Effect of *Bacteroides fragilis* cellular components on chemotactic activity of polymorphonuclear leukocytes towards *Escherichia coli*

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Summary. Chemotaxis of polymorphonuclear leukocytes (PMNL) in response to cell components of *Bacteroides fragilis* alone or in combination with *Escherichia coli* was evaluated. *E. coli* produced much more powerful chemotactic factors than *B. fragilis*. The culture filtrate (CF), outer membrane (OM) preparation, and lipopolysaccharide (LPS) of *B. fragilis* slightly stimulated chemotactic activity of PMNL. The culture filtrate and OM preparation were capable of inhibiting the chemotaxis of PMNL in response to the chemotactic factors of *E. coli* but LPS of *B. fragilis* was not able to do so. Reduction by *B. fragilis* of PMNL chemotaxis in response to *E. coli* was not specific for *B. fragilis* but also occurred in the presence of facultative bacteria. In parallel with chemotaxis, lysozyme release, but not β -glucuronidase release, by PMNL was significantly stimulated by *E. coli* but not by *B. fragilis*.

Introduction

Clinical and experimental observations of infections in which *Bacteroides* spp. are involved have led to the conclusion that bacteroides infections are characterised by abscess formation. *B. fragilis* is often associated in mixed infections with aerobic bacteria and often seems to play a key role in their pathogenesis, since its elimination by prophylactic chemotherapy prevents the establishment of infection (Hofstad and Kristofferson, 1970; Gorbach and Bartlett, 1974). Recent work has shown that anaerobic bacteria inhibit the phagocytosis and killing of aerobic bacteria (Ingham *et al.*, 1977; Jones and Gemmell, 1982; Namavar *et al.*, 1983; Connolly *et al.*, 1984; Vel *et al.*, 1985). Much work has been done to determine possible virulence factors of *B. fragilis*. This micro-organism produces lipopolysaccharide (LPS) with low biological toxicity (Kasper, 1976; Sveen *et al.*, 1977) and also a capsular polysaccharide (Kasper and Seiler, 1975) whose role in virulence is still controversial (MacLaren *et al.*, 1984). Inhibition of chemotaxis might be an important virulence factor in the early stages of *B. fragilis* infection; the development of abscesses suggests that it does not play a significant role in the later stages.

In our previous study (Namavar *et al.*, 1984), we found that the culture filtrates (CF) of *B. fragilis* inhibited polymorphonuclear leukocyte (PMNL) chemotaxis stimulated by *Proteus mirabilis*. Showell *et al.* (1976) and Becker (1976) have demonstrated the release of granule enzymes from cytochalasin B-treated PMNL exposed to synthetic chemotactic peptides, and found a close correlation between the ability of chemotactic stimuli to cause enzyme release and migration.

In this study, we report the chemotactic and antichemotactic effects of CF, outer membrane (OM) preparations and LPS of *B. fragilis* on the chemotactic stimulus of *Escherichia coli*. The effects of CF of *B. fragilis*, *E. coli* and a synthetic peptide upon exocytosis of PMNL were tested. Specificity of chemotaxis inhibition by *B. fragilis* on *E. coli* was tested with culture filtrates of *Staphylococcus aureus* and *Klebsiella pneumoniae* instead of *B. fragilis*.

Materials and methods

Bacterial strains

The strains used in these experiments were *E. coli* EB1 (serotype O8 K43) and *B. fragilis* BE1. Both strains were isolated from a mixed aerobic-anaerobic wound infection. In some experiments *S. aureus* NCTC 6571 and a clinical isolate of *K. pneumoniae*, 52K2, were used.

Media

The BM broth (Shah *et al.*, 1976) normally used for the growth of *Bacteroides* spp. was itself chemotactic for human PMNL. Therefore, to assess the chemotactic response of PMNL towards bacteria, a non-stimulating medium was used which had previously been described for measuring the chemiluminescence response of leukocytes (Namavar *et al.*, 1983). This was the minimal medium of Varel and Bryant (1974) for growth of *B. fragilis*.

Preparation of culture filtrates and cell suspension

B. fragilis BE1 was incubated for 24 h at 37°C in an anaerobic chamber in an atmosphere of N₂, 85%, H₂ 10%, CO₂ 5%. Facultative bacteria were incubated in the same medium at 37°C in a waterbath with shaking. The numbers of anaerobic and facultative bacteria were determined by optical density and adjusted to 10⁹ cfu/ml. Culture filtrates were prepared by centrifugation (1300 g, 15 min, 4°C) and filter sterilised through 0.45-µm membrane filters (Millipore). Cells were washed twice in phosphate-buffered saline (PBS) and resuspended in control medium to a concentration of 10⁹ cfu/ml.

Preparation of LPS and OM

LPS was extracted from *B. fragilis* BE1 by a modified Westphal procedure (Keleti and Lederer, 1974). LPS was assayed for protein by Bio-Rad protein assay (Bio-Rad Laboratories B.V., The Netherlands) with bovine plasma albumin as a standard. The LPS contained 2% protein. The OM of the *B. fragilis* was isolated by the method of Kasper and Seiler (1975). In this study the OM was the crude material isolated by centrifugation at 80 000g at 4°C for 2 h; this was suspended in a minimal volume of distilled water and lyophilised. The OM preparation contained 25% protein.

Preparation of PMNL

PMNL were separated from heparinised blood of healthy adults by dextran sedimentation. They were washed once in TC medium 199 (Difco) containing 2 mM L-glutamine and resuspended in the same medium at a concentration of 10⁷ PMNL/ml. The pH of the medium was adjusted to 7.4 with NaHCO₃ 7.5% w/v solution.

Chemotactic assay

The chemotaxis of PMNL was determined by the agarose chemotactic assay as previously described (Namavar *et al.*, 1984). The media contained agarose 1.2% w/v, 10 ml of 10× TC medium 199, 1 ml of sodium bicarbonate 7.5% w/v, 10 ml of inactivated new-born calf serum and 79 ml of distilled water. A 10-ml volume of the agarose medium was delivered into each tissue culture plate (60 × 15 mm; No. 3002, Falcon, CA, USA). Four

series of three wells, 3 mm in diameter and 3 mm apart were made in the agarose gel. The centre well of each three-well series received 10 µl of PMNL suspension (10⁷/ml), the outer well 10 µl of chemotactic factor and the inner well 10 µl of control medium. The plates were incubated at 37°C in a humidified atmosphere containing CO₂ 5% in air. After incubation for 3 h the plates were flooded with 5 ml of methanol for 30 min and then with formalin 4% for 30 min. The agarose was removed and the cells stained with Wright's stain. Each migration figure was magnified 25 times under a microscope and chemotaxis was measured by subtracting the spontaneous migration from the directed migration. The chemotaxis of the CF of *E. coli* was considered to be 100%.

Induced lysosomal enzyme release

A 0.5-ml volume of washed PMNL (10⁷/ml) was preincubated for 10 min at 37°C with or without 0.5 ml of cytochalasin B (CB) 10 µg/ml. The CB was diluted in control medium from a stock solution containing CB 5 mg/ml in dimethylsulphoxide. The dimethylsulphoxide remaining after dilution was shown not to effect enzyme release (Showell *et al.*, 1976). In duplicate, 0.5 ml of cell suspension was added to 0.5 ml of CF of EB1, BE1 or 10⁻⁷M N-formyl-methionyl-leucyl-phenylalanine (F-Met-Leu-Phe, Sigma Chemical Co., St. Louis, MO, USA). The mixture was then incubated for 10 min at 37°C and the tubes centrifuged at 1500g for 5 min at 4°C. The CF was removed and samples taken for measurements of lysozyme and β-glucuronidase. The β-glucuronidase was measured with the substrate 4-nitrophenyl glucuronide (Boehringer Mannheim GmbH). Lysosyme was determined by the rate of lysis of *Micrococcus luteus* measured by decrease in absorbance at 450 nm (Boehringer Mannheim, 1981).

Statistical analysis

Statistical analysis of the data was by analysis of variance and Student's *t*-test.

Results

Chemotactic factors of *B. fragilis* and *E. coli*.

The mean chemotactic activities of the PMNL from 10 different healthy donors, when stimulated by the CF and washed cells of *E. coli* and *B. fragilis*, are compared in fig. 1. Washed cells and CF of *E. coli* each induced chemotaxis equally. Chemotactic factors produced by *E. coli* were a property of viable cells because heat killed (30 min, 70°C) cells did not produce chemotactic factors. Chemotactic factors were, however, heat stable because heat-treated CF did not show a significant difference in chemotactic stimulation from the unheated CF. The washed cells and CF of *E. coli* contained more

Fig. 1. Chemotactic activity of PMNL induced by CF, CF heated for 30 min at 70°C, viable cells and killed cells (30 min, 70°C) of *E. coli* EB1 (□) and *B. fragilis* BE1 (▨). Values expressed as mean of 10 experiments; bars represent 1SD.

powerful chemotactic factors than those of *B. fragilis*.

Induction of chemotaxis by LPS and OM

The LPS and OM preparation of *B. fragilis* BE1 were assayed at various concentrations to measure possible chemotactic activity for PMNL (fig. 2).

Fig. 2. Dose response of PMNL to *B. fragilis* BE1 OM and LPS. Values expressed as mean percentages of five experiments; bars represent 1SD.

LPS and OM of *B. fragilis* slightly stimulated chemotactic activity of PMNL. A proportional increase in chemotaxis was observed with increasing amounts of LPS and OM. Chemotactic stimulus by 500 μg of the OM preparation of *B. fragilis* BE1 (25 ± 4.1) was almost the same as by CF (28 ± 3.8).

Effect of *E. coli* and *B. fragilis* on lysosomal enzyme release

Table 1 shows the effects of chemotactic factors on lysosomal enzyme release from PMNL. In the absence of CB, PMNL did not show significant differences in β-glucuronidase release when stimu-

Table 1. Lysosomal enzyme release from PMNL, untreated and treated with cytochalasin B (CB).

Added factors	Enzyme release (units/ml) (SD)*			
	lysozyme		β-glucuronidase	
	without CB	with CB	without CB	with CB
Control medium	115 (13)	188 (57)	1.9 (0.8)	2.1 (0.3)
Culture filtrate:				
<i>E. coli</i>	244 (43)†	390 (56)†	2.8 (0.7)‡	4.0 (0.6)†
<i>B. fragilis</i>	160 (48)†	225 (35)†	2.4 (0.8)‡	3.2 (0.7)†
f-Met-Leu-Phe, 10 ⁻⁷ M	194 (14)†	519 (84)†	2.7 (0.8)‡	3.9 (1.0)†

* Values expressed as mean (SD) of five experiments.

† $p < 0.05$, significantly larger than control medium.

‡ $p > 0.05$, not significant.

lated with CF of *E. coli* or *B. fragilis*, or 10^{-7} M f-Met-Leu-Phe. However, the three chemotactic factors significantly stimulated β -glucuronidase release from CB-treated PMNL ($p < 0.05$). No significant differences were found in β -glucuronidase release between the three chemotactic factors. In the absence of CB, all three chemotactic factors stimulated significantly more lysozyme release than did the control medium ($p < 0.05$) but the differences between *E. coli*, *B. fragilis* and 10^{-7} M f-Met-Leu-Phe preparations were not significant. Cytochalasin B-treated PMNL showed a significant increase in lysozyme release with all chemotactic factors in comparison with the control medium. The chemotactic peptide f-Met-Leu-Phe stimulated significantly more lysozyme release than *E. coli* CF, and *E. coli* CF significantly more than *B. fragilis* CF ($p < 0.05$).

Induction of chemotaxis by combination of *E. coli* CF, and *B. fragilis* cells, subcellular components or CF

The CF of *B. fragilis* significantly reduced the chemotactic activity of PMNL towards *E. coli* (fig. 3) ($p < 0.05$). The effects of combining *E. coli* CF with the OM, LPS or CF of *B. fragilis* on the chemotactic activity of PMNL are shown in table II. The LPS preparation, up to a concentration of 500 μ g, had no significant effect on the chemotactic stimulus of *E. coli* CF. However, 125 μ g of crude

Fig. 3. Chemotactic activity of PMNL induced by CF and washed cells of *E. coli* EB1 alone, or in combination with CF and washed cells of *B. fragilis* BE1. The CF or washed-cell preparations of *E. coli* were diluted 1 in 2 in control medium (□) or in the CF or washed cells of *B. fragilis* (▨). Values were expressed as mean percentages of 10 experiments; bars represent 1SD.

Table II. Effect of *B. fragilis* cell components on chemotactic activity of PMNL towards *E. coli* CF.

Reaction mixture	Index of chemotactic activity
<i>E. coli</i> + control medium†	100
<i>E. coli</i> + <i>B. fragilis</i> LPS:	
500 μ g	86 (10.0)
250 μ g	88 (6.2)
125 μ g	94 (6.7)
<i>E. coli</i> + <i>B. fragilis</i> OM:	
500 μ g	71 (9.4)‡
250 μ g	74 (10.0)‡
125 μ g	77 (6.8)‡
62.5 μ g	88 (10.9)
<i>E. coli</i> + <i>B. fragilis</i> CF†	75 (10.7)‡

* Values expressed as mean percentages of control values (SD) $\pm$ standard deviation of five experiments.

† CF of *E. coli* diluted 1 in 2 in control medium or *B. fragilis* CF.

‡ $p < 0.05$, significantly lower than control value.

OM significantly reduced ($p < 0.05$) the chemotactic activity of PMNL towards *E. coli* CF. These results suggest that the inhibitory effect of *B. fragilis* BE1 on chemotactic activity of PMNL towards *E. coli* can be caused by CF and OM.

Specificity of chemotaxis inhibition by *B. fragilis*

To assess the specificity of the effect of *B. fragilis* CF on chemotaxis of PMNL by *E. coli*, these experiments were repeated with *S. aureus* NCTC 6571 and *K. pneumoniae* 52K2 culture filtrates instead of *B. fragilis* CF (fig. 4). In the same conditions as described in fig. 3, the chemotaxis of PMNL to *E. coli* CF was significantly reduced ($p < 0.05$). These findings indicate that reduction by *B. fragilis* of PMNL chemotaxis towards *E. coli* was not specific for *B. fragilis* but also occurs in the presence of facultative bacteria.

Fig. 4. Chemotactic activity of PMNL induced by CF of *E. coli* (□), *B. fragilis*, *S. aureus* and *K. pneumoniae* (■). The CF of *E. coli* was also diluted 1 in 2 in the CF preparations of *B. fragilis* and of the facultative bacteria (▨). Values are expressed as mean percentages of 10 experiments; bars represent 1SD.

Discussion

In our previous studies we found that *E. coli* strain EB1 was killed by PMNL when normal serum was present. However, *B. fragilis* strain BE1 was not only killed but also significantly inhibited the killing of *E. coli* (Vel *et al.*, 1985). Pathogenic synergy was also observed when these two strains were tested in mice (Verweij-van Vught *et al.*, 1985). The aim of the present study was to evaluate how these two strains alone or in combination affected the chemotaxis of PMNL. The chemotaxis-inducing factors of *E. coli* EB1 were extracellular, heat stable and potent. Only slight to moderate chemotaxis was induced by the LPS, OM and CF of *B. fragilis* BE1.

These observations are in keeping with the results of Adamu and Sperry (1981). Because there are reports (Becker and Showell, 1974; Showell *et al.*, 1976) indicating that chemotactic stimuli, such as chemotactic peptides, stimulate enzyme release from human and rabbit neutrophils, we evaluated the effect of CF of *E. coli* and *B. fragilis* and the chemotactic peptide f-Met-Leu-Phe on lysosomal enzyme release from human leukocytes. Smith *et al.* (1980) have reported that chemotactic peptides stimulate enzyme release from human PMNL in the presence, but not in the absence, of the CB. Cytochalasin B is believed to curtail such PMNL activities as phagocytosis by interfering with contractile microfilament function (Davis *et al.*, 1971). The microfilamentous system is located below the cell membrane and may restrain lysosomal granules from fusing with the plasma mem-

brane, which would abrogate the extracellular release of granulase-associated enzyme. Cytochalasin B, however, by curtailing this restraining effect of microfilaments would facilitate the interaction of cell membrane and cytoplasmic granules. In the absence of CB, we found that lysosomal enzyme release was similar whichever chemotactic factors were used.

The amount of lysozyme release exceeded the amount of β -glucuronidase in all conditions. In parallel with its effect upon chemotaxis, the supernate of *E. coli* stimulated significantly more lysozyme release than the *B. fragilis* supernate but not more β -glucuronidase release.

The CF of *B. fragilis* significantly inhibited the chemotactic response of PMNL to *E. coli*. This antichemotactic activity was found in the OM and the CF preparations of *B. fragilis* but not in the LPS component. We did not test the effect of the OM on serum complement, but other investigators have shown that components of OM preparations of *B. fragilis* react with serum complement to inhibit locomotion of PMNL (Adamu and Sperry, 1981). The inhibiting effect of *B. fragilis* CF on chemotaxis of PMNL by *E. coli* was non-specific. The CF of *S. aureus* and *K. pneumoniae* also significantly inhibited the chemotaxis of PMNL towards *E. coli*. Non-specificity of the effect of *B. fragilis* on phagocytosis and killing of *E. coli* by PMNL also has been reported (Vel *et al.*, 1985; Wade *et al.*, 1983). It seems, therefore, that *B. fragilis* does not stimulate a particularly strong PMNL chemotactic response when compared with *E. coli*.

Kasper (1976) has reported the lack of local Schwartzman phenomena in rabbits to concentrations of *B. fragilis* endotoxin and OM of up to 1 mg per rabbit. *B. fragilis* LPS and OM also failed to kill 10-day-old chick embryos in doses up to 200 μ g/egg.

Wade *et al.* (1983) have found no deleterious effect by the capsular polysaccharide of *B. fragilis* on chemotaxis by PMNL. In some preliminary experiments we studied in-vivo chemotaxis of *B. fragilis* BE1 and *E. coli* EB1 and *Proteus mirabilis* in subcutaneously implanted poly-allomer chamber in guinea-pigs by the method of Veale *et al.* (1974). The results of these experiments showed that *E. coli* and *P. mirabilis* culture filtrates strongly stimulated the accumulation of PMNL into the chambers after 6 h, but *B. fragilis* culture filtrate did not (unpublished data). Therefore, it is possible that *B. fragilis* avoids early contact with the PMNL by inhibiting chemotaxis of PMNL for long enough to allow sufficient growth of *B. fragilis* and accompanying facultative bacteria eventually to overwhelm phagocytosis and establish infection.

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