Pathogenic synergy between *Escherichia coli* and *Bacteroides fragilis* or *B. vulgatus* in experimental infections: a non-specific phenomenon

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Summary. The virulence of *Bacteroides fragilis* and *B. vulgatus* for mice was compared in a skin-infection model. These strains were also tested for pathogenic synergy in mixed infections with *Escherichia coli*. Strains of *B. fragilis* were generally more virulent than strains of *B. vulgatus* and, with one exception, the effect of *Bacteroides* strains in mixed infections merely reflected their inherent virulence.

Introduction

The normal microbial intestinal flora determines the initial inoculum in intra-abdominal infections from which with careful laboratory handling an average of four to five species may be isolated (Finegold, 1977). The pathogenic significance of each isolate remains for the most part unclear.

The frequent presence of *Escherichia coli* and *Bacteroides* species of the fragilis group in these infections has led to the concept of pathogenic synergy between them (Gorbach and Bartlett, 1974b; MacLaren *et al.*, 1984). With various experimental animal models, evidence has been presented to support this theory (Onderdonk *et al.*, 1976; Kelly, 1978; Verweij-van Vught *et al.*, 1985). No satisfactory explanation has been given for the higher incidence of *B. fragilis* in mixed intra-abdominal infections compared with other species of the fragilis group, i.e., *B. vulgatus*, *B. distasonis*, *B. ovatus* and *B. thetaiotaomicron*, despite the lower prevalence of *B. fragilis* in the intestinal flora (Gorbach and Bartlett, 1974a).

The higher virulence of *B. fragilis* as documented by Onderdonk *et al.* (1977) and Maskell (1981) may explain the higher incidence of this species in infections. Evidence has been given for an important role of the capsular polysaccharide of *B. fragilis* as a virulence factor (Onderdonk *et al.*, 1977; Brook and Walker, 1984). On the other hand, the possibility remains of a synergistic mechanism specific for *B. fragilis*. In this paper, differences in pathogenic synergy between *E. coli* and *B. fragilis* and between *E. coli* and *B. vulgatus* are described. These differ-

ences have been studied in relation to the virulence of strains of *Bacteroides* in infections caused by a single species.

Materials and methods

Bacterial strains

The *Bacteroides* strains used in this study were collected from intra-abdominal wound infections of patients at the Academic Hospital of the Vrije Universiteit (BE strains 1, 12, 13, 21, 43 and 53) or from faeces from the same patient population (BE strains 16, 17, 18, 19 and 20). *E. coli* strain EB 1 was isolated together with *B. fragilis* strain BE 1 from an intra-abdominal wound infection. *Staphylococcus aureus* strain BE 50 was also isolated from a wound infection. The *E. coli* strain was identified with the API 20E system (API S.A., Montalieu Vercieu, France) and the *Bacteroides* strains with the BBL Minitek® Numerical Identification system (BBL Microbiology Systems, Becton Dickinson, USA).

Animals

Swiss Albino mice (TNO, Zeist, The Netherlands) weighing 20–25 g were used. The mice were raised in conventional conditions.

Inoculum preparation

E. coli strain EB 1 and *S. aureus* strain BE 50 were cultured aerobically in Nutrient Broth (No. 2, Oxoid) until in the late logarithmic phase of growth. Cells were harvested by centrifugation, washed in phosphate-buffered saline (PBS), pH 7.4, and held at 4°C. The next day an appropriate dilution, based on the viable count of the suspension, was made in PBS. *B. fragilis* and *B. vulgatus*

were grown in BM medium (Shah *et al.*, 1976) supplemented with haemin 5 mg/L and menadione 2 mg/L at 37°C in an anaerobic jar with an atmosphere of N₂ 80%, H₂ 10% and CO₂ 10%. To prevent excessive acid production, glucose was omitted from the medium and K₂HPO₄ was added at a concentration of 5 g/L. A 20-h culture, diluted 1 in 20 in fresh medium and incubated for *c.* 4 h, gave a logarithmic-phase culture. These cells were harvested by centrifugation and resuspended in PBS. A 0.1-ml volume of appropriate dilution of the bacteria, alone or in combination, was injected subcutaneously in the backs of mice about 1 cm from the midline after the skin had been shaved.

Evaluation of abscess formation and clearance

At different times after injection, animals were killed by chloroform inhalation, the skin was cleaned with ethanol 70% v/v and allowed to dry. Skin fragments including the site of the lesion were excised and examined for pus formation. Only pus-containing abscesses were regarded as positive. The fragments were incised through the centre and homogenised (Thomas Tissue Grinder, Philadelphia, USA) in PBS (5 ml). Viable counts were made: for *E. coli* on nutrient agar incubated aerobically; for *Bacteroides* spp. on BM medium supplemented with haemin 5 mg/L, menadione 2 mg/L and nalidixic acid 50 mg/L incubated anaerobically. In this way, clearance of bacteria was determined.

Results

In previous studies on the influence of *B. fragilis* on the clearance of *E. coli* from the skin of mice, stationary-phase cultures incubated for 18–20 h were used. However, comparison of viable and total counts of these cultures revealed that their viability varied between 10 and 100%. Because the presence of a variable and unknown number of dead bacteria may influence the course of infection by *Bacteroides* spp. and the clearance of *E. coli* in mixed infections, only cells from early logarithmic-phase cultures were used in further experiments. With these cultures, discrepancies between viable and total counts were never observed.

Virulence of *B. fragilis* and *B. vulgatus*

The virulence of strains of *Bacteroides* was assessed in a mouse model. The decrease in numbers of bacteria in the skin of mice after subcutaneous injection of *c.* 2×10^8 cfu was followed for 3 days. For five of six strains of *B. fragilis*, numbers decreased relatively slowly by factors of 10^1 – 10^3 after 3 days (fig. 1). One strain of *B. fragilis* and five strains of *B. vulgatus* were cleared more effectively, their numbers decreasing by factors of 10^5 – 10^7 in the same period (fig. 1).

Fig. 1. — The clearance of *B. fragilis* (—) and *B. vulgatus* (---) from the skin of mice at 1, 2 and 3 days after injection of $(2-4) \times 10^8$ cfu. Each point represents the mean of 6–25 lesions.

Attempts to determine LD₅₀ values were less satisfactory, indicating values greater than 10^9 cfu for *B. fragilis* strain BE 1 and *B. vulgatus* strain BE 18.

Pathogenic synergy between *E. coli* and *Bacteroides* species

After injection of either *E. coli* strain EB 1 (*c.* 5×10^6 cfu) or *B. fragilis* strain BE 1 (*c.* 2×10^8 cfu), each species was cleared effectively from the skin of mice (fig. 2). Their injection together resulted in abscess formation in all mice and after 6 days large numbers of *E. coli* (2.0×10^7 – 4.3×10^8 cfu) and *B. fragilis* (3.5×10^6 – 5.5×10^7 cfu) were present in the abscesses. When *B. vulgatus* strain BE 18 and *E. coli* strain EB 1 were injected together, their clearance

Fig. 2. — The clearance of *E. coli* strain EB 1 (●) and *B. fragilis* strain BE 1 (○) after injection alone (—) or together (---). Each point represents the mean of at least six lesions and bars indicate the standard error of the mean (SEM).

was initially delayed in all mice (fig. 3). Thereafter, the response varied. Thus, some mice developed abscesses whereas in others lesions tended to heal during the course of the experiment so that, after 6 days, only 50% of the mice had developed abscesses. The numbers of bacteria in the lesions, varied correspondingly, ranging from 5.0×10^3 to 1.6×10^7 cfu for *E. coli* and from 0 to 2.0×10^6 cfu for *B. vulgatus*.

Injection of a smaller dose (2×10^6 cfu) of *E. coli* strain EB 1 along with $c. 2 \times 10^8$ cfu of *B. fragilis* strain BE 1 or *B. vulgatus* strain BE 18 resulted in the formation of fewer abscesses. Thus, with *B. fragilis*, only 50% of the mice developed abscesses after 6 days whereas with *B. vulgatus* abscesses were not found at all.

Each of four strains of *B. fragilis* and four strains of *B. vulgatus* ($c. 2 \times 10^8$ cfu) was tested along with *E. coli* strain EB 1 (2×10^6 cfu) and the numbers of bacteria in the skin lesions followed for up to 6 days. Control experiments included tests with each strain injected alone. The mean of the numbers of *E. coli* strain EB 1 in the lesions 1, 3 and 6 days after injection is given in fig. 4. Each strain of *Bacteroides* impaired the clearance of *E. coli* and, except for *B. fragilis* strain BE 17, this influence was significant at a level of $p=0.01$ (as tested by the non-parametric test of Wilcoxon or the U-test of Mann and Whitney). There was a strong correlation between the observed virulence of *Bacteroides* strains in infections when injected alone and their influence on the clearance of *E. coli*; this effect was most

Fig. 3. — The clearance of *E. coli* strain EB 1 (●) and *B. vulgatus* strain BE 18 (○) after injection alone (—) or together (---). Each point represents the mean of at least six lesions and bars indicate the SEM.

Fig. 4. – The influence of four strains of *B. fragilis* (—) and *B. vulgatus* (---) on the clearance of *E. coli* (control *E. coli* alone ■—■). Each point represents the mean of at least six lesions. The numbers of abscesses at day 6 are given in parentheses.

marked with *B. fragilis* strains BE 1, BE 21 and BE 43 and *B. vulgatus* strain BE 19. The clearance of *Bacteroides* strains in mixed infections with *E. coli* was also impaired in these four combinations. The data with regard to the numbers of *Bacteroides* strains at day 6 are shown (fig. 5). In the other combinations where *Bacteroides* strains influenced but little the clearance of *E. coli*, their own clearance was enhanced by the presence of *E. coli*.

That the observed pathogenic synergy was not specific for *Bacteroides* species was seen in tests with *S. aureus* together with *E. coli*. Although the clearance of *S. aureus* strain BE 50 was only slightly influenced by the presence of *E. coli*, the effect of *S. aureus* on the clearance of *E. coli* was comparable to that of *B. fragilis* (data not shown).

Discussion

The higher incidence of *B. fragilis* in combination with *E. coli* in mixed infections, despite its lower prevalence in the normal intestinal flora may be explained either by the higher virulence of this species or by invoking some mechanism involving pathogenic synergy specific for *B. fragilis*.

The virulence of *Bacteroides* strains was readily

Fig. 5. – The numbers of *B. fragilis* and *B. vulgatus* in the skin of mice 6 days after injection alone (□) or together with *E. coli* strain EB 1 (▨). The significance is indicated as ** at a level of $p = 0.01$ and as * at a level of $p = 0.05$.

measured by following their clearance from the subcutaneous tissue of mice as long as logarithmic-phase bacteria were used. Lack of reproducibility in experiments with stationary-phase cultures of *Bacteroides* was attributable to the presence of variable numbers of non-viable cells in stationary-phase cultures. The finding that *B. fragilis* was generally more virulent than *B. vulgatus* is in full agreement with that of Maskell (1981) who also used a mouse-skin model. It has been proposed that the capsular polysaccharide of *B. fragilis* is an important virulence factor (Onderdonk *et al.*, 1977). However, the presence of a capsule is not a unique character of *B. fragilis* strains. Other authors have reported that other members of the fragilis group produce capsules (Babb and Cummins, 1978) which also may be important virulence factors (Brook and Walker, 1984). The strains of *B. fragilis* and *B. vulgatus* used in this study also showed varying degrees of capsulation when observed in India-ink preparations and it was surprising that correlation between degrees of encapsulation and virulence for mice was not observed in our experiments, a situation that clearly requires further study.

Four strains each of *B. fragilis* and *B. vulgatus*, tested for their infectivity for mice in combination with *E. coli*, tended to show an 'all-or-nothing' effect. At certain doses, abscesses developed containing large numbers of both species; at other doses the lesions, containing relatively small numbers of bacteria, tended to heal. This observation suggests

that a critical dose of both species must be maintained for at least a certain period for successful abscess formation. Depending on dose, both *B. fragilis* and *B. vulgatus* caused abscess formation along with *E. coli* whereas, injected alone, they did not. The influence of *Bacteroides* strains other than strain BE 19 merely reflected their own virulence. From these findings we conclude that the influence of *Bacteroides* strains on the clearance of *E. coli* is not specific for *B. fragilis* but rather is a non-specific phenomenon dependent on the ability of the anaerobe to maintain itself for at least a few days and results with *S. aureus* confirmed this idea. The clearance of *Bacteroides* strains in mixed infections is reduced in infections resulting in abscess formation. The presence of *E. coli* obviously created a niche favourable for *Bacteroides* spp. In other circumstances the presence of *E. coli* in mixed infections is rather disadvantageous for *Bacteroides* spp.

One mechanism of pathogenic synergy between *E. coli* and *B. fragilis* that has been studied *in vitro* is the inhibition of phagocytosis of *E. coli* in the

presence of the anaerobe which competes for complement factors (Tofte *et al.*, 1980; Namavar *et al.*, 1983; Vel *et al.*, 1985). Mouse serum also showed a reduced capacity for opsonisation of *E. coli* and Zymosan after incubation with *B. fragilis* strain BE 1 or *B. vulgatus* strain BE 18; in this respect, differences were not observed between these strains (data not shown). These observations may mean that both *B. fragilis* and *B. vulgatus* can act synergistically with *E. coli* in mice by competing for complement but that other factors determine their virulence and, therefore, the actual number of cells and the length of time they are available in the tissues for complement consumption. Thus, *B. vulgatus* may be unable to express *in-vivo* synergy with *E. coli*. It still remains to be proven, however, that complement depletion observed *in vitro* also occurs *in vivo*. The factors responsible for the higher virulence of *B. fragilis* strains are currently under investigation.

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