PATHOGENIC SYNERGY BETWEEN *ESCHERICHIA COLI* AND *BACTEROIDES FRAGILIS*: STUDIES IN AN EXPERIMENTAL MOUSE MODEL

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SUMMARY. An animal model is described for quantitative evaluation of pathogenic synergy between *Escherichia coli* and *Bacteroides fragilis* in which adjuvants were not required for abscess formation. Two sets of strains of *E. coli* and *B. fragilis* isolated from human wound infections were tested. Pathogenic synergy was observed in only one of the two combinations and was dependent on properties of *E. coli*.

INTRODUCTION

The frequent presence of more than one bacterial species in infections such as intra-abdominal abscesses led to the concept of pathogenic synergy between bacteria (Meleney, 1931; Finegold, 1977; Kelly and Warren, 1978). Pathogenic synergy, especially between *Escherichia coli* and *Bacteroides fragilis* has been described by several authors (Onderdonk *et al.*, 1976; Kelly, 1978; Mayrand and McBride, 1980; Carlson, 1982).

Various animal models have been developed to evaluate this synergy. The intra-abdominal sepsis model of Onderdonk *et al.* (1976) in rats clearly illustrated synergy between *E. coli* and *B. fragilis* in abscess formation and Kelly (1978) demonstrated this also in a guinea pig cutaneous wound model. Ingham *et al.* (1977) and Namavar *et al.* (1983) observed inhibition of phagocytosis of *Proteus mirabilis* by anaerobic bacteria, which may be one explanation of pathogenic synergy. This inhibition was not observed *in vivo* by Reznikov, Finlay-Jones and McDonald (1981), who studied the peritoneal clearance of *E. coli* in mice in the presence of *B. fragilis*, and Rodloff *et al.* (1981) were unable to demonstrate synergy between *E. coli* and *B. fragilis* in a mouse model of haematogenous infection.

The discrepancy in the findings of different authors may be due to differences in the models, the use of different bacterial strains or the presence or absence of adjuvants. The aim of the present investigation was to develop an animal model for evaluation of pathogenic synergy between *E. coli* and *B. fragilis*, if possible without the use of adjuvants.

MATERIALS AND METHODS

Bacterial strains. The two pairs of *E. coli* and *B. fragilis* strains studied were isolated from wound infections in patients in the Academic Hospital of the Vrije Universiteit. *E. coli* strain EB1 and *B. fragilis* strain BE1 were isolated from the same abscess; no other bacterial species was cultured. *E. coli* strain BE4 and *B. fragilis* strain BE4, isolated from another patient, were accompanied by three other anaerobes: *Acidaminococcus fermentans*, *Eubacterium lentum* and *B. distasonis*. The strains of *E. coli* were identified by the API 20E (API system S.A., Montalieu Vercieu, France) and the anaerobic strains by 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 under conventional conditions.

Inoculum preparation. Strains of *E. coli* were cultured aerobically in nutrient broth (Blood Agar Base No. 2, Oxoid) until in late logarithmic phase. Cells were harvested by centrifugation, washed in phosphate buffered saline (PBS), pH 7.4, and kept at 4°C. The next day an appropriate dilution was made in PBS, based on the viable count of the suspension. *B. fragilis* was grown in BM medium as described by Shah *et al.* (1976), supplemented with haemin 5 µg/ml and menadione 2 µg/ml at 37°C in an anaerobic jar in an atmosphere of N₂ 80%, H₂ 10% and CO₂ 10%. Cultures were harvested after 20 h by centrifugation and resuspended in PBS. In some experiments whole cultures were used. A volume of 0.1 ml of appropriate dilutions of the bacteria alone or in combination was injected subcutaneously in the back of mice at about 1 cm from the midline after shaving the skin (Simoons-Smit *et al.*, 1984).

Evaluation of abscess formation

At different times animals were killed by chloroform inhalation, the skin was cleaned with alcohol 70% v/v and allowed to dry. Skin fragments, including the site of the lesion, were excised. For evaluation of the infection, three different methods were used (see below).

Minimal abscess-forming doses. Ten-fold dilutions of the strains of *E. coli* and two-fold dilutions of the strains of *B. fragilis* were injected alone and in various combinations with each other. Excised skin fragments of mice were examined macroscopically after 7 days for redness, swelling and pus formation. Only abscesses with pus formation were regarded as positive. The lowest dose producing abscesses in at least 50% of the mice was determined (minimal abscess-forming dose).

Growth index, a semiquantitative estimation of the number of bacteria in the skin lesion, was determined as described before (Simoons-Smit *et al.*, 1984). Skin fragments were excised from dorsal to ventral and stretched. After macroscopic examination 5-mm parallel cuts at 4-mm interval were made starting from the periphery and working to the centre. Each lesion was sampled by rubbing a loop along both walls of each cut and plating on nutrient agar plates. After incubation at 37°C overnight the growth was graded as: 8 (confluent growth), 4 (semiconfluent growth), 2 (> 20 colonies), 1 (< 20 colonies) or 0 (no growth). For each strain, mean values of the growth indices in the separate cuts of the skin lesions were calculated.

Viable count of abscess content. At intervals after injection of bacteria, excised skin fragments including the whole abscesses were incised through the centre and homogenised in 5 ml of PBS (Thomas Tissue Grinder, Philadelphia, USA). A viable count was made aerobically on nutrient agar for *E. coli*. For all experiments the results are based on at least eight infection sites.

RESULTS

The formation of abscesses in mice after subcutaneous injection of bacteria was strongly dependent on the dose used. Injection of *E. coli* 5×10^7 cfu resulted in subcutaneous swelling with pus, which after a few days developed into a poorly delineated abscess. Spontaneous drainage through the skin occurred sometimes after 8 or 10 days with complete healing of the abscess. Only at high doses did some mice die

TABLE
Minimal abscess-forming doses (cfu) of *E. coli* and *B. fragilis* alone or in combination

Combination of strains	Minimal abscess-forming doses of				
	<i>E. coli</i>	<i>B. fragilis</i>	<i>E. coli</i> + <i>B. fragilis</i>		
EB1/BE1*	5×10^6	$> 8 \times 10^8$	$\begin{cases} 5 \times 10^5 \\ 5 \times 10^4 \end{cases}$	+	$\begin{cases} 2.5 \times 10^7 \\ 2 \times 10^8 \end{cases}$
EB4/BE4*	5×10^6	$> 8 \times 10^8$	5×10^6	+	$1-8 \times 10^8$
EB1/BE1†	5×10^7	$> 8 \times 10^8$	$\begin{cases} 5 \times 10^5 \\ 5 \times 10^4 \end{cases}$	+	$\begin{cases} < 1 \times 10^8 \\ 2 \times 10^8 \end{cases}$
EB4/BE4†	5×10^7	$> 8 \times 10^8$	5×10^7	+	$1-8 \times 10^8$

* Bacteria were suspended in BM medium.

† Bacteria were suspended in PBS.

shortly after injection of the bacteria, probably due to the toxicity of the bacteria. With *B. fragilis*, abscesses developed only at doses of $> 8 \times 10^8$ cfu a few days after injection, and were more firm and sharply delineated, quite different from the abscesses caused by *E. coli*. Injection of a combination of these two species of bacteria resulted in abscesses resembling those produced by *E. coli* alone. The number of *E. coli* needed for abscess formation was much lower in the presence of *B. fragilis*, suggesting pathogenic synergy; this was evaluated in three different ways.

Determination of the minimal abscess-forming doses. For the two pairs of *E. coli* and *B. fragilis*, minimal abscess-forming doses were determined for the bacteria alone and in combination. The strains of *E. coli* were suspended in BM medium; undiluted cultures of *B. fragilis* in BM medium were used (table). The minimal abscess-forming dose of *E. coli* EB1 in the presence of *B. fragilis* BE1 was much lower than the dose of *E. coli* injected alone, whereas the number of *B. fragilis* BE1 used in combination with *E. coli* was not sufficient for abscess formation, when used alone. *B. fragilis* strain BE4 had no influence on the frequency of abscess formation by *E. coli* strain EB4. To study the effect of BM medium on the minimal infective dose the same experiments were performed with all bacterial suspensions in PBS (table). Although BM medium had some effect on the infectivity of the bacterial strains, pathogenic synergy when present was also shown in the absence of this medium.

Enumeration of the viable bacteria in the abscess. Determination of the number of bacteria in the abscess might possibly give some information about the degree of bacterial synergism. We used for this purpose the *growth index*, a semi-quantitative bacterial count as described by Maskell (1981) and Simoons-Smit *et al.* (1984). Mice were given injections of 0.1 ml of *E. coli* (1×10^6 cfu) alone or of a mixture of *E. coli* (1×10^6 cfu) and *B. fragilis* (5×10^7 cfu). Only suspensions of bacteria in PBS were used. With this method pathogenic synergy between *E. coli* strain EB1 and *B. fragilis* strain BE1 was established, whereas with the other pair again no synergy was found at the dose used (fig. 1). Pairing of the *E. coli* strains EB1 and EB4 with the *B. fragilis* strains BE4 and BE1 respectively revealed that synergy was dependent on properties of *E. coli* strain EB1. Both strains of *B. fragilis* enhanced the pathogenicity of *E. coli* strain EB1, but not that of *E. coli* strain EB4. The course of the infection was followed during 7

FIG. 1.—Concentration profiles of *E. coli* in skin lesions in mice after injection of *E. coli* alone (—) or in combination with *B. fragilis* (----). Left: *E. coli* strain EB1 alone (●—●) or in the presence of *B. fragilis* strain BE1 (●-----●) or strain BE4 (*-----*). Right: *E. coli* strain EB4 alone (○—○) or in the presence of *B. fragilis* strain BE4 (○-----○) or strain BE1 (Δ-----Δ). Each point represents the mean of results of at least eight lesions.

days (fig. 2). Whereas *E. coli* when injected alone, decreased rapidly after the first day, the growth index of *E. coli* injected together with *B. fragilis* showed no decline during the experiment.

Determination of the viable count of *E. coli* in the abscess (fig. 3) after injection of the same doses (*E. coli* 1×10^6 cfu $\pm$ *B. fragilis* 5×10^7 cfu) confirmed the semiquantitative counts but gave more information on the degree of synergy. Both strains of *E. coli* were cleared rapidly from the skin with a reduction factor of 10 000-fold after 7 days. In

FIG. 2.—Progression of the infectious process of *E. coli* strain EB1 alone (●—●) or in combination with *B. fragilis* strain BE1 (●-----●). Each point represents the mean growth index value for *E. coli* of at least eight lesions.

FIG. 3.—Numbers of viable *E. coli* in the wound homogenates after injection of *E. coli* strain 25 alone (—) or in combination with *B. fragilis* (---). The results with strains EB1 + BE1 and strains EB4 + BE4 are given in figs A and B respectively. The doses of *E. coli* were 1×10^6 cfu (●) or 1×10^7 cfu (▼); the doses of *B. fragilis* were always 5×10^7 cfu. Each point represents the mean of results of at least eight lesions. SEM are indicated by bars.

the presence of *B. fragilis* strain BE1 counts of *E. coli* strain EB1 were enhanced and stayed high for at least 7 days (fig. 3A). The differences between *E. coli* strain EB1 alone and in combination with *B. fragilis* strain BE1 were significant (Student's *t* test, $p < 0.001$). The presence of *B. fragilis* strain BE4 only had a marginal effect on the clearance of *E. coli* strain EB4. The differences were only significant (Student's *t* test) at $t = 1$ h ($p < 0.02$) and $t = 4$ h ($p < 0.05$). Injection of a higher dose of *E. coli* strain EB4 (1×10^7 cfu) again resulted in a rapid clearance of *E. coli* from the skin of mice. However, a more pronounced effect of *B. fragilis* strain BE4 on the clearance was observed at this dose (fig. 3B). Abscesses which resulted from this combination usually healed within the 7 days of the experiment.

DISCUSSION

The aim of the present study was the development of a simple, reliable, economical animal model to evaluate pathogenic synergy between *E. coli* and *Bacteroides* spp., not dependent on the presence of adjuvants. A cutaneous animal model has been used for virulence studies by several authors (Joiner *et al.*, 1980; Brook and Walker, 1983). High doses of *B. fragilis* were needed for abscess formation in the skin of mice in our

experiments. This is in agreement with the observations of Brook and Walker (1983), who used a virtually identical model. Lower infective doses for *B. fragilis* have been reported, but always in the presence of adjuvants such as blood (Onderdonk *et al.*, 1976), sutures (Kelly, 1978), sterile caecal contents and barium sulphate (Onderdonk *et al.*, 1976) and croton oil and agar (Berti *et al.*, 1982). The animal model described here clearly points to pathogenic synergy between *E. coli* strain EB1 and *B. fragilis* strains BE1 and BE4 but not between *E. coli* strain EB4 and *B. fragilis* strain BE4. The enhancement of growth of only one strain of *E. coli* indicates that this phenomenon depends on properties of these strains. The presence of growth medium markedly influenced the minimal infective doses but we do not agree with Rodloff (1983) that synergy is dependent solely on the presence of growth medium, as the effect of *B. fragilis* remained in the absence of the medium. Because the medium was not a prerequisite for abscess formation its further use was avoided.

The kinetic experiments showed a rapid clearance of both strains of *E. coli* when injected alone. At a dose of 1×10^6 cfu of *E. coli* a substantial influence on the clearance by *B. fragilis* was only observed for *E. coli* strain EB1. At a higher dose this effect on the clearance was also observed for *E. coli* strain EB4, but did not necessarily result in long lasting abscesses. The differences between the strains of *E. coli* observed here may be due to differences in virulence factors; this is the subject of further research. Such differences may also partly explain discrepancies between the present results and those of other workers.

The mechanism of pathogenic synergy remains unclear. Some authors have reported inhibition of phagocytosis of facultative aerobes in the presence of anaerobes (Ingham *et al.*, 1977; Namavar *et al.*, 1983). Other mechanisms such as growth stimulation of *B. fragilis* by *E. coli* as suggested by Hagen *et al.* (1982) may also be involved. The fate of the bacteria during the first 24 h of the experiments has been shown to be crucial for the outcome of infection (Miles *et al.* 1957; Gorrill, 1965). Our model shows that in the presence of *B. fragilis* the clearance of *E. coli* from the skin of mice was reduced and multiplication occurred *in vivo*. The initial multiplication might be enough to establish the infection, but it is not yet known whether both organisms are needed after this initial period. Both organisms were invariably present when pus was cultured, even after 3 weeks. Further experiments will be needed to determine the exact roles of *E. coli* and *B. fragilis* during initiation and progression of the infection. The mouse model described here provides us with a simple and reproducible model for evaluation of pathogenic synergy, which is independent of adjuvants and discriminates between strains.

The skilful technical assistance of Mr W. Schouten was greatly appreciated.

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