Bacteraemia and seeding of capsulate *Bacteroides* spp. and anaerobic cocci

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Summary. The effect of capsulation on the ability of *Bacteroides fragilis*, *B. asaccharolyticus* and anaerobic gram-positive cocci to induce bacteraemia and seeding to various organs was investigated. The test species were injected into mice subcutaneously alone, or mixed with other aerobic or facultative organisms. Capsulate anaerobes were isolated more frequently from the blood, spleen, liver, and kidneys of infected animals than were non-capsulate organisms. After injection of single anaerobic strains, capsulate organisms were recovered from 163 (39%) of 420 animals; non-capsulate anaerobes were recovered from only 14 (3%) of 420 animals. After injection of *B. fragilis* mixed with aerobic or facultative organisms, the capsulate *B. fragilis* strain was isolated more often and for longer periods than the non-capsulate strain. Capsulate *B. fragilis* was also recovered more often 5 days after injection with other organisms, than when injected alone. These data demonstrate that capsulate *Bacteroides* spp. and anaerobic gram-positive cocci are more virulent than non-capsulate strains in single and mixed infections.

Introduction

Anaerobic bacteria are isolated from 5–15% of cases of bacteraemia (Finegold, 1977) and are particularly common in polymicrobial bacteraemia associated with abscesses (Brook *et al.*, 1980). Recent studies have established the increased virulence of capsulate *Bacteroides* spp. (Kasper, 1976; Onderdonk *et al.*, 1977; Brook *et al.*, 1983; Brook and Walker, 1984a), in comparison with non-capsulate strains, and the role of anaerobic gram-positive cocci (AGPC) (Brook and Walker, 1984b) in subcutaneous abscesses.

The role of capsular material in the systemic spread of anaerobic bacteria has not been defined. For this reason a subcutaneous abscess model in mice was used to determine the effect of capsulation on the ability of *Bacteroides* spp. and AGPC to induce bacteraemia and seeding to various organs. The effect of capsulation on previously non-capsulate strains was determined by injecting each of these alone or mixed with aerobic or facultative organisms.

Materials and methods

Organisms

All organisms were recent clinical isolates that had been stored frozen in skimmed milk at -70°C . The anaerobic organisms included one strain each from seven bacterial species: *Peptostreptococcus magnus*, *Pstr. asaccharolyticus*, *Pstr. prevoti*, *Pstr. micros*, *Pstr. anaerobius*, *Bacteroides fragilis* and *B. asaccharolyticus*. The facultative or aerobic organisms were one strain each of group-A β -hemolytic *Streptococcus*, group-D *Streptococcus*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Escherichia coli*, *Proteus mirabilis*, *Pseudomonas aeruginosa* and *Haemophilus influenzae* type b. They were identified by conventional methods (Lennette *et al.*, 1980; Sutter *et al.*, 1980).

Capsular staining of the test strains

The presence of capsules was established by Hiss's stain (Lennette *et al.*, 1980) and confirmed by electron microscopy after staining with ruthenium red (Kasper, 1976). The strains of *Bacteroides* spp. and AGPC were originally <1% capsulate (defined in this study as non-capsulate). Cultures in which at least 50% of the organisms had capsules (defined as capsulate) were prepared from the non-capsulate organisms by injecting them subcutaneously with *K. pneumoniae* to produce abscesses as previously described (Brook *et al.*, 1983). Pus from these abscesses was removed and cultured 10 days after injection and were found to contain at least 50% capsulate

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forms of these organisms. *K. pneumoniae*, *S. aureus*, groups A and D streptococci, and *H. influenzae* were capsulate before and after their injection into the mice. Ruthenium red staining demonstrated a homogenous polysaccharide capsule external to the cell wall. *E. coli*, *P. mirabilis*, and *P. aeruginosa* were non-capsulate.

Animals

Male Swiss albino mice weighing 20–25 g were obtained from the Naval Medical Research Institute mouse colony (NMRI/NIH-CV). They were raised in conventional conditions.

Abscess formation

The bacteria were grown anaerobically or aerobically on sheep-blood-agar plates made with Brain Heart Infusion Base (BHI; Difco). Mice were each given subcutaneous injections in the right groin of 0.1 ml of the appropriate bacteria suspended in saline (10^8 bacteria). This dose of a single organism would induce subcutaneous abscesses almost without mortality (Brook *et al.*, 1983; Brook and Gober, 1983). Sizes of individual abscesses were determined during necropsy on days 1, 3, 5 and 7 after injection, at which time the abscesses had neither decreased in size nor resolved. Although the volume of abscesses could not be determined accurately, their size was estimated by measuring with calipers two perpendicular diameters representing maximum length and width. Assuming that the abscess is an irregular prolate spheroid, the product of the length and width is approximately proportional to the outer surface of the abscess. This product, expressed as mm², was arbitrarily selected as the means of comparison.

Examination of abscesses, organs and blood

Animals were killed by cervical dislocation. The spleen, liver, and the left kidney were removed aseptically; the spleen, kidney, and one lobe of the liver were immediately homogenised and inoculated on to media. Blood was obtained by open heart puncture and 0.3–0.5 ml was immediately plated. The site and histology of the primary abscesses were confirmed in two animals in each experimental group by staining with haematoxylin and eosin. Pus was diluted in 1 ml of enriched BHI broth and inoculated on to media. Specimens were inoculated within 5 min of collection on to reduced as well as pre-reduced BHI-agar plates (Sutter *et al.*, 1980). One plate from each source was incubated aerobically and the other was incubated anaerobically in an anaerobic jar at 37°C. Bacterial growth on the plates was estimated semi-quantitatively. Colonies were identified by Gram's stain and biochemical tests 48 h and 96 h after inoculation (Lennette *et al.*, 1980; Sutter *et al.*, 1980).

Experimental design

The ability of each bacterial strain to induce subcutaneous abscesses, bacteraemia and seeding of organs was

monitored by injecting each bacterial strain into 25 mice. The effect of capsulation in mixed infections was studied by injecting each of the capsulate and non-capsulate *B. fragilis* strains in combination with aerobic and facultative bacteria into 30 mice. On days 1, 3, 5 and 7, five randomly selected mice from each group were killed and cultures of their spleens, livers, left kidneys, blood and abscesses, if present, were made. Cultures were not done of mice that were found dead because of the uncertainty of the effect of delay on bacterial growth. To evaluate mortality, each bacterial strain or combination of strains was injected into 20 mice. All experiments were done three times.

Statistical analysis

Analysis of the data was done with the χ^2 -square test.

Results

Induction of abscesses

After injection of single organisms, abscesses were induced by all the aerobic and facultative bacteria as well as by the capsulate anaerobic bacteria. However, these organisms did not produce abscesses on every occasion. Abscesses were observed in 456 (76%) of the 600 animals given aerobic and facultative bacteria and in 462 (88%) of the 525 given capsulate anaerobic bacteria. Abscesses developed in only 21 (4%) of the 525 animals given the non-capsulate anaerobes alone.

Abscesses were induced in 564 (94%) of the 600 mice given mixtures of the capsulate *B. fragilis* and other bacteria, compared with 504 (84%) of the 600 animals given the non-capsulate strain and other bacteria. Abscesses formed within 24–36 h and their size increased gradually. Without antibiotic therapy, the abscesses caused by pure cultures at day 5 had outer surface areas of 80–200 mm², and the outer surface areas of abscesses caused by two organisms were 90–300 mm².

Histopathological examination of abscesses

A fibrous encapsulated collection of material that contained bacteria and polymorphonuclear leukocytes was observed in abscesses caused by all single organisms as well as by all combinations of organisms.

Recovery of organisms from abscesses

Anaerobic bacteria were recovered from 434 (94%) of 462 abscesses in the animals given pure cultures of capsulate anaerobes, and from 18 (86%)

of the 21 abscesses in animals given non-capsulate anaerobes. Aerobic or facultative bacteria were isolated from 438 (96%) of the 456 abscesses induced by single aerobic or facultative bacteria.

From abscesses induced by mixtures of capsulate *B. fragilis* and aerobic or facultative bacteria, *B. fragilis* was recovered from 446 (79%) of the 564 abscesses, and the aerobic or facultative species were isolated from 485 (86%). From abscesses induced by mixtures of non-capsulate *B. fragilis* and aerobic or facultative bacteria, *B. fragilis* was recovered from 312 (62%) of the 504 abscesses, and the aerobic or facultative species were isolated from 454 (90%). The *B. fragilis* recovered from these abscesses was capsulate in 262 (84%) of the 312 instances.

Recovery of anaerobic bacteria from blood, spleen, liver and kidney

In most animals from which organisms were recovered, identical bacteria were recovered concomitantly from all of the sites cultured (blood, spleen, liver and kidney). For this reason, the number of animals rather than the number of sites was chosen to represent the data. When injected alone, capsulate *Bacteroides* spp. and AGPC were isolated more frequently and for longer periods

than were the non-capsulate strains (table I). Capsulate strains were recovered from 163 (39%) of the 420 animals, compared with recovery of non-capsulate strains from 14 (3%). Capsulate *B. fragilis* was recovered for longer periods after injection with facultative or aerobic bacteria, than when capsulate *B. fragilis* was injected alone (table II). Five days after injection, capsulate *B. fragilis* was isolated from 78 of the 120 mice given *B. fragilis* and other flora, compared with its recovery from 5 of the 15 animals given *B. fragilis* alone ($p < 0.05$).

A similar difference, although not statistically significant, was observed on the seventh day after injection; capsulate *B. fragilis* was recovered from 32 of the 120 mice given *B. fragilis* and other bacteria, compared with from 2 of the 15 given capsulate *B. fragilis* alone ($p < 0.1$). No increase in

Table I. Recovery of bacteria from the organs (liver, spleen and kidney) of mice given injection of single capsulate or non-capsulate strains

Test species		Number of mice* from which bacteria were recovered on days 1-7 after injection				
		1	3	5	7	Total
<i>B. fragilis</i>	cap +	12	13	5	1	31
	cap -	2	1	0	0	3
<i>B. asaccharolyticus</i>	cap +	8	10	5	2	25
	cap -	1	0	0	0	1
<i>Pstr. magnus</i>	cap +	11	10	1	0	22
	cap -	2	2	0	0	4
<i>Pstr. asaccharolyticus</i>	cap +	11	13	5	0	29
	cap -	2	1	0	0	3
<i>Pstr. prevotii</i>	cap +	9	7	0	0	16
	cap -	1	0	0	0	1
<i>Pstr. micros</i>	cap +	12	10	6	0	28
	cap -	1	1	0	0	2
<i>Pstr. anaerobius</i>	cap +	9	3	0	0	12
	cap -	0	0	0	0	0

cap + = Capsulate organisms; cap - = non-capsulate organisms.

* A total of 15 mice were killed in each group each day.

Table II. Recovery of bacteria from the organs (liver, spleen and kidney) of mice given injections of *B. fragilis* and facultative or aerobic bacteria

Bacteria injected	Number of mice* from which <i>B. fragilis</i> /other bacteria were recovered on day			
	1	3	5	7
BF (+) alone	12	13	5	1
BF (-) alone	2	1	0	0
<i>S. aureus</i> alone	12	6	1	0
<i>S. aureus</i> & BF (+)	11/14	13/10	9/6	4/3
<i>S. aureus</i> & BF (-)	0/14	2/13	0/4	0/2
Gr. A <i>Streptococcus</i> alone	10	3	0	0
Gr. A <i>Streptococcus</i> & BF (+)	11/10	12/5	11/4	4/0
Gr. A <i>Streptococcus</i> & BF (-)	0/10	0/4	0/0	0/0
Gr. D <i>Streptococcus</i>	11	8	5	0
Gr. D <i>Streptococcus</i> & BF (+)	9/12	12/6	10/8	3/1
Gr. D <i>Streptococcus</i> & BF (-)	1/13	0/7	0/5	0/0
<i>H. influenzae</i> alone	7	3	1	0
<i>H. influenzae</i> & BF (+)	9/10	8/10	10/3	3/0
<i>H. influenzae</i> & BF (-)	0/8	0/7	0/2	0/0
<i>K. pneumoniae</i> alone	10	9	8	4
<i>K. pneumoniae</i> & BF (+)	12/12	13/11	10/10	5/8
<i>K. pneumoniae</i> & BF (-)	0/10	0/10	0/6	0/3
<i>E. coli</i> alone	8	6	4	0
<i>E. coli</i> & BF (+)	10/12	9/11	7/13	4/2
<i>E. coli</i> & BF (-)	2/13	0/8	0/6	0/0
<i>P. mirabilis</i> alone	12	8	5	1
<i>P. mirabilis</i> & BF (+)	8/12	6/10	10/9	3/2
<i>P. mirabilis</i> & BF (-)	0/11	1/10	1/6	0/0
<i>Ps. aeruginosa</i> alone	15	13	10	6
<i>Ps. aeruginosa</i> & BF (+)	10/14	13/10	11/10	6/4
<i>Ps. aeruginosa</i> & BF (-)	0/15	1/12	0/11	0/5

BF (+) = Capsulate *B. fragilis*; BF (-) = non-capsulate *B. fragilis*.

* A total of 15 mice were killed from each group each day.

the recovery rate of non-capsulate *B. fragilis* was observed when it was injected with other bacteria.

Facultative or aerobic bacteria were generally recovered more often and for longer periods after injection with capsulate *B. fragilis*, than when they were injected alone (table II). Higher rates of recovery of facultative or aerobic bacteria after injection mixed with capsulate *B. fragilis* were observed only with *S. aureus*, group-A *Streptococcus*, *K. pneumoniae* and *E. coli* ($p < 0.05$). A significant increase in the recovery rate after injection mixed with capsulate *B. fragilis* compared with the rate after injection mixed with non-capsulate *B. fragilis* was observed only with *E. coli* and *K. pneumoniae*.

Mortality

When mortality was noted, it occurred within the first 72 h after injection. No deaths occurred after injection of the *Bacteroides* spp., *H. influenzae*, Group-D streptococci, or AGPC alone. The mortality after injection of *S. aureus* alone was 3 (5%) of 60, group-A streptococci 2 of 60 (1%), *K. pneumoniae* 4 of 60 (7%), *E. coli* 8 of 60 (13%), *P. mirabilis* 5 of 60 (8%) and *Ps. aeruginosa* 5 of 60 (8%).

No increase in mortality was noted after injection of mixtures of non-capsulate anaerobic organisms

and the other organisms (table III). However, a significant increase in mortality was noted when capsulate *B. fragilis* was injected mixed with other organisms in 6 of 8 combinations; similar results were obtained with capsulate *B. asaccharolyticus* in 7 of 8 instances. An increase in mortality was noted with 7 of the 40 combinations of the capsulate AGPC and the other organisms. The aerobic or facultative bacteria most often associated with increased mortality were group-D streptococci in 5 of 7 combinations with anaerobes, *Ps. aeruginosa* in 4 of 8, and *S. aureus* in 3 of 8. Although in many combinations there were no significant individual differences in the mortality rates, comparison of the total mortality for each facultative or aerobic species was significant in all instances (table III).

Discussion

This study highlights the importance of capsulate *Bacteroides* spp. and AGPC in increasing the mortality associated with bacteraemia and seeding to different organs. The presence of capsular material made these organisms more virulent than their non-capsulate counterparts. Similar virulence characteristics have also been observed in other bacterial species, such as *Str. pneumoniae* (Wood and Smith, 1949) and *H. influenzae* (Chandler *et al.*,

Table III. Mortality of mice after inoculation with anaerobic bacteria and facultative or aerobic bacteria*

		Number of deaths* in mice given the test anaerobe plus							
Anaerobic organism		<i>S. aureus</i>	Group A <i>Streptococcus</i>	Group D <i>Streptococcus</i>	<i>H. influenzae</i>	<i>K. pneumoniae</i>	<i>E. coli</i>	<i>P. mirabilis</i>	<i>Ps. aeruginosa</i>
<i>B. fragilis</i>	cap +	11	16†	13†	6	19†	19†	17†	22†
	cap -	4	5	2	3	6	8	5	7
<i>B. asaccharolyticus</i>	cap +	12†	14†	10†	9	15†	21†	15†	20†
	cap -	2	4	3	2	5	7	7	8
<i>Pstr. magnus</i>	cap +	2	3	9†	6	7	10	9	7
	cap -	1	0	0	1	6	8	8	4
<i>Pstr. asaccharo.</i>	cap +	13†	9	7†	3	6	11	3	14
	cap -	3	1	0	0	3	10	4	6
<i>Pstr. prevoti</i>	cap +	10	1	6†	1	9	10	5	15
	cap -	5	2	0	1	4	7	6	9
<i>Pstr. micros</i>	cap +	14†	8	5	1	3	12	3	15†
	cap -	2	3	2	2	1	8	3	6
<i>Pstr. anaerobius</i>	cap +	7	5	6	2	4	8	8	17†
	cap -	2	0	1	0	2	6	7	4
Total capsulate anaerobes		69‡	56‡	46‡	28‡	63‡	91‡	60†	110‡
Total non-capsulate anaerobes		19	15	8	9	27	54	40	44

cap + = Capsulate strain; cap - = non-capsulate strain.

* A total of 60 mice was given each combination.

† = $p < 0.05$ compared with non-capsulate strains.

‡ = $p < 0.001$ compared with non-capsulate strains.

1937); however, this phenomenon has not been previously described in *Bacteroides* spp. and AGPC. Onderdonk *et al.* (1974) detected bacteraemia due to *B. fragilis* only during the first few hours after intraperitoneal injection of *B. fragilis* and *E. coli* in rats. However, their model represented an intra-abdominal infection, whereas ours was a subcutaneous infection. Bennion *et al.* (1984) induced colonic ischaemia in dogs and this caused prolonged and persistent bacteraemia due to *B. fragilis* and other anaerobic bacteria.

Several studies provide support for the pathogenic role of capsulate anaerobes in infectious processes. A higher recovery rate of capsulate anaerobic bacteria was noted from acutely inflamed tonsils (Brook and Gober, 1983) and orofacial abscesses (Brook, 1986) than from the normal oral flora.

Onderdonk *et al.* (1977), Brook *et al.* (1983) and Brook and Walker (1984a, 1984b) correlated the virulence of *Bacteroides* spp. and AGPC with the presence of capsules. Injection of capsulate anaerobes alone resulted in abscess formation in most animals, whereas non-capsulate species seldom caused abscesses unless they were combined with an aerobic organism. Heat-killed capsulate strains of *B. fragilis* or purified capsular polysaccharide alone, but not capsular polysaccharide from *E. coli*, resulted in formation of intra-abdominal abscesses (Onderdonk *et al.*, 1977). Simon *et al.* (1982) showed that capsulate strains of *Bacteroides* were more resistant to neutrophil-mediated killing than were non-capsulate strains. These investigations provide evidence that the capsular polysaccharide of *B. fragilis* is an important virulence factor.

We have previously observed the emergence of capsulate *Bacteroides* spp. and AGPC in mixed infections with other flora (Brook *et al.*, 1983; Brook and Walker, 1984a, 1984b). With few exceptions, possession of a capsule made these organisms more important *in vivo* than their aerobic counterparts. Although non-capsulate organisms did not induce abscesses, many of the strains (which had only minimal numbers of capsulate organisms) when injected mixed with other bacteria, survived in the abscesses and became predominantly capsulate. These capsulate strains were thereafter able to induce abscesses when injected alone. This phenomenon may help to explain how non-pathogenic organisms that are part of the normal host flora can become pathogens. It may also explain the importance of anaerobic organisms in chronic infections and in abscess formation, because the process of capsulation took 10–14 days. The non-capsulate strains used in our study were clinical strains that

had probably lost their capsules after in-vitro passages and storage, but were able to re-acquire the capsule after animal passage.

The present study confirms our previous observation of the synergy between *Bacteroides* spp. and AGPC and the other flora present with them in mixed infections (Brook *et al.*, 1984). Synergy between aerobic and anaerobic bacteria has long been recognised in various clinical infections (Gorbach and Bartlett, 1974; Finegold, 1977). In several animal studies, a mixed inoculum of anaerobic and aerobic bacteria produced sepsis that could not be induced by either component alone (Gorbach and Bartlett, 1974; Onderdonk *et al.*, 1974; Kelly, 1977, 1978). The role of capsulation was, however, not considered in these studies.

Altmeier (1942) demonstrated the pathogenicity of bacterial isolates isolated from peritoneal cultures after rupture of the appendix. Pure cultures of individual isolates were relatively innocuous when implanted subcutaneously in animals, but combinations of facultative anaerobic and anaerobic strains greatly increased virulence. Similar observations were reported by Meleney *et al.* (1932) and Hite *et al.* (1949). Several hypotheses have been proposed to explain microbial synergy. When this phenomenon occurs in mixtures of aerobic and anaerobic flora, it may be due to protection from phagocytosis and intracellular killing (Ingham *et al.*, 1977, 1981), supply of essential growth factors (Lev *et al.*, 1971), and lowering of oxidation-reduction potential in host tissues (Mørgenhausen *et al.*, 1958).

The increased virulence of capsulate anaerobic bacteria and their synergic potential with other flora is believed to be mediated through increased resistance of these bacteria to phagocytosis and killing by polymorphonuclear leukocytes and macrophages (Ingham *et al.*, 1977, 1981; Namavar *et al.*, 1983, 1984; Connolly *et al.*, 1984). Furthermore, the capsulate bacteria have been shown to decrease phagocytosis of other organisms associated with them in mixed infections.

Ingham *et al.* (1977, 1981) investigated the effect of *Bacteroides* spp. on the phagocytic killing of facultative species. Killing of *B. fragilis* and *P. mirabilis* in mixtures *in vitro* was impaired when the concentration of *B. fragilis* was $> 10^7$ cfu/ml in the phagocytic system. Connolly *et al.* (1984) provided evidence that correlated the inhibition of in-vitro phagocytosis of *P. mirabilis* with the capsular polysaccharide of *B. fragilis*. *B. gingivalis* cells or culture supernates were shown to possess the greatest inhibitory effect amongst the *Bacteroides* species (Namavar *et al.*, 1984). Supernates of cultures of *B. fragilis* group, *B. melaninogenicus* group, and *B.*

gingivalis were shown to be capable of inhibiting the chemotaxis of leukocytes to the chemotactic factors of *P. mirabilis* (Namavar *et al.*, 1983). Capsular material from *B. melaninogenicus* also inhibited phagocytosis and phagocytic killing of other microorganisms in an in-vitro system (Okuda and Takazoe, 1973).

Further studies are necessary to explain the increased virulence of capsulate *Bacteroides* spp. and AGPC, as expressed by persistent bacteraemia and seeding to different organs.

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