Reciprocal synergy between *Escherichia coli* and *Bacteroides fragilis* in an intra-abdominal infection model

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Summary. The synergic relationship between *Escherichia coli* and *Bacteroides fragilis* was examined in a model of intra-abdominal abscess formation. The addition of *B. fragilis* to *E. coli* in the fibrin clot inoculum increased abscess weight and residual numbers of *E. coli* in the abscess at 7 days. In a reciprocal fashion, *E. coli* was capable of enhancing *B. fragilis* persistence in abscesses. Neither heat-killed *E. coli* nor heat-killed *B. fragilis* was able to mimic the synergic effect of its live counterpart. Furthermore, *B. fragilis* culture filtrate was unable to reproduce the ability of live *B. fragilis* to act synergically with *E. coli*. For *B. fragilis* to act synergically with *E. coli*, it had to be inoculated locally with *E. coli* in the peritoneal cavity, indicating that an effect on systemic resistance by *B. fragilis* was an unlikely mechanism for the production of bacterial synergy. These studies suggest that the synergic relationship between bacteria in polymicrobial infections is a complex one, resulting from intimate interactions between bacteria and the host in the local milieu of the infection.

Introduction

The microbial flora of peritonitis resulting from perforation of the lower gastrointestinal tract consists of a mixture of aerobic and anaerobic bacteria (Lorber and Swenson, 1975). Several studies have demonstrated the importance of directing antimicrobial therapy against both components of the polymicrobial flora in the management of intra-abdominal infection (Berne *et al.*, 1982; Gorbach, 1984). However, much remains to be learned about the interactions between aerobic and anaerobic bacteria in determining the detailed pathogenesis of these infections.

The purpose of these studies was to examine potential mechanisms of pathogenic synergy between *Escherichia coli* and *Bacteroides fragilis* *in vivo*. Most studies of bacterial synergy have employed subcutaneous infection models (Kelly, 1978; Brook *et al.*, 1984; MacLaren *et al.*, 1984; Verweij-van Vught *et al.*, 1985). Host defence mechanisms in these infections differ markedly from those active in the peritoneal cavity (DeLong and Simmons,

1982; Dunn *et al.*, 1987). As a result, conclusions derived from these studies may not pertain directly to the setting of intra-abdominal infection. Onderdonk *et al.* (1976) clearly established the synergic relationship between *E. coli* and *B. fragilis* in an intra-abdominal infection model. However, subsequent studies have focused on the mechanisms by which *B. fragilis* capsular polysaccharide induces abscess formation (Onderdonk *et al.*, 1977; Shapiro *et al.*, 1982, 1986). In the present studies, the mechanisms of *E. coli*-*B. fragilis* interactions were examined in an intra-abdominal model of mixed infection to closely mimic the clinical setting. Using this model, we have previously shown bacterial synergy between *E. coli* and *B. fragilis* with respect to lethality and abscess formation (Rotstein *et al.*, 1987; Rotstein and Kao, 1988). The initial inoculum of *E. coli* in the bacterial mixture was the prime determinant of whether synergic lethality or abscess formation occurred. With a high *E. coli* inoculum ($>10^8$ cfu), the addition of *B. fragilis* to *E. coli* resulted in an enhanced mortality rate compared to that resulting from inoculation of either organism alone. At a lower *E. coli* inoculum ($<10^8$ cfu), the combination of *E. coli* plus *B. fragilis* rarely caused death but resulted in synergic abscess formation. In this report, the reciprocal nature of the synergic relationship between *E. coli* and *B. fragilis* is

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explored and the characteristics of this interaction are further defined.

Materials and methods

Bacterial strains and growth conditions

A serum-resistant strain of *E. coli*, 00, isolated from a patient at the University of Minnesota Hospitals and a strain of *B. fragilis*, VPI 9032, obtained from Dr T. D. Wilkins, Virginia Polytechnic Institute and State University (VPI), Blacksburg, VA, were used in these studies. Techniques of storage, growth conditions, medium ingredients and methods of enumeration have been previously described (Namavar *et al.*, 1983; Rotstein *et al.*, 1987).

For inoculation into the fibrin clot, the *B. fragilis* culture was pelleted by centrifugation at 1600 g for 10 min, and resuspended in pre-reduced Hanks's Balanced Salts Solution (HBSS, Gibco, Grand Island, NY) supplemented with cysteine hydrochloride (Sigma Chemical Company, St Louis, MO) 0.5 mg/L.

Incorporation of bacteria into fibrin clots

Human fibrinogen (Sigma) 2 g/L was sterilised with ultraviolet light for 10 min. For preparation of infected clots, 1.4 ml of fibrinogen was added to a 75 × 12 mm polystyrene tube in an anaerobic chamber. Either *E. coli* in 0.2 ml of saline, *B. fragilis* in 0.2 ml of pre-reduced HBSS, or both, were added to the fibrinogen solution before clotting with thrombin (Parke-Davis, Morris Plains, NJ) 6 U in 0.2 ml. In monomicrobial clots, the appropriate control vehicle was used to replace the synergic bacterial partner. In studies examining the ability of *B. fragilis* culture supernates to act synergically with *E. coli*, 0.2 ml of filter-sterilised 24-h cultures of *B. fragilis* or sterile culture broth were substituted for the inoculum of *B. fragilis*. For some studies, bacteria (either *E. coli* or *B. fragilis* VPI 9032) were heat-killed by boiling for 15 min. Killing was confirmed by aerobic and anaerobic culture. Bacterial numbers were adjusted by counting in a Petroff-Hauser chamber followed by appropriate dilution.

Fibrin-clot peritonitis model

Male Wistar rats, weighing 200–250 g, were inoculated intraperitoneally (i.p.) with infected fibrin clots as previously described (Rotstein *et al.*, 1985). Briefly, a single clot was inserted i.p. into a rat under halothane-nitrous oxide anaesthesia via a midline incision. The incision was closed with clips. Total anaesthesia time was 4–6 min. Animals were weighed daily and deaths were recorded.

Quantitation of viable bacteria in abscesses

On day 7, rats were killed by CO₂ asphyxiation. At laparotomy, the peritoneal cavity was inspected for the

presence of abscesses. Abscess formation in this model was defined as the presence of a single walled-off collection of purulent material. Swabs of the peritoneal cavity were always sterile. The abscess was excised aseptically, weighed, and carefully minced into a tube containing prereduced HBSS and sterile glass beads held under a VPI anaerobe apparatus (Holdeman *et al.*, 1977; Wells *et al.*, 1985). The tube was stoppered, vortex mixed, and transferred to an anaerobic chamber. The tube contents were then serially diluted in prereduced HBSS and surface plated in duplicate on supplemented Brain Heart Infusion Agar (BHIA; Difco Laboratories, Detroit, MI) and nutrient agar (NA). The supplemented BHIA plates were incubated anaerobically at 35°C for 48 h and the NA plates were incubated aerobically at 37°C for 24 h. The numbers of viable bacteria were quantitated and converted to log₁₀ values. The limit of detection of bacteria in abscesses was 2.7 log₁₀ cfu/ml.

Statistical analysis

Data on abscess weights and viable bacterial numbers in abscesses were analysed by a one-way analysis of variance and differences between groups were tested by Student's *t* test.

Results

Mortality

The mortality rate throughout these studies was low and varied according to the bacterial inoculum. Monomicrobial *E. coli* inocula (2×10^6 or 2×10^7 cfu/clot) resulted in no deaths amongst 76 animals and *B. fragilis* 2×10^9 cfu/clot did so on only one occasion (~5%). The combination of *E. coli* 2×10^6 or 2×10^7 cfu/clot plus *B. fragilis* 2×10^9 cfu/clot caused death in 4 out of 31 animals (13%). The simultaneous inoculation of *E. coli* 2×10^7 cfu i.p. within a fibrin clot with *B. fragilis* 2×10^7 cfu administered either free i.p., subcutaneously, intravenously or in a separate i.p. fibrin clot resulted in a mortality rate of 19% (11 out of 58). Lower inocula of *B. fragilis* enmeshed with *E. coli* 2×10^7 cfu/clot did not cause death in 24 animals. Finally, *E. coli* 2×10^7 cfu mixed with either dead *B. fragilis*, sterile culture medium or *B. fragilis* culture filtrate caused a 12% mortality rate (6 out of 49). Animals who died before the 7-day evaluation were not assessed for abscess formation and were thus excluded from further analysis.

Effect of mixed infection on abscess formation and bacterial numbers

An inoculum of *E. coli* 2×10^7 cfu/clot produced abscesses in 80% (41 out of 52) of animals. These

abscesses were generally small and contained low numbers of *E. coli* ($3.2 \pm 0.5 \log_{10}$ cfu/ml). *B. fragilis*-infected clots, at the highest inoculum tested (2×10^9 cfu/clot), frequently caused abscesses (94%, 17 out of 18) and these contained large numbers of bacteria ($7.8 \pm 0.6 \log_{10}$ cfu/ml) (table I). At all the lower inocula tested, *B. fragilis* did not produce true abscesses. Any abscesses seen were small (0.03 – 0.1 g) and consisted of a residual fibrin nidus which was frequently sterile, rather than the well-defined abscesses with a central purulent area and a collagenous capsule produced by high *E. coli* or *B. fragilis* inocula.

The addition of high doses of *B. fragilis* (2×10^9 cfu) to *E. coli* (2×10^7 cfu) in the fibrin clot inoculum resulted in significantly larger abscesses than were produced with either *E. coli* or *B. fragilis* alone ($p < 0.05$). The abscesses were quantitatively more purulent than monomicrobial abscesses and had higher numbers of *E. coli* ($7.4 \pm 0.3 \log_{10}$ cfu/ml *vs* $3.2 \pm 0.5 \log_{10}$ cfu/ml; $p < 0.0001$). *B. fragilis* numbers were increased in mixed abscesses but differences were not statistically significant.

At intermediate doses of *B. fragilis* a reciprocal synergic relationship between *E. coli* and *B. fragilis* was demonstrated. There were significantly greater numbers of viable *E. coli* in abscesses initiated with a mixture of *E. coli* and *B. fragilis* (2×10^7 cfu/clot or 2×10^5 cfu/clot) than in abscesses initiated by *E. coli* alone ($5.4 \pm 0.3 \log_{10}$ cfu/ml and $5.5 \pm 0.2 \log_{10}$ cfu/ml respectively for mixed abscesses *vs* $3.2 \pm 0.5 \log_{10}$ cfu/ml for *E. coli* alone; $p < 0.005$). Furthermore, whereas *B. fragilis* inoculated alone

at either of these doses resulted in small, frequently sterile abscesses, the presence of *E. coli* in the initial inoculum resulted in abscesses containing large numbers of *B. fragilis*.

The combination of *E. coli* plus *B. fragilis* at the lower inocula (2×10^3 cfu/clot and 2×10^2 cfu/clot) produced abscesses of similar size and with similar *E. coli* viable counts as those caused by *E. coli* alone. The ability of *E. coli* to increase survival of *B. fragilis* inoculated at 2×10^3 cfu/clot persisted, but the effect was less evident than at higher *B. fragilis* inocula.

Effect of bacterial viability on E. coli-B. fragilis synergy

To determine the role of bacterial viability on the production of *E. coli-B. fragilis* synergy, heat-killed *B. fragilis* were added to live *E. coli* in the fibrin clot inoculum (table II). Dead *B. fragilis* at inocula of 2×10^9 particles or 2×10^{10} particles did not increase the weight of, or *E. coli* viable counts in, abscesses compared to *E. coli* alone, although mixed inocula containing 2×10^{10} dead *B. fragilis* did result in significantly higher *E. coli* counts than those produced with 2×10^9 dead *B. fragilis* plus *E. coli* ($p < 0.01$). Inoculation of dead *B. fragilis* alone did not result in abscess formation (6 animals were used for each dose). The data from two animals (one given *E. coli* plus 2×10^9 dead *B. fragilis* and one given *E. coli* plus 2×10^{10} dead *B. fragilis*) were excluded from analysis because quantitative abscess counts revealed the presence of live *B. fragilis*.

Table I. Abscess formation following intraperitoneal inoculation of fibrin clots infected with *E. coli* or *B. fragilis* or both

Bacterial inoculum (cfu)		Number of rats with abscesses/number inoculated (%)	Mean abscess weight, g (SD)	Mean bacterial viable counts, $\log_{10}$ cfu/ml (SD)	
<i>E. coli</i>	<i>B. fragilis</i>			<i>E. coli</i>	<i>B. fragilis</i>
2×10^7	0	41/52 (80)	0.28 (0.15)	3.2 (0.5)	...
0	2×10^9	17/18 (94)	0.31 (0.19)	...	7.8 (0.6)
0	2×10^7	8/20 (40)	0.10 (0.02)	...	0.9 (0.9)
0	2×10^5	3/4 (75)	0.03 (0.03)	...	NG
0	2×10^3	0/4 (0)	...	...	...
2×10^7	2×10^9	22/23 (96)	0.62 (0.35)*	7.4 (0.3)†	8.9 (0.2)
2×10^7	2×10^7	20/20 (100)	0.51 (0.19)*	5.4 (0.3)†	7.9 (0.4)‡
2×10^7	2×10^5	8/8 (100)	0.47 (0.09)*	5.5 (0.2)†	8.5 (0.2)‡
2×10^7	2×10^3	8/8 (100)	0.38 (0.11)*	3.0 (0.9)	4.9 (1.4)
2×10^7	2×10^2	8/8 (100)	0.43 (0.16)	1.2 (1.5)	0.9 (0.9)

NG = no growth.

* $p < 0.05$ *vs* monomicrobial *E. coli* inoculum.

† $p < 0.005$ *vs* monomicrobial *E. coli* inoculum.

‡ $p < 0.0001$ *vs* monomicrobial *B. fragilis* inoculum at same dose.

Table II. Effect of dead *B. fragilis* on *E. coli*-*B. fragilis* abscess formation

Bacterial inoculum*	Number of abscesses evaluated	Mean abscess weight, g (SD)	Mean <i>E. coli</i> viable counts in abscesses, log ₁₀ cfu/ml (SD)
<i>E. coli</i> alone	7	0.32 (0.26)	3.6 (1.6)
<i>E. coli</i> plus live <i>B. fragilis</i> 2 × 10 ⁹ cfu/clot	9	0.59 (0.27)§	6.6 (1.5)†
<i>E. coli</i> plus dead <i>B. fragilis</i> 2 × 10 ⁹ /clot	7	0.27 (0.21)	2.4 (1.1)
<i>E. coli</i> plus dead <i>B. fragilis</i> 2 × 10 ¹⁰ /clot	10	0.22 (0.32)	4.7 (1.9)‡

* *E. coli* 2 × 10⁷ cfu/clot in all cases.

† *p* < 0.02 vs all other groups.

‡ *p*, 0.01 vs *E. coli* plus dead *B. fragilis* 2 × 10⁹; not significantly different from *E. coli* alone.

§ *p* < 0.02 vs both groups containing *E. coli* plus dead *B. fragilis*.

Interestingly, these abscesses were large and contained high numbers of both *E. coli* and *B. fragilis*.

In a reciprocal fashion, synergy was not demonstrated when 2 × 10⁷ heat-killed *E. coli* and 2 × 10⁷ cfu of live *B. fragilis* were inoculated together i.p. within a fibrin clot (data not shown).

Effect of B. fragilis culture filtrates on *E. coli* abscess formation

Filter-sterilised, 24-h culture filtrates of *B. fragilis* were added to *E. coli* fibrin clot inocula to determine whether a soluble by-product of *B. fragilis* was responsible for *E. coli*-*B. fragilis* synergy. As shown in table III, *B. fragilis* filtrates did not increase abscess size or final *E. coli* numbers in abscesses when compared to *E. coli* alone or to *E. coli* combined with sterile culture medium.

Effect of the location of B. fragilis on its ability to act synergically with *E. coli*

To determine whether the mechanism of *E. coli*-

B. fragilis synergy required direct interaction between *E. coli* and *B. fragilis*, *B. fragilis* inocula were administered by various routes simultaneously with the i.p. insertion of a fibrin clot containing *E. coli* 2 × 10⁷. Table IV shows that synergy occurred only when *E. coli* and *B. fragilis* were both inoculated i.p. whether in the same clot, in separate clots or when *B. fragilis* was given free i.p. in the presence of an *E. coli* clot, although in the latter case figures did not reach statistical significance. In the 10 rats given *B. fragilis* and *E. coli* clots simultaneously i.p., there was always a single abscess present at 7 days and in nine of these animals the abscess contents contained both *E. coli* and *B. fragilis* in high numbers. In one animal, the abscess contained only *E. coli*; the abscess was small and *E. coli* viable counts were low (3.3 log₁₀ cfu/ml). When an *E. coli*-infected clot was co-inoculated i.p. with a sterile fibrin clot, single abscesses were present in three out of four animals. These were small (0.16 SD 0.09 g) and contained low numbers of *E. coli* (2.2 SD 2.0 log₁₀ cfu/ml).

Table III. Effect of *B. fragilis* culture filtrates on *E. coli* abscess formation

Bacterial inoculum*	Number of abscesses evaluated	Mean abscess weight, g (SD)	Mean <i>E. coli</i> viable counts in abscesses, log ₁₀ cfu/ml (SD)
<i>E. coli</i> alone	4	0.18 (0.12)	2.1 (1.4)
<i>E. coli</i> plus <i>B. fragilis</i> 2 × 10 ⁹ cfu/clot	3	0.45 (0.12)†	7.2 (1.4)‡
<i>E. coli</i> plus sterile medium	8	0.27 (0.11)	3.7 (1.4)
<i>E. coli</i> plus <i>B. fragilis</i> culture filtrate	7	0.20 (0.11)	3.3 (1.3)

* *E. coli* 2 × 10⁶ cfu/clot in all groups.

† *p* < 0.05 vs all other groups.

‡ *p* < 0.01 vs all other groups.

Table IV. Effect of the location of *B. fragilis* inoculation on production of *E. coli*-*B. fragilis* synergy

Bacterial inoculum*	Number of rats with abscesses/number inoculated (%)	Mean bacterial viable counts, log ₁₀ cfu/ml (SD)	
		<i>E. coli</i>	<i>B. fragilis</i>
<i>E. coli</i> alone in clot	3/6 (50)	2.4 (1.0)	...
<i>B. fragilis</i> alone in clot	1/4 (25)	...	NG
<i>E. coli</i> plus <i>B. fragilis</i> in same clot	6/6 (100)	5.6 (0.7)‡	8.4 (0.7)
<i>E. coli</i> in clot plus <i>B. fragilis</i> free i.p.†	4/4 (100)	4.9 (0.9)	7.3 (0.8)
<i>E. coli</i> plus <i>B. fragilis</i> in separate clots	10/10 (100)	5.5 (0.5)‡	6.9 (0.5)
<i>E. coli</i> in clot plus separate sterile clot	3/4 (75)	2.2 (2.0)	NG
<i>E. coli</i> in clot plus <i>B. fragilis</i> i.v.†	4/4 (100)	1.1 (0.9)	NG
<i>E. coli</i> in clot plus <i>B. fragilis</i> s.c. in clot	2/3 (66)	3.8 (1.2)	NG

NG = No growth.

* *E. coli* 2×10^7 cfu/clot in all cases; *B. fragilis* 2×10^7 cfu/clot.† *B. fragilis* 2×10^7 cfu in 0.2 ml pre-reduced HBSS.‡ $p < 0.05$ vs *E. coli* alone.

These data suggest that the increased *E. coli* counts in abscesses initiated by double clot insertion were more likely to be due to bacterial interaction than the presence of a second intra-abdominal clot. In contrast, simultaneous intravenous or subcutaneous inoculation of *B. fragilis* did not act synergically with *E. coli*-containing clots given i.p. Abscesses were uniformly small and never contained *B. fragilis*.

Discussion

The importance of synergic bacterial interactions in determining the pathogenicity of mixed infections has been demonstrated clearly in several model systems. Several mechanisms have been proposed to account for this relationship (Mackowiak, 1978). One bacterial species may promote the survival of its bacterial partner in a mixed infection by providing growth factors, impairing host defence mechanisms, optimising the local micro-environment, or by transferring virulence factors. Correlation between these proposed mechanisms and the in-vivo setting has received relatively little attention, particularly in relation to intra-abdominal infections. Reznikov *et al.* (1981) demonstrated that co-injection of *B. fragilis* with *E. coli* into the peritoneal cavity had no effect on the clearance of *E. coli*. These authors concluded that impaired

phagocytosis of *E. coli* due to the presence of *B. fragilis*, a well defined in-vitro mechanism, was unlikely to be active *in vivo*. Studies by Dunn *et al.* (1985) demonstrated that non-viable *B. fragilis* were able to impair clearance of non-viable *E. coli* from the peritoneal cavity. However, non-viable *E. coli* were able to mimic the effect of *B. fragilis*, thereby implying that impaired clearance was a nonspecific effect mediated by large numbers of particles rather than a demonstration of *E. coli*-*B. fragilis* synergy. The inability to demonstrate synergy in these two studies may have been due to the rapid clearance of bacteria from the peritoneal cavity via the diaphragmatic lymphatics (Dunn *et al.*, 1987), thereby not allowing the intimate interaction required for microbial synergy. Adjuvant materials such as sterile faeces and barium (Onderdonk *et al.*, 1976), fibrin (Wells *et al.*, 1985; Rotstein and Kao, 1988), and cellulose fibres (Hagen *et al.*, 1983) which retard bacterial clearance, are generally required to demonstrate bacterial synergy in the peritoneal cavity.

Onderdonk *et al.* (1976) demonstrated synergy between *E. coli* and *B. fragilis* with respect to abscess formation using a model in which the bacterial inoculum was mixed with barium sulphate and sterilised rat faeces. These studies also showed that mortality in mixed infections correlated with the dose of *E. coli* in the inoculum. Subsequent

studies with the fibrin clot peritonitis model confirmed these findings and, furthermore, demonstrated that *B. fragilis* could modulate the mortality rate (Rotstein *et al.*, 1985). The ability of *Bacteroides* spp. to do so did not correlate with capsulation (Rotstein *et al.*, 1987). The inoculum size and the particular strain of *E. coli* in the infecting inoculum may also determine whether *B. fragilis* is able to modulate *E. coli*-fibrin clot lethality (Wells *et al.*, 1985; Rotstein and Kao, 1988).

In the present studies, we used the fibrin clot model of intra-abdominal infection to examine the synergic interactions between *E. coli* and *B. fragilis* with respect to abscess formation *in vivo*. This model closely mimics the clinical scenario in which an infected fibrinopurulent exudate represents an important precursor to abscess formation. In this model, the addition of *B. fragilis* to *E. coli* in the infecting inoculum approximately doubled the weight of the 7-day abscess and increased the residual number of *E. coli* by 2–4 log₁₀. In a reciprocal fashion, *E. coli* enhanced the persistence of *B. fragilis*. This was particularly evident with the lower *B. fragilis* inocula which alone resulted in a low rate of abscess formation with small abscesses and low bacterial numbers. The addition of *E. coli* to these inocula resulted in consistent formation of abscesses containing high numbers of *B. fragilis*. With higher inocula of *B. fragilis*, the degree of purulence and the weight of the abscesses was again increased by the addition of *E. coli*. However, final *B. fragilis* numbers remained the same, probably because of limitation of bacterial growth within an abscess rather than a failure to demonstrate synergy.

The addition of *B. fragilis* at low inocula (10²–10³ cfu) to *E. coli* in the fibrin clot resulted in abscesses no larger and containing no more *E. coli* than monomicrobial *E. coli* inocula. This threshold level of *B. fragilis* for synergic abscess formation (>2 × 10³ cfu) was several log₁₀ values lower than that reported for synergic lethality with *E. coli* in this model (>3 × 10⁷ cfu; Rotstein *et al.*, 1987). The explanation for this difference probably lies in the different endpoints studied. Synergic lethality was characterised by an increase in mortality within the first 24–48 h. Thus, high *B. fragilis* inocula were required to facilitate the increased *E. coli* bacteraemia and subsequent increased mortality. In contrast, the prolonged interaction resulting in synergic abscess formation may have permitted lower initial inocula of *B. fragilis* to act synergically.

Several mechanisms have been invoked as being important in the pathogenesis of aerobic-anaerobic bacterial synergy (Mackowiak, 1978). One mechanism, the ability of *Bacteroides* spp. to impair the

phagocytic killing of aerobes by neutrophils, has received particular attention. Viable *Bacteroides* spp. are able to affect this phenomenon, whereas dead *Bacteroides* particles vary in their ability to do so. This depends on the method used to kill the bacteria. Boiling or killing with metronidazole removes the ability of *Bacteroides* spp. to impair phagocytic killing, but autoclaving at 115°C for 20 min has no effect (Ingham *et al.*, 1981; Jones and Gemmell, 1982). To examine the requirements for live bacteria in the production of bacterial synergy *in vivo*, washed heat-killed *E. coli* or *B. fragilis* particles were substituted for live bacteria in mixed inocula with their live co-pathogens. Neither was able to reproduce the synergic effect of its viable counterpart. Since one might argue that 2 × 10⁹ dead *B. fragilis* was an inappropriate substitute for 2 × 10⁹ live *B. fragilis* because of the potential growth *in vivo* of the latter, we also studied the combination of 2 × 10¹⁰ dead *B. fragilis* with live *E. coli* and were unable to demonstrate synergy. These findings are in agreement with those of Hite *et al.* (1949) in which heat killing of either '*Bacteroides*', now *Fusobacterium*, *necrophorum* or *Streptococcus liquefaciens* before their combined inoculation at a subcutaneous site prevented bacterial synergy. The failure of *B. fragilis* heat-killed by boiling at 100°C for 10 min to act synergically with *E. coli* *in vivo* corresponds well with in-vitro studies demonstrating that *B. fragilis* cells killed by boiling lost their ability to impair the phagocytic killing of *E. coli* by neutrophils (Ingham *et al.*, 1981). Further studies with *B. fragilis* killed by other methods are required to verify this correlation between the in-vivo and in-vitro setting. A number of studies have demonstrated that soluble factors in *Bacteroides* culture supernates may impair host defence mechanisms (Van Dyke *et al.*, 1982; Namavar *et al.*, 1983; Rotstein *et al.*, 1986) but in the present study *B. fragilis* culture filtrates were unable to produce *E. coli*-*B. fragilis* synergy. This does not rule out the possibility that these factors diffused out of the fibrin mesh and were, therefore, unable to exert their synergic effect with *E. coli* in the fibrin clot. Conceivably, live *B. fragilis* mixed with *E. coli* might provide a local milieu in which these soluble factors are present in high concentrations for prolonged periods and are able to exert their effects.

A synergic relationship between *E. coli* and *B. fragilis* was evident when *B. fragilis* was inoculated within the peritoneal cavity together with an *E. coli*-infected clot, but not when *B. fragilis* was inoculated intravenously or subcutaneously. These results argue against inhibition of systemic host defences by *B. fragilis* as a mechanism for synergy

as suggested by Rodloff *et al.* (1986). The consistent presence of both bacterial species within the abscesses when both were inoculated i.p. suggests the requirement for intimate local interaction between bacteria in the production of synergy. Interestingly, in the single animal which had a small abscess following i.p. inoculation with separate clots containing *E. coli* and *B. fragilis*, the abscess was monomicrobial, containing small numbers of *E. coli*. While it is unclear why cross-seeding of clots with bacteria resulting in synergy did not occur in this animal, the results further demonstrate the requirement of local interaction between bacteria in the production of synergy.

In conclusion, these studies have demonstrated a reciprocal synergic relationship between *E. coli* and *B. fragilis* in a relevant model of intra-abdominal infection. Studies examining the mechanisms underlying this phenomenon illustrate the complex nature of the local interactions between bacteria and their environment in determining the ultimate virulence of mixed infections. Further studies should attempt to examine mechanisms of synergy *in vivo* to verify their importance.

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