bacterial cells failed to protect against intracerebral challenge.

Intracerebral injection of other fusobacteria (*F. nucleatum*, *F. varium* and *F. necrogenes*) and of 22 strains belonging to 10 *Bacteroides* spp. was without apparent effect on mice, except for a slight transient illness in some animals given *B. fragilis*. This organism (five strains) differed from the other *Bacteroides* spp. tested, which included eight strains belonging to the fragilis group, in being eliminated more slowly from the mouse brain – a point that may be relevant to the special pathogenicity of *B. fragilis* in endogenous infections in man. There was no evidence that *B. fragilis* multiplied in the brain or that intravenous vaccination with a large dose of heat-killed homologous culture affected the rate at which it was eliminated.

INTRODUCTION

In recent years the use of improved technical methods has revealed that gram-negative anaerobes are frequently concerned in infections of man. In animals *Fusobacterium necrophorum* has long been recognized as a pathogen in such diseases as bovine liver abscess and foot rot, and in calf diphtheria. The part played by *Bacteroides nodosus* in sheep foot rot is likewise well known. Several recent studies suggest that in domestic animals, as in man, infections with *Bacteroides* and *Fusobacterium* spp. are more common than hitherto suspected (Berkhoff, 1978; Sørensen, 1978; Hirsh, Biberstein & Jang, 1979; Love *et al.* 1979; Prescott, 1979).

During the first four months of 1979 an outbreak of necrobacillosis caused the death of more than 200 Red-necked wallabies (*Macropus rufogriseus*) in the Zoological Society's Collection at Whipsnade Park (Ashton, 1982). The sites most commonly affected were the stomach wall and the face. Such lesions are well known in captive wallabies, but their aetiology is the subject of debate, having been

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variously ascribed to *Nocardia* spp., *Actinomyces* spp., *F. necrophorum*, *Bacteroides* spp., *B. ruminicola* subsp. *brevis*, and other organisms (Fox, 1923; Beveridge, 1934; Watts & McLean, 1956; Finnie, 1976; Keane *et al.* 1977; Burton, 1981). In our experience, gathered from a detailed study of 27 cases, the main pathogen is *F. necrophorum*, sometimes alone but often accompanied by other organisms, especially *Bacteroides* spp. (*B. oralis*, *B. bivius*, *B. fragilis* and others) and *F. nucleatum* (Oliphant, Parsons & Smith, 1984).

This report is concerned with studies on experimental infections in mice, made with possible vaccine development in mind. *F. necrophorum* is known to produce lethal infections in mice and other animals by the intraperitoneal, intravenous and subcutaneous routes, but immunization against the necessarily large infecting doses is not easy (Beveridge, 1934; Wilkins & Smith, 1974; Maestrone *et al.* 1975; Cameron & Fuls, 1977; Garcia, Charlton & McKay, 1977; Langworth, 1977; Abe, Holland & Stauffer, 1978). Our purpose was to examine the pathogenicity, by intracerebral inoculation, of a number of fusobacterial and bacteroides strains from wallabies with necrobacillosis and from other sources, paying particular regard to *F. necrophorum* because of its obvious pathogenicity for wallabies, and to *B. fragilis* by reason of its well-established reputation as the most pathogenic member of the genus *Bacteroides* for man.

MATERIALS AND METHODS

Organisms

Fifteen strains were isolated, seldom in pure culture, from animals in the Zoological Society's collections at Regent's Park and Whipsnade. They were identified by the methods of Duerden *et al.* (1980). These strains comprised: one (A6) of *F. necrophorum* from a Roan antelope (*Hippotragus equinus*) with a subcutaneous abscess, two (A24 and A51) from wallabies with facial necrobacillosis, and one (A42) from a wallaby with necrobacillosis of the leg and face; one (A7) of *B. oralis* from a giraffe (*Giraffa camelopardalis*) with a purulent lesion of the face, and three (A25, A30 and A44) from wallabies with facial necrobacillosis; one (A5) of *B. bivius* from a Roan antelope with a subcutaneous abscess, and two (A27 and A49) from wallabies with facial necrobacillosis; two (A17 and A46) of *B. fragilis* from wallabies with necrobacillosis of the spleen and stomach wall respectively; one (A22) of *B. distasonis* from a wallaby with a liver abscess; and one (A31) of *B. thetaiotaomicron* from a wallaby with a purulent infection of the foot. These strains had not undergone more than 10 laboratory subcultures since isolation.

The National Collection of Type Cultures supplied 14 strains, namely: *F. necrophorum* NCTC 10576 (from a bovine liver abscess); *F. varium* NCTC 10560 (human faeces); *F. nucleatum* NCTC 10562 (human gingivitis); *F. necrogenes* NCTC 10723 (duck caecum); *B. fragilis* NCTC 8560 (human post-appendectomy infection), NCTC 9343 (human appendix abscess), NCTC 9344 (human post-operative sepsis); *B. vulgatus* NCTC 10583 (human faeces), NCTC 11154 (origin unknown); *B. thetaiotaomicron* NCTC 10582 (human faeces); *B. eggerthii* NCTC 11155 (human faeces); *B. ovatus* NCTC 11153 (origin unknown); *B. melaninogenicus* NCTC 9336 (human gingivitis); *B. ureolyticus* (formerly *corrodens*) NCTC 10939 (human vaginitis). *B. distasonis* strain ATCC 8503 (origin unknown)

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was supplied by Dr Ella M. Barnes, ARC Food Research Institute, Norwich. The number of laboratory subcultures undergone by the NCTC and ATCC strains was unknown.

Culture media

Freshly prepared BM medium (Deacon, Duerden & Holbrook, 1978), with the addition of sodium succinate 0·25% before autoclaving, was used for liquid cultures. Solid medium consisted of Columbia Blood Agar Base (Oxoid CM 331) with defibrinated Horse Blood (Oxoid SR 50) 7%.

Anaerobic methods

Anaerobic jars (Baird & Tatlock Ltd and Don Whitley Scientific Ltd) were used with a low-temperature catalyst (Oxoid BR 42). They were de-oxygenated twice by evacuating to at least 50 cm of mercury and filling with a mixture of H_2 90% and CO_2 10%. Cultures were used with a minimum of delay after removal from the anaerobic atmosphere and, wherever appropriate, laboratory manipulations were accompanied by 'gassing' of tubes and containers with CO_2.

Viable counts of cultures

The cultures were serially diluted 10^{-2}, 10^{-4}, 10^{-5} and 10^{-6} in BM medium. The 10^{-6} and 10^{-5} dilutions were sampled by spreading 0·04 ml volumes on the surface of blood agar plates. These plates had been thoroughly dried, and pre-reduced by overnight storage at room temperature in an anaerobic jar containing glycerol-soaked filter paper to remove moisture. The viable count plates were immediately returned to the anaerobic jar, and colonies were counted after incubation at 37 °C for 48 h.

Vaccines and vaccination

F. necrophorum vaccines ('whole culture' and 'washed cell') were prepared from 48 h BM culture of strain A 42, killed by heating at 56 °C for 30 min in a water bath, and stored for a few days at −20 °C before use. Whole culture vaccine had an opacity equivalent to that of Brown's tube 3. Washed cell vaccine consisted of a suspension (Brown's tube 4) in phosphate-buffered saline (PBS) of organisms from a heat-killed culture washed three times.

B. fragilis vaccine was prepared from a 24 h BM culture of strain A 46 (opacity, Brown's tube 4), killed by heating at 60 °C for 30 min and used immediately.

Mice were vaccinated intravenously with 0·25 ml doses three weeks before intracerebral challenge with the homologous organism. In the *F. necrophorum* and *B. fragilis* immunization experiments control mice received, respectively, PBS and BM medium.

Mice, intracerebral inoculation and brain cultures

Female Swiss white mice were obtained from an outbred closed colony. The animals weighed 18–20 g except in the immunization experiments, in which they were vaccinated at 16–18 g weight.

Mice, under inhalation anaesthesia induced by a 1:2:3 mixture of alcohol, chloroform and ether, were inoculated intracerebrally in the left side of the brain with 0·05 ml doses of 24 h BM culture, diluted if necessary with BM medium.

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Table 1. *Virulence of five strains of* Fusobacterium necrophorum *for mice by intracerebral inoculation*

Culture dilution	Death in groups of 8 mice inoculated with strain A6 (150)	A24 (11)	A42 (26)	A51 (50)	NCTC 10576 (8)
1/1	8	7*	8	8	7
10^{-1}	8	8	8	7	7
10^{-2}	5	6	7	5	1
10^{-3}	5	4	7	4	0
10^{-4}	0	1	2	1	0
10^{-5}	3	0	0	1	0
10^{-6}	ND	0*	0	0	0

Numbers in parentheses indicate viable organisms (millions) in largest dose.
* Seven mice in group.
ND = not done.

Cultures on solid medium were made from mice that died, or were killed with chloroform, by exposing the left side of the brain aseptically and plating out a small amount of tissue obtained with a wire loop. In the few instances in which a quantitative examination was required, the whole brain was removed aseptically and homogenized under CO_2 in 100 ml of BM medium in an Atomix (MSE) blender. Decimal dilutions were then prepared, and four (10^{-2} to 10^{-5}) sampled on blood agar as described under *Viable counts of cultures.*

In one experiment cultures were made from the brains of slaughtered mice by transferring tissue aseptically with Pasteur pipettes into 5 ml volumes of BM medium. The identity of the organisms recovered was checked by the methods of Duerden *et al.* (1980).

RESULTS

Intracerebral inoculation with F. necrophorum

Table 1 shows the results of decimal titrations of five strains. All readily produced lethal infections. The virulence of the strains varied, but two (A24 and A42) had LD50 values (Reed & Muench, 1938) as low as *ca.* 14000 and 8000 viable organisms, respectively. Depending on the dose, signs of illness first appeared after periods ranging from a few hours to 2 days; and mice died after 1–5 (usually 1–3) days. Mice that died almost always yielded a heavy growth of *F. necrophorum* on culture, but occasionally the yield was slight or nil. Brain cultures made from apparently healthy mice killed 5 or 6 days after inoculation invariably gave negative results.

Multiplication in the brain

Of 24 mice inoculated intracerebrally with 10000 living organisms of strain A42, 16 became ill. Of the sick mice, five and four were killed 24 and 48 h respectively after inoculation and the numbers of living organisms in the brain assayed.

The degrees of multiplication demonstrated in the individual mice were: (24 h) 1600, 925, 325, 25 and < 25 times; (48 h) 3250, 600, 450 and < 25 times.

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Table 2. *Comparison of the intracerebral method of producing* F. necrophorum (*strain A42*) *infection with three other methods*

Dose of viable organisms (10^6)	Route of inoculation	Deaths of mice 3 days after inoculation*
0·6	Intracerebral	16/19
1·2	Intraperitoneal	0/10
1·2	Subcutaneous	0/10
1·2	Intravenous	0/9†

* The results remained unchanged on the 8th day after inoculation.

† Two mice died from fusobacterial infection on days 9 and 14. All survivors were killed 21 days after infection and found to be normal.

Comparison of the intracerebral route with other routes

A 1-in-100 dilution of a 24 h BM culture of strain A42 was injected intraperitoneally, subcutaneously or intravenously into three groups of mice (dose 0·1 ml; $1{\cdot}2 \times 10^6$ viable organisms), and then intracerebrally into a further group (dose 0·05 ml; $0{\cdot}6 \times 10^6$ viable organisms).

Table 2 shows that 84 % of the mice inoculated intracerebrally died within 3 days. Of the mice inoculated by other routes, all were alive 8 days later, and 93 % rid themselves of infection without showing any effect.

Intracerebral inoculation with three other Fusobacterium *spp.*

Each bacterial strain was injected in culture dilutions of 1 in 1, 1 in 5, and 1 in 10 into three groups of eight mice. Four animals from each group were killed 2 days after inoculation for cultural examination of the brain on solid medium, and the remainder after a further 2 days. The organisms used were *F. varium* (NCTC 10560), *F. nucleatum* (NCTC 10562), and *F. necrogenes* (NCTC 10723).

None of the mice became ill; 68 of the 72 brain cultures gave negative results, the remaining four yielding slight growth ($\leqslant 5$ colonies).

Failure to immunize against F. necrophorum

Of 48 mice that received whole-culture vaccine intravenously, a number became ill within a few hours, presumably owing to toxic effects. Most recovered, but four died within 20 h of inoculation. Of 45 mice that received washed-cell vaccine, one was found dead 20 h later, but sick mice were not seen. Three weeks later the two vaccinated groups and the PBS-treated controls were each divided into subgroups of 12–15 mice for intracerebral challenge with $3{\cdot}5 \times 10^6$, $3{\cdot}5 \times 10^5$ and $3{\cdot}5 \times 10^4$ viable organisms of the homologous strain (A42).

In terms of death and survival neither vaccine gave any evidence of protection. The deaths produced by the smallest dose in mice that received whole-culture vaccine, washed-cell vaccine and PBS were, respectively: 9/15, 7/12 and 8/14. Mortality ranged from 80 to 100 % in the six subgroups of mice given the two larger doses. Observations made at frequent intervals after challenge showed, moreover, that the rates at which mice died were closely similar in the two groups of vaccinated animals and in the controls.

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Table 3. *Intracerebral inoculation of mice with* 21 *strains belonging to* 10 Bacteroides *species*

Strain no.	*Bacteroides* sp.	Viable count (10^6) in largest (1/1) inoculum	Positive brain cultures, 2 and 4 days after inoculation, in groups of 4 mice given stated culture dilutions: 2 days 1/1	2 days 1/5	2 days 1/10	4 days 1/1	4 days 1/5	4 days 1/10
A17	*fragilis*	205	4	4	3	4	3	3
A46	*fragilis*	205	4	4	4	4	4	4
NCTC 8560	*fragilis*	82	4	4	4	4*	3*	4*
NCTC 9343	*fragilis*	158	4	4	4	4	4	4
NCTC 9344	*fragilis*	152	4	4	3	4	4	4
A7	*oralis*	183	3	4	3	0	1	1
A25	*oralis*	255	3	0	0	0	0	0
A30	*oralis*	165	0	0	0	0	0	0
A44	*oralis*	160	1	0	0	2	1	0
A5	*bivius*	490	0	0	0	0†	0	1†
A27	*bivius*	220	0	0	0	0	0	0
A49	*bivius*	120	2	1	1	1	0	1
A22	*distasonis*	38	0	0	0	1	0	0
A31	*thetaiotaomicron*	150	1	0	0	2	1	0
NCTC 10582	*thetaiotaomicron*	191	4	4	4	2	2	0
NCTC 10583	*vulgatus*	74	3	3	3	0*	1*	1*
NCTC 11154	*vulgatus*	46	1	0	0	2	0	1
NCTC 9336	*melaninogenicus*	0·5‡	0	0	0	0*	0*	0*
NCTC 11153	*ovatus*	151	4	4	4	1†	2	2
NCTC 11155	*eggerthii*	36	4	3	3	0*†	0*	0*
NCTC 10939	*ureolyticus*	12	0	0	1	0*	0*	0*

* Mice killed 3 instead of 4 days after inoculation.
† Only three mice in group.
‡ Low count associated with clumping.

Intracerebral inoculation of 22 *strains belonging to* 10 *species of* Bacteroides, *including* B. fragilis

In the course of several tests all except one of the 22 strains were injected in culture dilutions of 1 in 1, 1 in 5, and 1 in 10 into three groups of eight mice. Four animals from each group were killed 2 days after inoculation, and the remainder after a further 1 or 2 days. The experimental details and results of brain cultures on solid medium from slaughtered mice are shown in Table 3.

Apart from transient signs of slight illness in a few of the mice that received *B. fragilis*, the injections were without apparent effect. Brain cultures showed, however, a difference between *B. fragilis* (five strains) and the other *Bacteroides* species. Of mice killed 3 or 4 days after receiving *B. fragilis* 95% gave positive cultures, whereas only 13% of those inoculated with other species still harboured infection; and *B. fragilis* cultures diluted 1 in 10 invariably gave a larger number of positive brain cultures than did undiluted cultures of the other *Bacteroides* species. The difference was also clear 2 days after infection except for five strains (A7, NCTC 10582, NCTC 10583, NCTC 11153, NCTC 11155).

The growth of *B. fragilis* from mouse brains as judged by plating with a wire

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Table 4. *Intracerebral inoculation of mice with five* B. fragilis *strains and eight fragilis-group strains*

Strain no.	*Bacteroides* sp.	Dose (10^6) of living organisms	Positive brain cultures* in groups of mice
A 17	*fragilis*	13	2/5
A 46	*fragilis*	9	3/5
NCTC 8560	*fragilis*	17	1/5
NCTC 9343	*fragilis*	14	4/6
NCTC 9344	*fragilis*	13	2/6
A 22	*distasonis*	85	0/6
ATCC 8503	*distasonis*	8	0/6
A 31	*thetaiotaomicron*	157	0/5
NCTC 10582	*thetaiotaomicron*	190	0/6
NCTC 10583	*vulgatus*	116	0/5
NCTC 11154	*vulgatus*	24	0/6
NCTC 11153	*ovatus*	ND	0/6
NCTC 11155	*eggerthii*	31	0/6

* Mice killed for examination 6 days after inoculation.
ND = not done.

loop varied from heavy to slight (a few colonies only). In general the weight of residual infection appeared to be related to the interval between inoculation and slaughter and to the size of dose, but many exceptions occurred.

In a confirmatory experiment the five strains of *B. fragilis* were compared with eight strains belonging to five fragilis-group species (Table 4). In this experiment the *B. fragilis* strains were inoculated as culture diluted 1 in 10, and the other strains as undiluted culture. The mice were killed 6 days later and their brains cultured in liquid medium. Positive results were given by some members of each of the five groups of mice that received *B. fragilis*, but all other groups were completely negative despite the fact that the choice of doses deliberately biased the experiment against the expected result.

Persistence of B. fragilis *in the brain*

Three *B. fragilis* strains, A 17, A 46 and NCTC 9343, were injected intracerebrally into groups of mice in doses of (millions) 202, 159 and 467, respectively. Brain cultures from mice killed 4, 7, 9 and 12 days after inoculation gave, respectively, the following positive results: 4/4, 2/4, 2/4, not done (strain A 17); 4/4, 3/4, 1/4, 0/2 (strain A 46); 4/4, 2/4, 2/4, 0/2 (strain NCTC 9343). By the 9th day after inoculation the infected brains never gave rise to more than a few colonies when cultured.

Intracerebral titration of B. fragilis

Strain A 46 was injected in graded doses into groups of eight mice. Four from each group were killed after 2 and 4 days, and brain cultures were made. All mice that received 8×10^6 and 8×10^5 viable organisms gave positive cultures. Doses of 8×10^4, 8×10^3 and 80 resulted in positive cultures (a few colonies only) in the following numbers of mice, respectively: 4/4, 3/4, 0/4 (2 days); and 1/4, 3/4, 0/4 (4 days).

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Table 5. *Failure of vaccination to influence the rate of clearance of* B. fragilis *from the mouse brain*

Challenge dose of viable organisms (millions)	Positive brain cultures at the stated intervals after intracerebral challenge in			
	vaccinated mice		control mice	
	2 days	4 days	2 days	4 days
12	12/12	10/13	12/12	6/11
1·2	11/11	3/11	9/11	3/11

Failure of B. fragilis *to multiply in the brain*

Two groups of mice were inoculated intracerebrally with strains A46 and NCTC 9343 in doses of 159×10^6 and 154×10^6 viable organisms respectively. The brains of three mice from each group killed 24 h after inoculation contained the following numbers (millions) of viable bacteria: 0·95, 0·7, 0·4 (strain A46); and 6·2, 0·6, 0·1 (strain NCTC 9343). The numbers (millions) for mice killed after a further 24 h were: 0·29, 0·11, 0·07 (strain A46); and 0·1 (strain NCTC 9343).

Failure of vaccination to influence the rate of clearance of B. fragilis *from the brain*

Intravenous vaccination with *B. fragilis* (A46) produced no obvious illness. Twenty days later immunized and control mice were subdivided and challenged with two doses of the homologous strain. The animals were killed 2 or 4 days later. The experimental details and results of brain cultures made from slaughtered mice are shown in Table 5.

Vaccination did not influence the rate at which *B. fragilis* was cleared from the brain. This is apparent from Table 5 and from the observation that there was no obvious difference between immunized and control animals in respect of the numbers of colonies that grew from their brains in culture.

DISCUSSION

The potential usefulness of vaccination against gram-negative anaerobe infections is greater in animals than in man. A short-lived immunity against sheep foot rot can be produced by means of a formolized *B. nodusus* vaccine (Egerton & Roberts, 1971; Egerton & Thorley, 1981). Attempts to immunize animals against infection with *F. necrophorum* have, however, been mainly disappointing (Cameron & Fuls, 1977). Immunization against this organism is complicated by the occurrence of a cell-wall lipopolysaccharide endotoxin, and one or more exotoxins associated with the cytoplasm and with haemolytic and leucocidal effects (see Langworth, 1977). Garcia *et al.* (1974) claimed to have reduced the incidence of *F. necrophorum* abscesses of the liver in cattle from 35% to 10% by vaccination with a toxoid prepared from the cytoplasmic fraction of the organism. Abe, Holland & Stauffer (1978) found that hyperimmunization by repeated intraperitoneal injections of killed culture partly protected mice from intraperitoneal challenge.

Our experiments have shown that intracerebral inoculation is much more

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effective than intraperitoneal, intravenous or subcutaneous inoculation as a means of infecting mice with *F. necrophorum*. Small doses multiply profusely in the brain and cause death within a few days. Intracerebral challenge has been used successfully in immunization experiments with typhoid bacilli (Norton & Dingle, 1935), *Bordetella pertussis* (Kendrick *et al.* 1947; Standfast, 1958), *Pasteurella haemolytica* (Smith, 1959) and *Neisseria gonorrhoeae* (Diena *et al.* 1978). We were not, however, successful with *F. necrophorum*. Large intravenous doses of whole-culture or washed-cell vaccine, killed by a minimal degree of heat, failed conclusively to protect mice against intracerebral challenge with graded doses that included one as small as 35000 organisms. Other methods of immunization should be tried, including the use of more than one dose of vaccine and the use of an adjuvant, but the results support the view that vaccination against *F. necrophorum* presents special difficulties.

Intracerebral infection of mice with large doses of strains belonging to three other *Fusobacterium* spp. and ten *Bacteroides* spp. was without apparent effect, except for a slight transient illness in some of the mice that received *B. fragilis*. The behaviour of five strains of *B. fragilis* differed, however, from that of the 17 other bacteroides strains (nine *Bacteroides* spp.), which included eight belonging to the fragilis group. The difference lay in a more prolonged survival in the mouse brain.

This unexpected finding seemed of interest in relation to the special pathogenicity of *B. fragilis* in endogenous infections of man. Although greatly outnumbered in the gut by other *Bacteroides* spp. of the fragilis group, *B. fragilis* predominates in gut-associated clinical infections (Kasper *et al.* 1977). Its polysaccharide capsule plays an important role in the experimental production of peritoneal abscesses in the rat (Onderdonk *et al.* 1977; Kasper & Onderdonk, 1982). *In vitro* experiments suggest, moreover, that it interferes with phagocytosis, not only of *B. fragilis* but also of accompanying facultative anaerobes (Ingham *et al.* 1977). The phagocytosis and killing of clinical isolates of *B. fragilis in vitro* require the participation of factors present in immune and normal serum (Casciato *et al.* 1975; Bjornson, Altemeier & Bjornson, 1976; Ellis & Barrett, 1982; Simon *et al.* 1982).

Possibly the prolonged survival of *B. fragilis* in the mouse brain was due to its polysaccharide capsule. There was no evidence that multiplication occurred, or that vaccination influenced the rate at which the organism was eliminated from the brain.

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