Aggregation by fragilis and non-fragilis *Bacteroides* strains *in vitro*

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Summary. *Bacteroides fragilis* is associated with the formation of intra-abdominal abscesses, whereas other *Bacteroides* species are rarely involved. Since bacterial clumping may contribute to the survival of bacteria in the face of host defence mechanisms, the hypothesis has been put forward that differences in aggregation between fragilis and non-fragilis strains of *Bacteroides* may account for their differences in survival *in vivo*. All seven *B. fragilis* strains tested formed aggregates within 4 h, but strains not associated with intra-abdominal sepsis—*B. vulgatus*, *B. thetaiotaomicron* and *B. distasonis*—did not form aggregates *in vitro*. Aggregation occurred at 37°C, but not at 4°C or 20°C. Treatment with pronase partially inhibited aggregation. Periodate treatment killed the cells and caused them to form clumps which were distinguishable from the control aggregates. Heat-killed *B. fragilis* cells formed similar distinct clumps, but cells killed by glutaraldehyde and formaldehyde did so to a lesser degree. No inhibition was found upon addition of carbohydrates, ethylenediaminetetraacetic acid or after treatment with trypsin. These results demonstrate that aggregate formation occurs with *B. fragilis* strains alone, and that surface proteins probably mediate this interaction.

Introduction

Microbiological studies of intra-abdominal infection following perforation of the colon reveal a mixture of aerobic and anaerobic bacteria (Lorber and Swenson, 1975). *Bacteroides fragilis* is the most frequently isolated anaerobe despite the fact that it ranks relatively low in frequency among the anaerobic bacteria present in the colonic flora (Finegold *et al.*, 1974; Gorbach and Bartlett, 1974). Several explanations have been advanced to account for the unique survival characteristics of this particular *Bacteroides* species. These include its ability to resist phagocytosis by host cells (Onderdonk *et al.*, 1977), its capacity to elaborate proteolytic enzymes (Rudek and Haque, 1976; Steffen and Hentges, 1981) and its enhanced oxygen tolerance (Tally *et al.*, 1977).

The ability of bacteria to form aggregates has

been considered to be a virulence factor as well as a protective mechanism against killing by host phagocytes (Costerton *et al.*, 1985), and against antimicrobial therapy (Nickel *et al.*, 1985). Furthermore, it may represent an efficient means of transferring nutrients and maintaining a micro-environment suitable for microbial survival (Onderdonk *et al.*, 1979). The purpose of the present study was to examine the ability of *B. fragilis* to aggregate *in vitro* as a potential mechanism for its persistence *in vivo* and its tendency towards abscess formation.

Materials and methods

Bacteria and growth conditions

The strains examined included: clinical isolates of *B. fragilis*—strains 9032, 6712, A-73, 36, A-460 and 484—and type strain ATCC 25385; clinical isolates of *B. vulgatus*—strains A-541, A-374, and 00—and type strain ATCC 29327; clinical isolates of *B. thetaiotaomicron*—strains 34 and 38—and type strain ATCC 29741; and one isolate of *B. distasonis*, strain 5346. All strains except *B. fragilis* 9032 were obtained from Dr R. Devlin, Department of Microbiology, Toronto General Hospital.

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B. fragilis 9032 was obtained from Dr T. Wilkins, Virginia Polytechnic Institute, Blacksburg, VA. Stock cultures of all strains were maintained at -70°C in Thioglycolate Broth (BBL Microbiology Systems, Cockeysville, MD, USA). Frozen cultures were thawed and 0.1 ml samples were inoculated into tubes containing 10 ml of minimal growth medium as previously described (Namavar *et al.*, 1983; Rotstein *et al.*, 1987). The tubes were incubated for 24 h at 35°C in an anaerobic chamber (Forma Scientific, Marietta, OH, USA) which maintained an atmosphere of N_2 85%, H_2 10%, CO_2 5%. The organisms were harvested by centrifugation at 3000 rpm for 25 min and were resuspended in pre-reduced phosphate-buffered saline (PBS, pH 7.1) to give concentrations ranging from 10^7 to 10^{10} cfu/ml. Viable counts were performed by serial dilution and plating on supplemented Brain Heart Infusion Agar. Plates were incubated anaerobically for 48 h at 35°C before colonies were counted.

Aggregation assay

A 500- μl volume of bacterial suspension was added to the well of a microtitration tray (24 wells; Nunclon, Denmark) and dispersed by passage through a 25-gauge needle (Neolus, Terumo, Japan). The bacteria were incubated either anaerobically (Gas Pak jar) or aerobically at 37°C in a shaker (100 rpm). An observer who was unaware of the identity of the strain under test measured aggregation by examination of the cells at various times under the $10\times$ lens of a dissecting microscope. The scoring system was based on a visual measurement of 0 (no aggregation; fig. 1)–4 (large macroscopic clumps; fig. 2), in a similar fashion to that reported previously (Reid *et al.*, 1988).

It is noteworthy that the morphology of various strains differed somewhat as determined by microscopy of Gram's stain. Although the strains illustrated in figs. 1

and 2 were both gram-negative bacilli, the *B. thetaiotaomicron* strain (fig. 1) was generally shorter, broader and more homogeneous than the *B. fragilis* strain, which was long and pleomorphic (fig. 2). This may account for the somewhat more rounded appearance of the strain illustrated in fig. 1. However, an electronmicroscopy study of *B. fragilis* 9032, *B. vulgatus* A541 and *B. vulgatus* 29327 showed no obvious morphological differences between strains (data not shown).

Effect of various agents on aggregation

The effect of lactose, glucose, galactose, mannose, methylglucopyranoside and glucuronic acid was studied by adding 250 μl of 0.18M solution of the sugar to 500 μl of a suspension containing 10^9 cfu of dispersed bacteria at the beginning of the incubation period. Aggregation was compared to that of the control in which 250 μl of PBS was substituted for the sugar solution. Ethylenediaminetetraacetic acid (EDTA), at a concentration of 2.5 mM and adjusted to pH 7.8 with NaOH, was also tested in the aggregation mixture. Trypsin and pronase were made up to 2 mg/ml in distilled water and the pH adjusted to 7.8 with NaOH; 1 ml of the bacterial suspension was centrifuged and resuspended in the protease solutions, shaken for 45 min at 37°C , centrifuged, and resuspended in PBS before assay. Bacterial suspensions were treated with sodium periodate (0.001 M, 0.01 M and 0.1 M respectively) for 30 min at room temperature, added to glycerin and then washed three times in PBS before assay.

Effect of bacterial killing on aggregation

The bacteria were heat killed by exposure to: (a) 121°C for 15 min; (b) 100°C for 30 min; and (c) 80°C for 60

Fig. 1. Micrograph of *B. thetaiotaomicron* 38 taken on a Zeiss microscope with a $\times 40$ objective lens. The bacteria are a little out of focus due to difficulties in photographing the wet mount preparation. There is no evidence of aggregation, although some dividing cells in twos and threes are seen. Bar = c. 2 μm .

Fig. 2. Micrograph of *B. fragilis* 9032 taken on a Zeiss microscope with a $\times 40$ objective lens. There is strong, macroscopically visible aggregation—score 3. Large clumps of aggregated cells are evident. Bar = c. $3\mu\text{m}$.

min; and then tested for aggregation. In addition, bacterial suspensions were killed by the addition of glutaraldehyde 10% for 45 min, and formaldehyde 5% and 10% for 20 min; they were washed three times and then tested for their ability to form aggregates.

Results

Aggregation of B. fragilis and non-fragilis Bacteroides

Aggregation of *B. fragilis* 9032 occurred in a dose dependent fashion, ranging from a score of 3 at 10^9 cfu/ml to a score of 1 at 10^8 cfu/ml and none at 10^7 cfu/ml. Aggregation was similar whether determined at 4 or 20 h and whether performed under aerobic or anaerobic conditions. Aggregation was also temperature dependent. *B. fragilis* incubated at 4°C demonstrated poor aggregation (score 1) and only moderate aggregation when incubated at 20°C (score 2).

To compare aggregation between several different *Bacteroides* strains, each strain, at a concentration of 10^9 cfu/ml was incubated for 20 h at 37°C in

anaerobic conditions. All seven *B. fragilis* strains tested produced dense aggregates at both 4 and 20 h. Conversely, only two non-fragilis strains aggregated: *B. vulgatus* A-541 produced moderate aggregation and the type strain of *B. vulgatus* demonstrated only minimal aggregation (table I). Viability checks of the aggregates confirmed the presence of living bacteria.

Effect of various agents on aggregation

The addition of carbohydrates or the calcium chelating agent, EDTA, had no effect on the ability of *B. fragilis* to aggregate (table II). Trypsin failed to inhibit aggregation, whereas pronase caused a marked reduction in the clumping effect. Of all the agents used, pronase caused the most inhibition. The effect of periodate on bacterial aggregation was difficult to score with the standard scoring system. Although bacteria clearly aggregated, the appearance of the aggregate differed from untreated *B. fragilis* in that treated cells produced large debris-like clumps which settled on the bottom of the well.

Table I. Aggregation scores for *Bacteroides* spp. tested *in vitro**

Strain	Aggregation score	Strain	Aggregation score
<i>B. fragilis</i> 9032	3	<i>B. vulgatus</i> A541	2
<i>B. fragilis</i> 6712	3	<i>B. vulgatus</i> 29327	1
<i>B. fragilis</i> A460	3	<i>B. vulgatus</i> A374	0
<i>B. fragilis</i> A73	3	<i>B. vulgatus</i> 00	0
<i>B. fragilis</i> 36	3	<i>B. thetaiotaomicron</i> 38	0
<i>B. fragilis</i> 25385	2	<i>B. thetaiotaomicron</i> 34	0
<i>B. fragilis</i> 484	3	<i>B. thetaiotaomicron</i> 29741	0
		<i>B. distasonis</i> 5346	0

*Based upon an inoculum of 500 μ l from a suspension of 10^9 cfu/ml incubated for 4 h at 37°C in an anaerobic environment (similar results were obtained aerobically).

Score 0 = no aggregation; 1 = small aggregates; 2 = larger aggregates; 3 = macroscopic aggregates; 4 = large clumps of macroscopic aggregates.

Periodate, at all concentrations tested, killed *B. fragilis*.

Aggregation of dead cells

To determine whether bacterial killing could account for the altered appearance of the aggregate,

Table II. The effect of various treatments on the ability of *B. fragilis* 9032 to form aggregates *in vitro* when the inoculum was a 10^9 cfu/ml suspension

Treatment	Aggregation score
Control 9032 in buffer	
at 37°C	3
at 20°C	2
at 4°C	1
Carbohydrates:	
Lactose	3
glucose	3
galactose	3
mannose	3
methylglucopyranoside	3
glucuronic acid	3
EDTA	3
Periodate	1*
Trypsin	3
Pronase	1
Glutaraldehyde 10%	2
Formaldehyde 5%	1
10%	1
Heat: 121°C, 15 min	0*
100°C, 30 min	0*
80°C, 60 min	0*

*Cell debris settled to bottom of the well, but appearance differed from aggregation.

The scoring system is described in the footnote to table I.

B. fragilis cells were killed by various techniques and tested for their ability to aggregate. Heat treatment caused the dead cells to settle on the bottom of the well, as a large clump similar to that noted for periodate-treated cells. There was no difference between the three heat treatment regimens. Glutaraldehyde and formaldehyde pretreatments caused clumping to a lesser degree so that they could still be assessed for their effect on aggregation. Glutaraldehyde partially inhibited aggregation, while formaldehyde-killing completely inhibited the aggregation.

Discussion

Strains of *B. fragilis* are the most frequently recovered anaerobic micro-organisms in intra-abdominal infections despite being greatly outnumbered in the gastrointestinal tract by non-fragilis strains (Finegold *et al.*, 1974). This phenomenon suggests that *B. fragilis* has some unique survival characteristics which account for its persistence in these infections. Compared to non-fragilis strains, strains of *B. fragilis* have demonstrated enhanced resistance to phagocytosis by neutrophils *in vitro* (Onderdonk *et al.*, 1977), and greater adherence to peritoneal surfaces *in vivo* (Onderdonk *et al.*, 1978). These properties have generally been attributed to capsulation and, in particular, to the possession of a unique polysaccharide capsule. However, studies by Pruzzo *et al.* (1984) have suggested that fimbriae present on non-capsulate *Bacteroides* strains are important mediators of adherence. *B. fragilis* strains also exhibit enhanced oxygen tolerance compared to non-fragilis strains, probably as a result of their ability to produce superoxide dismutase (Tally *et al.*, 1977). This enzyme is capable of degrading

oxygen radicals toxic to the cell in an aerobic environment.

One further potential mechanism for survival of fragilis strains is their ability to form clumps, a means by which bacteria can resist killing by antimicrobial agents as well as phagocytic killing by host cells (Costerton *et al.*, 1985; Nickel *et al.*, 1985). In addition, clumping of bacteria may create optimal conditions locally for survival in an aerobic environment such as the peritoneal cavity. Coaggregation of bacteroides actinomyces and streptococci has been reported in the oral cavity (Kolenbrander *et al.*, 1985) where bacterial interactions are associated with plaque formation (Ellen and Balcerzak-Rackowski, 1977). In support of the concept that aggregation may, at least in part, account for the persistence of fragilis strains of *Bacteroides* in the peritoneum, all seven fragilis strains tested aggregated *in vitro*, whereas only two of seven non-fragilis strains did so. Aggregation by *B. fragilis* 9032 was dose dependent in both aerobic and anaerobic conditions. This aggregation was probably not mediated via lectin binding because carbohydrates were not inhibitory. However, treatment with pronase impaired aggregation, which suggests a potential role for protein adhesins in the mechanism of aggregation. In keeping with this hypothesis, *B. fragilis*, killed by formaldehyde or glutaraldehyde, aggregated much less or not at all, presumably as a result of denaturation or fixation of surface proteins.

Sodium periodate was tested to determine the contribution of surface polysaccharide to the aggre-

gation process. At all concentrations tested, periodate killed the bacteria, causing them to clump at the bottom of the well in a pattern which was clearly different from aggregation. Vel *et al.* (1986) have reported that similar concentrations of periodate prevented haemagglutination of several *Bacteroides* strains. However, these authors did not report on the viability of cells following periodate treatment.

All strains grew well in the culture medium used in these studies, but there was variation among strains. The effect of growth conditions on aggregation was not investigated in the present studies. However, these have been demonstrated to affect bacterial morphology and adhesion (Cook *et al.*, 1988) and will be the subject of future studies related to *Bacteroides*.

The present studies demonstrated a significant degree of aggregation among *B. fragilis* strains but the phenomenon was largely absent among non-fragilis strains. There was some evidence to suggest that the aggregation process appeared to be mediated via a surface protein adhesin. Whether aggregation itself serves to enhance survival of fragilis strains over non-fragilis strains by impeding host clearance of these bacteria, or by some other mechanism, will be the subject of further study.

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