

Killing of *Escherichia coli* by human polymorphonuclear leucocytes in the presence of *Bacteroides fragilis*

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SUMMARY The inhibitory effect of *Bacteroides fragilis* on the in vitro killing of *Escherichia coli* by polymorphonuclear leucocytes was studied with two pairs of *E coli* and *B fragilis* isolated from human wound infections. Both *B fragilis* strains behaved similarly: they inhibited the killing of one *E coli* strain, while the killing of the other *E coli* strain was not affected. The different behaviour of the two *E coli* strains depended on their need for fresh serum in the killing by the polymorphonuclear leucocytes. The inhibitory effect of the *B fragilis* strains could be completely accounted for by their effect on complement.

The early bacteriologists who had to convince a sceptical world of the role of bacteria in disease laid great emphasis on isolating pure cultures from infected lesions. This need for pure cultures is implicit in the famous postulates of Koch. Although it was soon realised that bacterial species seemed to assist each other in producing infections—for example, Meleney's synergistic gangrene and foot rot in sheep—most attention was paid to identifying pure cultures as causative micro-organisms. Recently, the increasing importance of commensal infections, such as wound infections and infections after gastrointestinal surgery, has stimulated interest in bacteria which seem to play a synergistic role with each other. An understanding of their interplay is important not only for elucidating the pathogenesis of the infection but also for optimal diagnosis and treatment. The well known synergy between members of the genus *Bacteroides* and enterobacteria has been variously explained. One possible explanation is the inhibitory effect that these anaerobic bacteria have on the in vitro phagocytosis of enterobacteria, as described by several authors.¹⁻⁵

In 1981 Tofte *et al* suggested that the inhibitory effect of *B fragilis* on the killing of *E coli* by polymorphonuclear leucocytes could be explained by competition for opsonins.⁶ The results of the in vitro experiments mentioned above support this theory. On the other hand, whereas synergy bet-

ween *E coli* and *B fragilis* has been shown in animal models,⁷⁻⁹ in vivo Reznikov *et al*¹⁰ found no inhibition of phagocytosis of aerobes by anaerobes. Thus a direct relation between in vivo observations and in vitro findings is still missing.

While most of the work in animal models has been carried out with *B fragilis* and *E coli*, the combination most often encountered in abdominal abscesses,¹¹ most in vitro work has adopted rather arbitrary combinations of *B fragilis* or asaccharolytic *Bacteroides* species and *Proteus mirabilis* or *E coli*. The purpose of this study was to investigate the in vitro inhibitory effect of the anaerobes on the killing of the aerobes by polymorphs in two pairs of *E coli* and *B fragilis* which had been isolated from wound infections. In a parallel study these pairs were tested for their in vivo synergistic effect.⁹

Material and methods

BACTERIAL STRAINS

The two pairs of *E coli* and *B fragilis* used in this study were collected from infected wounds in patients of the Academic Hospital of the Vrije Universiteit. *E coli* EB1 and *B fragilis* BE1 were isolated from the same abscess; no other bacterial species was cultured. *E coli* EB4 and *B fragilis* BE4 were found in an abscess of another patient together with three other species: *Acidaminococcus fermentans*, *Eubacterium lentum*, and *B distasonis*. The *E coli* strains were identified with API 20 E (API sys-

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tem SA, Montalien Vercien, France) and the anaerobic strains with the BBL Minitek numerical identification system (BBL Microbiology systems, Becton and Dickinson, USA).

E coli strains were cultured aerobically in nutrient broth (Lab Lemco, Oxoid, England). *B fragilis* strains were grown in liquid BM medium¹² supplemented with haemin (5 µg/ml, BDH, Poole, England) and menadione (2 µg/ml, E Merck AG, Darmstadt, West Germany) at 37° in an anaerobic chamber (Coy's Manufacturing Co, Ann Arbor, Michigan, USA) in an atmosphere of 85% N₂, 10% H₂, and 5% CO₂. Bacteria were maintained as a mixture of 1 ml of pure culture and 1 ml of 60% glycerol and frozen at -70°C.

For each experiment the *E coli* strains from the frozen stock culture were grown on a nutrient plate, and a single colony was inoculated on to nutrient broth and cultured until in late log phase. Bacteria were washed three times and resuspended in phosphate buffered saline (PBS). Viable counts were performed by plating serial dilutions on nutrient agar plates. The bacterial suspension was kept overnight at 4°C, and the next day adjusted to 10⁸ colony forming units per millilitre.

Anaerobic bacteria from the frozen stock culture were cultured on a BM agar plate supplemented with 5% sheep blood, haemin, and menadione. A pure culture from a single colony was grown in liquid BM medium with haemin and menadione at 37°C in the anaerobic chamber. After 18 h the cultures contained 2 - 5 × 10⁸ colony forming units per millilitre, as determined by viable counts. Unless the whole culture was used, the bacteria were washed once in PBS and resuspended to the original volume.

In some experiments bacteria were killed by incubating them for 1 h at 70°C or by shaking the bacteria for 30 min with 0.5% formalin. In both cases the bacteria were washed afterwards and resuspended in PBS to the original volume.

In some experiments *Staphylococcus aureus* (Oxford strain) NCTC 6571 was used; it was cultured and treated as described for *E coli*.

POLYMPHONUCLEAR LEUCOCYTES

Polymorphs were separated from heparinised blood of healthy donors by dextran sedimentation. They were washed three times with Hank's balanced salt solution (HBSS) (Gibco Laboratory, Grand Island, NY) with 0.1% gelatin, buffered with bicarbonate at pH 7.3 (gel-HBSS), and suspended to a concentration of 10⁷ cells/ml.

SERUM

Pooled human serum from healthy human donors

was used in all experiments as the source of opsonins. In experiments with preopsonised *E coli* 10⁸ bacteria were incubated with 50% serum in 1 ml PBS in a shaking waterbath at 110 rpm and 37°C. After 30 min the bacteria were washed and resuspended in PBS to the same concentration. In experiments in which preabsorbed serum was used, equal volumes of serum and bacterial suspension, or for controls PBS, were mixed. The mixture was incubated with gentle shaking at 37°C or at 4°C. After 30 min the serum was filter sterilised and used in the killing experiments at a final concentration of 10% (vol/vol). Antibody titres against the four *E coli* and *B fragilis* strains in normal and absorbed sera were measured with indirect immunofluorescence according to standard procedures, using FITC labelled rabbit antihuman immunoglobulins (Bio-Merieux, Charbonnières les Bains, France). Complement depletion in these sera was measured by determining 50% haemolytic units (CH₅₀) as described by Mayer.¹³

KILLING EXPERIMENT

Reaction mixtures were prepared in sterile polypropylene tubes (12 × 75 mm, Rofa Mavi, Beverwijk, The Netherlands), each containing 0.5 ml of the polymorph suspension (10⁷ cells/ml), 0.1 ml serum, 0.1 ml *E coli*, 0.1 ml *B fragilis* and 0.2 ml gel-HBSS, unless otherwise stated. The reaction mixture was incubated in a Julabo Paramix shaking apparatus (Rofa Mavi) at 400 rpm in a CO₂ incubator with a humidified atmosphere of 95% air and 5% CO₂ at 37°C. After 0, 30, 60, and 90 min 0.1 ml of the reaction mixture was suspended in 9.9 ml of distilled water to lyse the polymorphs. Viable counts of *E coli* were carried out by plating serial dilutions on nutrient agar plates, which were incubated aerobically. Viable *B fragilis* were counted by plating serial dilutions on BM agar supplemented with haemin and menadione and 50 µg/ml nalidixic acid, which inhibited the growth of *E coli*. The plates were incubated for 48 h in the anaerobic chamber.

Statistical analysis was performed with Student's *t* test.

Results

E coli EB1 and *E coli* EB4 were both killed by polymorphs when normal serum was present. *B fragilis* BE1 was not killed, whereas about 70% of *B fragilis* BE4 was killed after 90 min. None of these strains was affected by serum alone, and the anaerobes survived well the 90 min aerobic incubation in the killing experiments.

Both *B fragilis* BE1 and BE4 significantly inhibited the killing of *E coli* EB1 (Fig. 1; *p* < 0.01 at

30, 60, and 90 min), but not of *E coli* EB4 (Fig. 2). The inhibitory capacity of *B fragilis* was found with whole cultures, with washed cells (as mentioned above), and with cells that had been killed by incubation for 1 h at 70°C (Table 1). A slightly smaller, but still significant, inhibition was found when washed cells which had been killed by formalin treatment were used. The supernatant of a BE1 culture and the BM medium itself had an inhibitory or rather a delaying effect on the killing of *E coli* EB1 (Table 1); therefore, in further experiments washed cells of *B fragilis* were used.

The inhibition of the killing was the result of the influence of *B fragilis* on the serum. Incubation of the serum with the *E coli* and *B fragilis* strains resulted in loss of complement activity, as measured by CH₅₀ determination (Table 2). *B fragilis* BE1 had no inhibitory effect on the killing of preopsonised *E coli* EB1. On the other hand, killing of EB1 in the presence of serum preincubated with *B fragilis* BE1 was significantly inhibited (Fig. 3; $p < 0.01$ at 30, 60, and 90 min). In serum absorbed with EB1 itself, with *B fragilis* BE4, and, for instance, with

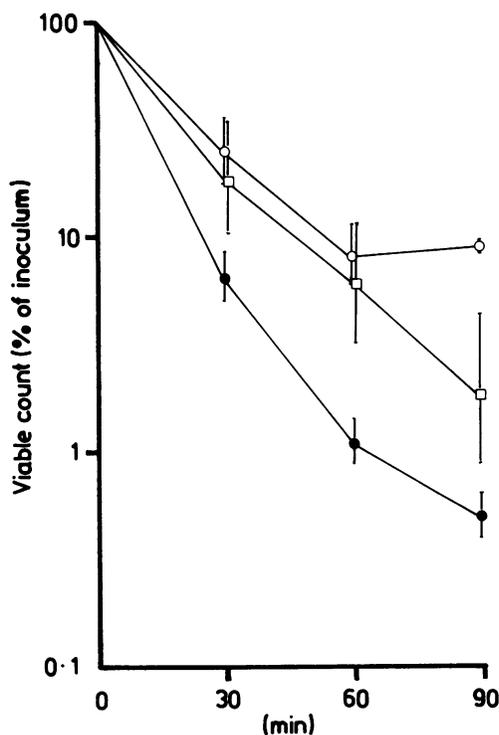


Fig. 1 Killing of *E coli* EB1 alone (●—●), in the presence of *B fragilis* BE1 (○—○) or *B fragilis* BE4 (□—□). Each point represents the mean of at least four experiments and vertical bars indicate SEM.

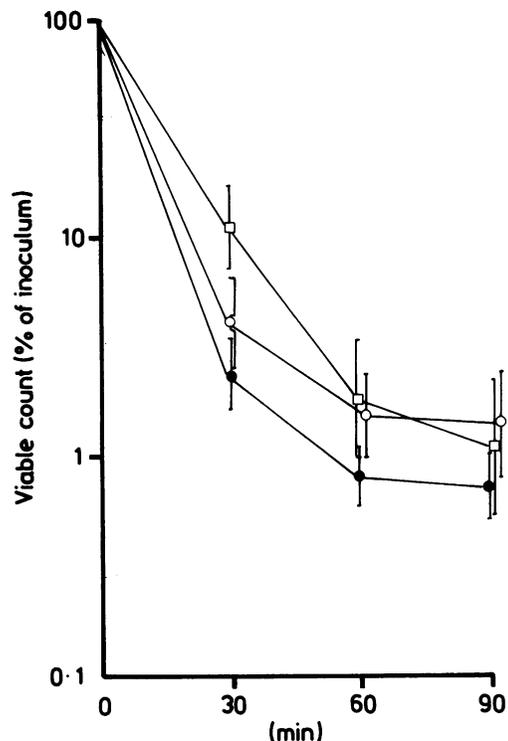


Fig. 2 Killing of *E coli* EB4 alone (●—●), in the presence of *B fragilis* BE1 (○—○) or *B fragilis* BE4 (□—□). Each point represents the mean of at least four experiments and vertical bars indicate SEM.

Table 1 Killing by polymorphonuclear leucocytes of *E coli* EB1 in the presence of *B fragilis* BE1

	% of inoculum surviving after		
	30 min	60 min	90 min
<i>E coli</i> alone	16.9 (3.0)	3.8 (0.9)	1.4 (0.1)
with <i>B fragilis</i> whole culture	81.5 (13.8*)	38.6 (12.7*)	ND
with <i>B fragilis</i> washed cells	61.2 (6.5*)	35.7 (7.1*)	37.2 (9.6*)
with <i>B fragilis</i> heat killed	77.7 (8.9*)	44.0 (12.4*)	40.8 (17.2*)
with <i>B fragilis</i> formalin killed	67.2 (13.4*)	16.4 (8.9*)	9.9 (3.8*)
with <i>B fragilis</i> supernatant	90.3 (7.3*)	11.5 (1.8*)	4.1 (1.1†)
with BM medium	83.7 (7.7*)	25.7 (14.1*)	5.1 (1.7‡)

Values represent mean (standard error) of at least four experiments.

ND = not determined.

Significance of difference from the killing of *E coli* alone at the same incubation time: * $p < 0.001$, † $p < 0.1$, ‡ $p < 0.01$.

Staphylococcus aureus killing of EB1 was completely inhibited (Table 3). In serum, absorbed with *E coli* EB4, which, as shown in Table 2, uses com-

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Table 2 Depletion of complement in normal human serum by *E coli* and *B fragilis*

Serum preincubated with	% of CH ₅₀ after		
	5 min	15 min	30 min
PBS (control)	89.6 (3.6)	84.8 (6.3)	84.8 (3.0)
<i>E coli</i> EB1	77.4 (6.1)*	69.0 (5.0)†	44.2 (5.8)‡
<i>E coli</i> EB4	80.0 (18.0)*	65.0 (11.0)†	55.0 (1.0)§
<i>B fragilis</i> BE1	60.5 (8.1)†	40.0 (3.4)‡	33.0 (3.7)‡
<i>B fragilis</i> BE4	50.3 (8.7)†	6.5 (6.2)‡	0.3 (0.3)‡

Values represent mean (standard error) of at least three experiments.

Significance of difference from control: *not significant, †p < 0.1, ‡p < 0.001, §p < 0.01.

Table 3 Killing of *E coli* EB1 in the presence of serum preincubated with *E coli*, *B fragilis*, and *Staphylococcus aureus*

Serum pretreated with	% of inoculum surviving after		
	30 min	60 min	90 min
PBS (control)	29.1 (9.8)	2.8 (1.6)	1.5 (0.8)
<i>E coli</i> EB1	121.0 (4.6)*	111.0 (33.0)*	196 (86.0)†
<i>E coli</i> EB4	105.0 (6.5)†	88.2 (31.0)‡	56.2 (6.8)‡
<i>B fragilis</i> BE4	123.0 (6.5)*	196.0 (3.3)*	430.0 (118.0)*
<i>Staph aureus</i>	131.0 (13.0)*	145.0 (1.9)*	324.0 (100.0)*

Values represent mean (standard error) of at least three experiments.

Significance of difference from control: *p < 0.001, †p < 0.01, ‡p < 0.01.

paratively little complement, killing was also inhibited, but not completely. When serum was treated with *B fragilis* at 4°C instead of 37°C, no inhibition of the killing of EB1 was found (Fig. 3). Therefore, competition for cross reacting antibodies could not have been responsible for the inhibition. This was confirmed when antibodies were determined with immunofluorescence: the antibody titre against each of the four strains was not affected by absorption with any of the other three, and only slightly reduced by absorption with the strain itself (data not shown). Complement was essential for the killing of *E coli* EB1 but not of EB4, which was killed almost

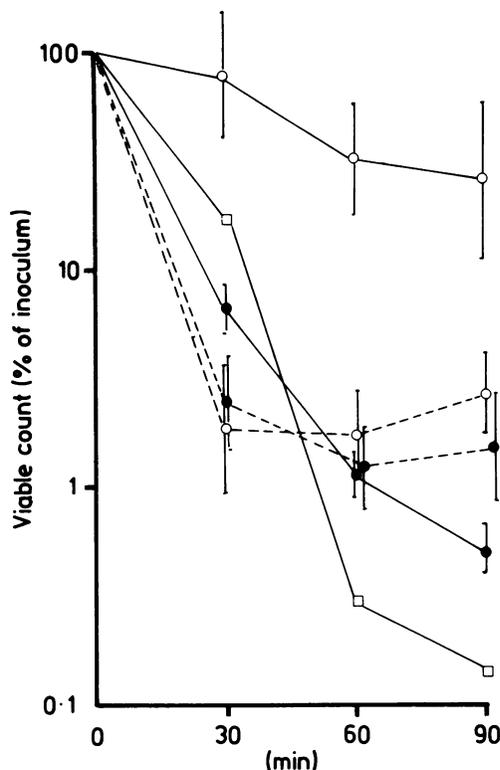


Fig. 3 Effect of *B fragilis* BE1 on the killing of *E coli* EB1. Killing of *E coli* alone (●—●) and in the presence of serum pretreated with *B fragilis* at 37°C (○—○) or at 4°C (□—□). Killing of preopsonised *E coli* alone (●—●) or in the presence of *B fragilis* (○—○). Each point represents the mean of at least four experiments, except the points indicating the killing in the presence of serum treated with *B fragilis* at 4°C, which are the means of two experiments. Vertical bars indicate SEM.

as well in the absence of serum as in its presence (Table 4).

Thus of the two clinically isolated pairs of *E coli* and *B fragilis* only one pair showed synergy in the

Table 4 Killing by polymorphonuclear leucocytes of *E coli* EB1 and *E coli* EB4: effect of serum

	% of inoculum surviving after		
	30 min	60 min	90 min
<i>E coli</i> EB1 with normal serum	16.9 (3.0)	3.8 (0.9)	1.4 (0.4)
without serum	131.0 (27.5)	150.6 (39.7)	206.4 (74.5)
with inactivated serum*	136.8 (14.7)	167.0 (18.5)	331.3 (32.8)
<i>E coli</i> EB4 with normal serum	6.1 (1.7)	2.0 (0.8)	1.5 (0.6)
without serum	25.2 (10.1)	8.1 (4.0)	3.6 (1.9)
with inactivated serum*	21.0 (10.3)	3.2 (1.9)	1.2 (0.4)

Values represent mean ± standard error of at least four experiments.

*Serum was inactivated by heating at 56°C during 30 min.

sense that the anaerobe inhibited the killing of the aerobe by polymorphs. The occurrence of this inhibition depended on the properties of the aerobe—namely, on its requirement of complement for killing.

Discussion

In this study the inhibitory effect of two strains of *B fragilis* on the killing of their aerobic partners was studied in vitro. Our results agree with the conclusions drawn when the pathogenic properties of these paired strains were tested in mice.⁹ *B fragilis* BE1 and BE4 both have a substantial effect on the clearance of a low dose of subcutaneously injected *E coli* EB1, and abscesses are the result. This phenomenon was not found with an equal dose of *E coli* EB4, which shows that the synergy is dependent on the *E coli* strain. Likewise, in vitro inhibition of killing is found for *E coli* EB1, but not for EB4. This dependence of the inhibition of the killing on the *E coli* strain used has also been described earlier by Wade *et al.*⁵ These authors do not state, however, whether *E coli* 2882, the strain for which killing was not inhibited by *B fragilis* in their experiments, was killed by polymorphs in the absence of serum, a phenomenon which explained the lack of inhibition of the killing of our strain *E coli* EB4. The failure of Reznikov *et al.*¹⁰ to show any effect of *B fragilis* on the clearance of *E coli* in mice could also be due to the *E coli* strain tested.

The results in this study are in agreement with the hypothesis that inhibition of the killing is the result of competition for opsonins and confirm the findings of other authors.^{1,3,4,5} The dependence of the inhibitory power of *B fragilis* on low redox potential, which was noted by some authors,^{1,3} was not confirmed. As in the report of Wade *et al.*,⁵ suspensions of washed cells of *B fragilis* in saline without addition of any reducing agent had the inhibitory effect. Perhaps this difference is related to the bacteria used as inhibitory organisms. The findings of Ingham *et al.*¹ and Jones and Gemmell³ refer to *B melaninogenicus*, a species which is much more sensitive to oxygen than *B fragilis*. Namavar *et al.*⁴ found an inhibitory effect by culture filtrates but not by washed cells of *B gingivalis* on the killing of *Proteus mirabilis*. It therefore seems likely that in the inhibition of the killing of aerobic bacteria by different species of bacteroides different mechanisms are concerned. According to Ingham *et al.*¹ merely increasing the numbers of the aerobe, or using other aerobes as inhibiting species, does not have the same effect. Our results confirm those of Wade *et al.*,⁵ who found inhibition by *Staph aureus* of *E coli* killing. It seems that any complement depleting organism can

inhibit serum dependent killing of *E coli* and that this in vitro phenomenon is achieved not only by anaerobes. Data are not given in the above mentioned reports, however, to indicate whether the inhibiting species themselves are killed by polymorphs. This might be important, since wasting of large amounts of complement on bacteria which are not killed subsequently would be more disadvantageous to the host than the use of complement for the destruction of a second infecting species.

It is not clear what part of the bacteroides cell is responsible for the inhibitory effect. The possession of a capsule is considered to be of great importance for virulence,^{14,15} and encapsulation is reported to impair the phagocytosis of bacteroides cells.^{16,17} The role of the capsule in the inhibition of the killing of other species is controversial, since experiments with purified capsular material give conflicting results.^{5,18} Moreover, the occurrence of capsules among different bacteroides species, within one species, and even within one strain is variable.¹⁹ From the report by Conolly *et al.*¹⁸ and from our own observations encapsulation does not seem to be related to inhibitory effects on the killing. Nevertheless, if the killing inhibiting effect is non-specific, as indicated by Wade *et al.*⁵ and by this paper, the question remains unanswered why *B fragilis* is encountered much more often in mixed abdominal infections than would be expected from its prevalence in the gut.

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