Epidemiology of the *Bacteroides fragilis* group in the colonic flora in 10 patients with colonic cancer

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Summary. We report the relative frequencies of members of the *Bacteroides fragilis* group in the faeces, in colon lavage fluid obtained pre-operatively, and in colonic tissue specimens obtained at operation from 10 patients with colonic cancer. *B. vulgatus* was the most and *B. fragilis* and *B. ovatus* were the least frequently isolated *Bacteroides* spp. in the faeces of the 10 subjects. *B. uniformis* and *B. thetaiotaomicron* ranked second and third in the faeces. The relative frequencies of all species except *B. fragilis* were lower in the lavage fluid and in cultures of mucosa. The relative frequency of *B. fragilis* increased from 4% in faeces to 39% in the final lavage fluid and to 42% in the colonic mucosa culture. Our results suggest that *B. fragilis* has a more intimate association with the gut mucosa than other members of the *B. fragilis* group, which might be one explanation for the high incidence of this species in gut-associated intra-abdominal infections.

Introduction

The *Bacteroides fragilis* group consists of at least six species—*B. fragilis*, *B. ovatus*, *B. distasonis*, *B. vulgatus*, *B. thetaiotaomicron* and *B. uniformis* (Johnson, 1978). Members of this group, especially *B. fragilis*, play an important role in a wide variety of pure and mixed infections in man (Gorbach and Bartlett, 1974; Duerden, 1980; McGowan and Gorbach, 1981; Finegold *et al.*, 1986). Strains of the *B. fragilis* group are common inhabitants of the human alimentary tract and constitute a major portion of the microbial flora of faeces. Some of the most extensive studies dealing with the isolation and characterisation of anaerobes under optimal conditions have been made by Holdeman and Moore and their co-workers (Moore and Holdeman, 1974; Holdeman *et al.*, 1976). In two studies, they found that *Bacteroides* spp. accounted for 20–30% of the total cultivable flora; *B. vulgatus* was the most frequently encountered species. *B. thetaiotaomicron* and *B. distasonis* were also detected in high numbers, whereas *B. fragilis* and *B. ovatus* were isolated only occasionally.

The difficulty of obtaining samples has meant that much of the work to define the microbial flora of the large intestine has been done with faecal

samples, whereby it is assumed that this material is representative of the colonic contents from which it is derived. However, little is known about local differences in prevalence of bacteria within the large intestine itself. We have studied the relative frequencies of members of the *B. fragilis* group in faeces, in irrigation fluid before colon surgery, and in the flora associated with colonic epithelium in patients with cancer of the colon.

Materials and methods

Patients

The group studied comprised 10 patients with cancer of the colon (six women and four men, aged 32–77 years, average age 61·7 years). Seven patients had cancers of the sigmoid colon, two of the transverse colon (patients nos. 7 and 8), and one had cancer of the descending colon (patient no. 4).

Specimen processing

Stool specimens were collected in sterile plastic containers from all patients before whole gut irrigation and transported to the laboratory immediately for bacteriological examination. Whole gut irrigation was performed the day before operation. Lavage was continued until the effluent from the rectum became completely clear. Three separate samples of c. 5 ml of lavage fluid

were obtained—lavage 1 at the beginning of irrigation (effluent contained faecal material), lavage 2 during irrigation (turbid effluent without faecal material), and lavage 3 at the end of irrigation (completely clear effluent). The initial stool specimens and the lavage-1 specimens were each thoroughly mixed before estimation of the numbers of bacteria present. A 1-g sample of each was then transferred to 9 ml of saline (NaCl 0.9%). After vigorous shaking to disperse the sample completely, serial 10-fold dilutions were made for bacterial viable counts. Ten-fold dilutions of lavages 2 and 3 were prepared in saline and used for bacterial counts. Mucosal specimens of c. 3 cm² were taken from apparently healthy portions of the colon which had been removed during wide resection of the tumour. Tissue specimens were washed by immersion and agitation in three separate volumes of sterile fresh saline and a 1-g sample was homogenised in 1 ml of saline in a grinder (Heidolph, Electro Abi, Haarlem, Netherlands) within 30 min of excision. Serial dilutions of the homogenate were prepared for determining the number of viable organisms/g of tissue.

Media

A selective medium for the *B. fragilis* group containing ammonium sulphate (2 g/L) and gentamicin (50 mg/L) (BFAG) was used in this study. This medium was developed by Ushijima *et al.* (1983) for the isolation and presumptive identification of the *B. fragilis* group. Viable counts of bacteria were performed by plating serial dilutions on BFAG agar, Wilkins-Chalgren Anaerobe Agar (Oxoid), and blood agar (Blood Agar Base No. 2, Oxoid) with sheep blood 5%, haemin (BDH Chemicals Ltd, Poole, England) 5 µg/ml and menadione (Merck, Darmstadt, West Germany) 2 µg/ml. Plates were incubated in an anaerobic chamber (Coy Laboratory Products, USA) in an atmosphere of N₂ 80%, H₂ 10%, CO₂ 10% for 3 days at 37°C.

The total numbers of the *B. fragilis* group present were determined by plating serial dilutions of faeces, lavage fluid and tissue homogenate on BFAG agar and incubating in the anaerobic chamber for 3 days. The total number of bacteria are given as colony-forming units (cfu)/1 g of tissue or faeces or 1 ml of lavage fluid.

Identification procedure

To assess the relative occurrence of the different species belonging to the *B. fragilis* group, we picked randomly 50 discrete colonies from each sample on BFAG plates (i.e., 250 colonies from each patient). In view of the large number of colonies to be examined (2500 in total) we first found it necessary to develop a rapid plate method based on the identification scheme of Bergey's Manual of Systematic Bacteriology (Holdeman *et al.*, 1984). The basal medium for identification tests was Peptone Yeast Extract (PY) agar (Holdeman *et al.*, 1977), pH 7.1, with bromocresol purple (Merck) 0.0015% w/v. Arabinose, trehalose, rhamnose, salicin and maltose were added to

PY agar at a final concentration of 1% as recommended by Holdeman *et al.* (1977). Xylan fermentation was determined by the method of Salyers *et al.* (1977). For the indole reaction, tryptophan 0.1% was added to PY agar and the modified spot indole test with para-dimethyl-aminocinnamaldehyde was used (Dowell and Lombard, 1981). Each bacterial colony to be identified was transferred to 1.5 ml of saline; from this suspension a gram-stained film was made and it was plated out on bouillon agar (Blood Agar Base No. 2, Oxoid) and PY agar for aerobic incubation to exclude contamination by facultative anaerobes. From the remaining suspension, a series of PY-carbohydrate agar plates was inoculated with a multipoint inoculator type A400 (Denley, Sussex). On each plate 11 samples were tested—nine unknown bacterial isolates and two type culture collection strains (*B. fragilis* ATCC strain 23745 and *B. ovatus* ATCC strain 8483) as controls. Plates were incubated in the anaerobic chamber for 48 h. Fermentation was considered to be positive when a yellow colour developed around the colony and negative when the purple colour remained unchanged.

The validity of this identification procedure was tested blindly with 25 strains of the *B. fragilis* group which had previously been identified with the Minitek system (BBL, Becton Dickinson, USA) and five type culture collection strains—*B. fragilis* ATCC 23745, *B. vulgatus* ATCC 8482, *B. distasonis* ATCC 8503, *B. thetaiotaomicron* ATCC 29148 and *B. ovatus* ATCC 8483. Correlation to species level of the two systems was 94%. Statistical analysis was performed by Student's *t* test.

Results

To test the plating efficiency of the BFAG medium, 24-h cultures in BM medium (Shah *et al.*, 1976) of 15 strains belonging to the *B. fragilis* group were washed in saline and resuspended to a pre-determined optical density; their growth was tested on BFAG medium and, in parallel, on non-selective Wilkins-Chalgren and blood-agar media (table I). Each strain tested produced virtually the same number of cfu on BFAG and non-selective media. Before we attempted to isolate strains of the *B. fragilis* group from specimens from the 10 patients on BFAG agar, the selectivity of this medium was tested by culturing one human faecal sample. BFAG agar plates were inoculated with serial dilutions of faeces. From the dilution yielding discrete colonies, 97 colonies were examined and 92 isolates were identified as belonging to the *B. fragilis* group; five strains could not be identified with our identification scheme nor with the Minitek system (table II). Thus BFAG medium was highly selective for the *B. fragilis* group and this selectivity and high plating efficiency made it eminently suitable for the present study.

Table I. Plating efficiency of selective medium BFAG

Strains	Number tested	Selective BFAG medium	Viable count*—cfu $\times 10^9$ /ml (SD)—on	
			non-selective media	
			Wilkins-Chalgren agar	Blood agar
<i>B. fragilis</i>	3	2.3 (0.35)	2.4 (0.07)	2.4 (0.10)
<i>B. distasonis</i>	3	2.3 (0.35)	2.3 (0.42)	2.3 (0.6)
<i>B. thetaiotaomicron</i>	3	1.3 (0.28)	1.3 (0.21)	1.4 (0.35)
<i>B. vulgatus</i>	3	1.9 (0.28)	2.3 (0.77)	1.9 (0.14)
<i>B. ovatus</i>	3	1.8 (0.24)	1.8 (0.07)	1.8 (0.14)

* Counted after anaerobic incubation for 3 days.

Table II. Prevalence of species of *B. fragilis* group on BFAG inoculated with faeces of one healthy individual

Species	Number of isolates (%) (n=97)
<i>B. thetaiotaomicron</i>	31 (31.9)
<i>B. uniformis</i>	35 (36.1)
<i>B. vulgatus</i>	13 (13.4)
<i>B. fragilis</i>	6 (6.1)
<i>B. distasonis</i>	5 (5.1)
<i>B. ovatus</i>	2 (2.1)
unknown	5 (5.1)

Table III shows the log viable counts of the *B. fragilis* group strains in the specimens from the 10 subjects. The total number of *B. fragilis* group organisms isolated from the specimens varied from patient to patient. The average number of this group of bacteria in faeces was 2.1×10^9 cfu/g, which was only slightly higher than the average number of 1.0×10^9 cfu/g found in lavage 1. Lavage samples 2 and 3 contained on average 9.3×10^7

cfu/ml and 7.6×10^6 cfu/ml respectively. The efficiency of lavage (whole gut irrigation) in removing this group of bacteria from the large intestine is shown by the fact that counts in lavage fluid 3 were only 0.3% of those in faeces. The number of the *B. fragilis* group found in colon homogenates from patients varied from 2.0×10^4 cfu/g to 4.0×10^7 cfu/g with an average number of 3.6×10^5 cfu/g. Thus only 0.01% of the total bacterial counts in faeces was found in tissue specimens. Statistical analysis (Student's *t* test) showed that the differences between the counts in faeces, lavages 1, 2 and 3, and mucosa of the 10 patients were significant ($p < 0.02$). The isolation of the individual species of the *B. fragilis* group was variable in the faeces of the patients. *B. vulgatus* was isolated from all patients, *B. thetaiotaomicron*, *B. uniformis* and *B. distasonis* from nine out of 10, *B. fragilis* from eight and *B. ovatus* was isolated from only four. As is shown in table IV, strains of *B. vulgatus* accounted for 45% of the *B. fragilis* group isolates and were the most frequently isolated bacteria in the faeces of the 10 patients. The numbers of this species were lower in lavage samples 1, 2 and 3—43.9%, 40.4% and 32.2% respectively. However, 25.6% of all *B. fragilis* group strains found in tissue homogenates

Table III. Viable counts of *B. fragilis* group in faeces, lavages, and the intestinal mucosa from 10 patients

Specimen	Viable count (log ₁₀ cfu/g or ml) in patient no.									
	1	2	3	4	5	6	7	8	9	10
Faeces	9.3	7.4	10.1	9.4	9.0	10.0	9.0	9.3	9.1	10.6
Lavage 1	8.9	7.5	10.0	8.9	8.8	9.9	8.7	9.0	8.8	9.4
Lavage 2	8.9	7.2	9.5	7.0	7.8	7.1	8.2	8.0	8.4	7.6
Lavage 3	6.2	6.2	8.5	7.0	6.0	6.2	7.0	6.6	8.3	6.8
Mucosa	5.9	5.5	7.6	4.7	5.9	5.7	5.4	5.3	5.3	4.3

were identified as *B. vulgatus*. *B. uniformis* (22.2%) and *B. thetaiotaomicron* (15.1%) were second and third in frequency in the faeces of the 10 subjects. The mean percentage of *B. uniformis* (21.6%) in lavage 1 did not differ from its percentage in faeces. There was no important difference in the proportion of *B. uniformis* isolated from lavages 2, 3 and colonic tissue homogenates; the relative number of this species in these specimens was c. 11.0%.

The numbers of strains of *B. thetaiotaomicron* were moderately lower in lavages 1 and 2 (10%) than in faeces (15.1%) and were slightly lower again in lavage 3 and in mucosal specimens. *B. distasonis* strains, with the average proportion of 9.0%, showed a constant pattern in all specimens except for lavage 3, in which they represented only 5.6% of the total bacteria. *B. fragilis* and *B. ovatus* strains, with average proportions 4.0% and 3.6% respectively, were least often isolated from the faeces of our subjects. The relative frequency of *B. ovatus* remained constant at 3.0% for all specimens. Interestingly, the proportion of *B. fragilis* strains in mucosa homogenates was significantly greater than in faeces ($p < 0.01$) (Student's *t* test). No significant differences were found in the proportions of other *B. fragilis* group strains in mucosa and faeces.

Discussion

Drasar and Barrow (1985) suggested that faecal flora is probably a final decadent phase of the intestinal flora and accurately represent only that of the rectum. The intestinal microflora can be divided into faecal and epithelium-associated microflora. The mucosal microflora growing in close association with the mucosal surface has been reported to differ quantitatively from the faecal

flora (Weidema and Van Den Bogaard, 1984). Our results are in agreement with this report. We had only two specimens from the transverse colon and one from the descending colon, but the incidence of the *B. fragilis* group did not differ significantly in these specimens from that of specimens from the sigmoid colon.

The absolute numbers of *B. fragilis* group strains in faeces were virtually identical to those of lavage 1 but were reduced through lavage samples 2 and 3. The relative frequencies of *B. vulgatus*, *B. uniformis* and *B. thetaiotaomicron* in mucosal samples were approximately half those in faeces. The relative numbers of *B. distasonis* and *B. ovatus* species were similar in all specimens tested. In contrast, the relative frequency of *B. fragilis* rose from 4.4% in faeces to 42.8% in colonic specimens. Although, in our study, mucosal specimens were taken from apparently healthy portions of the colon, we do not know whether there are differences in the ecology of mucosa-associated bacteria of the colon in normal and cancerous patients. There was a discrepancy in the frequency of *B. thetaiotaomicron* and *B. vulgatus* found in one healthy individual (table II) and in the mean frequencies in the 10 patients (table IV). This discrepancy may reflect differences between populations, such as between healthy individuals and patients. However, in the group of patients studied, striking variations were found between individuals, e.g., in patient 5 the relative frequency of *B. thetaiotaomicron* and *B. vulgatus* was 59% and 9% respectively, whereas patients 2 and 8 had equal number of *B. thetaiotaomicron* and *B. vulgatus* in their faeces. Be that as it may, the relative frequency of *B. fragilis* in all the patients rose from faeces to mucosal culture. We

Table IV. Prevalence of species of the *B. fragilis* group in faeces, lavages and the intestinal mucosa of 10 patients

Species	Percentage of <i>B. fragilis</i> group (range) in				
	Faeces*	Lavages			Mucosa
		1	2	3	
<i>B. fragilis</i>	4.4 (0-19)	11.4 (0-30)	22.7 (5-38)	39.1 (7-85)	42.8 (9-80)
<i>B. vulgatus</i>	45.0 (9-80)	43.9 (14-91)	40.4 (16-89)	32.2 (2-76)	25.6 (4-68)
<i>B. distasonis</i>	9.3 (0-21)	8.3 (0-22)	11.8 (0-22)	5.6 (0-14)	9.8 (0-23)
<i>B. uniformis</i>	22.2 (0-71)	21.6 (0-44)	13.1 (0-38)	11.0 (2-21)	10.3 (0-22)
<i>B. thetaiotaomicron</i>	15.1 (0-59)	10.1 (0-39)	10.3 (0-30)	7.4 (0-24)	8.0 (0-31)
<i>B. ovatus</i>	3.6 (0-12)	2.9 (0-12)	1.0 (0-3)	2.9 (0-19)	3.6 (0-26)

* = Percentage of *B. fragilis* group from 10 patients; the range is given in brackets.

have not yet investigated the location of the disproportionately high numbers of *B. fragilis* in the mucosal flora but Nelson and Mata (1970) studied the microflora of six colonic samples and found that *Bacteroides* spp. were located in the mucous lining, especially in the tags of mucus which were attached to the surface epithelium between the crypt orifices.

The important question remains why *B. fragilis* was found in relatively higher numbers than the other members of the *B. fragilis* group in the mucosal flora. A greater ability of *B. fragilis* to adhere to colonic epithelium may be a factor of importance. If this is so, several properties of *B. fragilis*, such as capsule, glycocalyx, haemagglutinins and fimbriae, may be responsible for its more efficient adherence to the epithelium of the intestine. Onderdonk *et al.* (1978) have shown that capsulate *B. fragilis* strains adhere better to the peritoneal surface of the rat than strains without an extracellular polysaccharide layer, which implicates the capsule in adherence. Glycocalyx production by *B. fragilis*, besides protecting the infecting organisms from phagocytosis and antibiotics, may possibly play a role in adherence (Lambe *et al.*, 1984). Adherence is often measured by haemagglutination. Vel *et al.* (1986) compared the haemagglu-

tinating property of five species of the *B. fragilis* group for erythrocytes of different origins. Only strains of *B. fragilis* agglutinated erythrocytes and different patterns of haemagglutination were observed.

Pruzzo *et al.* (1984) have shown that those strains of *B. fragilis* capable of adhering to human epithelial cells and a cultured human cell line (intestine 407) were haemagglutinating strains and possessed fimbriae-like structures. On the other hand, fimbriae have been demonstrated in *B. fragilis*, but did not appear to be associated with haemagglutination. However, different types of fimbriae are probably present (Van Doorn *et al.*, 1987). Thus it is clear that the factors mediating adherence in *B. fragilis* have not yet been defined and await further studies. The finding that *B. fragilis* was the only member of the group with a low prevalence in faeces and a proportionally higher prevalence in mucosal specimens would support the concept of a greater capacity to adhere to the epithelial lining of the intestine. Furthermore, these results indicate that *B. fragilis* has a more intimate association with the gut mucosa than do other members of the *B. fragilis* group, which might be one of the explanations for the high incidence of this species in gut-associated infections.

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