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Interactions between polymorphonuclear leucocytes, *Bacteroides* sp, and *Escherichia coli:* their role in the pathogenesis of mixed infection

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SUMMARY Five *Bacteroides fragilis* strains and five *Bacteroides vulgatus* strains were compared with regard to their ability to consume complement and to fix C3, their killing by polymorphonuclear leucocytes, and their ability to inhibit the bactericidal effect of serum and polymorphs on *Escherichia coli* strains. Complement consumption was positively related to C3 fixation, but no relation was observed between these variables and the killing of the anaerobes. Greatest inhibition of the killing of *E coli* by serum and polymorphs was achieved with the bacteroides strains that fixed most complement. The greater virulence of *B fragilis* in mixed infections with *E coli* was not reflected either by a greater ability to inhibit the killing of *E coli* or a greater resistance of the anaerobes themselves to the bactericidal effect of serum and polymorphs.

A mixture of aerobic and anaerobic bacteria can often be cultured from intra-abdominal infections that are the result of contamination of normally sterile tissues with bacterial flora from the gut. Although it is difficult to tell whether the bacteria cultured are mere contaminants or true pathogens in these infections, abundant evidence shows that combinations of aerobic and anaerobic bacteria acting in synergy are capable of causing infections like (abdominal) abscesses and peritonitis.[1-3] This synergy between aerobes and anaerobes has been studied extensively in vivo as well as in vitro.[4]

One of the possible explanations of this synergy—namely, the impairment of phagocytosis and killing of the aerobes by polymorphonuclear leucocytes in the presence of anaerobes—has been supported by several in vitro studies.[5-10] These studies show that competition for complement is an important mechanism by which anaerobic bacteria protect aerobic bacteria from killing by polymorphs. To date it is not known whether differences in ability to compete for complement and to inhibit phagocytosis exist between anaerobes that differ in virulence. Furthermore, our attention was drawn to whether or not complement consumption leads to effective opsonisation and killing of the anaerobes. If not, competition for complement by anaerobes in vivo could lead to a waste of serum factors.

In mixed abdominal infections *Bacteroides fragilis* is found much more often than *Bacteroides vulgatus,* the most common bacteroides species in the gut.[11] In addition, experimental infections in mice have shown that *B fragilis* is more capable of inhibiting the clearance of *E coli* than *B vulgatus.*[12] In this study we compared the properties of five *B fragilis* strains and five *B vulgatus* strains with respect to their consumption of complement, their killing by polymorphs, and their inhibition of the bactericidal effect of serum and polymorphs on *E coli* strains. Among the strains used were those which showed differences in virulence and synergistic effects in experimental mixed infections with *E coli.*[12] The purpose of this study was to discover whether the greater virulence of *B fragilis* (compared with that of *B vulgatus*) was reflected in, and possibly explained by, these properties.

Material and methods

BACTERIA

The *Bacteroides* strains used in this study were isolated from patients at Vrije Universiteit hospital and identified with Minitek (BBL, Becton Dickinson). *E coli* EB1 K^- was an unencapsulated mutant from *E*

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Table 1 *Composition of reaction mixtures in different killing experiments*

Type of experiment	*Volume (ml) in reaction mixture*					
	Polymorphs	*Serum**	*Bacteroides treated serum**	*Bacteroides*	*E coli*	*gel-HBSS*
Killing of *Bacteroides*	0·5	0·1		0·1		0·3
Serum sensitivity of *Bacteroides*		0·1		0·1		0·8
Growth of *Bacteroides*				0·1		0·9
Killing of *E coli* EB1:						
In untreated serum	0·5	0·1			0·1	0·3
In *Bacteroides* treated serum	0·5		0·2		0·1	0·2
In the presence of *Bacteroides*	0·5	0·1		0·1	0·1	0·2
Serum sensitivity of *E coli* EB1 K^-:						
In untreated serum		0·1			0·1	0·8
In *Bacteroides* treated serum			0·2		0·1	0·7

*The given volumes represent a serum concentration of 10% in the reaction mixture. In experiments in which other serum concentrations were used the volumes of serum and of gel-HBSS were adjusted.

coli EB1 which had been isolated from a mixed infection with *B fragilis* BE1 from a patient in the same hospital. All strains were maintained as a pure culture mixed with 30% glycerol frozen at −70°C

For each experiment strains from the frozen stock were cultured on an agar plate: *Bacteroides* strains on blood agar base No 2 (Oxoid) supplemented with 5% sheep blood, 5 mg/l haemin (BDH, Poole), and 2 mg/l menadione (Merck, Darmstadt, West Germany) and incubated at 37°C in an anaerobic chamber with an atmosphere of 85% nitrogen, 10% hydrogen and 5% carbon dioxide. From these plates single colonies were inoculated in liquid BM medium,[13] supplemented with haemin and menadione, and incubated as described above for 18 hours. Log phase cells were obtained by diluting the 18 hour culture 1/10 with fresh medium and growing it until the optical density at 690 nm reached a value of 0·2 in a Coleman junior 111 spectrophotometer. This value represented 10^8 bacteria/ml, as determined with a counting chamber (CA Hauser, Philadelphia, United States of America).

E coli was cultured on nutrient agar plates (Blood agar base No 2, Oxoid), and single colonies were inoculated into nutrient broth (Lab Lemco, Oxoid) and grown until in log phase. The cells were harvested by centrifugation, washed three times in phosphate buffered saline (pH 7·4), counted by viable count, and kept overnight at 4°C. The next day the suspension was adjusted to a concentration of 10^8 *E coli*/ml.

Bacteroides cells were also harvested by centrifugation and directly resuspended in phosphate buffered saline to a concentration of 10^8 cells/ml.

SERUM

In all experiments pooled serum from a large number of healthy donors was used. The serum was kept in aliquots frozen at −70°C.

POLYMORPHONUCLEAR LEUCOCYTES

Polymorphs were isolated from the blood of healthy volunteers by dextran sedimentation. The cells were washed three times in Hanks's balanced salt solution (Gibco, New York, United States of America) with 1% gelatin (gel-HBSS). The cells were counted in a counting chamber, and the suspension was adjusted to 10^7 cells/ml.

TREATMENT OF THE SERUM WITH BACTEROIDES

Bacterial suspension (0·5 ml) was mixed with 0·5 ml serum and incubated at 37°C in a shaking water bath. For determination of the complement depletion samples were taken after 10, 20, and 30 minutes, immediately cooled in ice, and centrifuged at 7000 *g* in a Sarsted MH2 centrifuge at 4°C to remove the bacteria. The remaining complement haemolytic activity was determined as described by Mayer[14] and expressed as the percentage of the CH50 value of untreated serum. Serum was also incubated with phosphate buffered saline as a control. Serum that had been incubated for 30 minutes with bacteroides and further treated as described above was used in some experiments for killing as "bacteroides treated serum."

KILLING EXPERIMENTS

Reaction mixtures were prepared in sterile polypropylene tubes (12 × 75 mm, Rofa Mavi, Beverwijk, The Netherlands) and incubated in a Julabo Paramix shaking apparatus (Rofa Mavi) at 400 rpm in a carbon dioxide incubator with a humidified atmosphere of 95% air and 5% carbon dioxide at 37°C. Table 1 lists the composition of the reaction mixture in the various experiments.

After 0, 30, 60, and 90 minutes 0·1 ml of the reaction mixture was suspended in 9·9 ml of distilled water to lyse the polymorphs. Viable counts of the bacteria were carried out by plating serial dilutions on appropriate agar plates. Statistical analysis was performed using the Spearman rank correlation test.

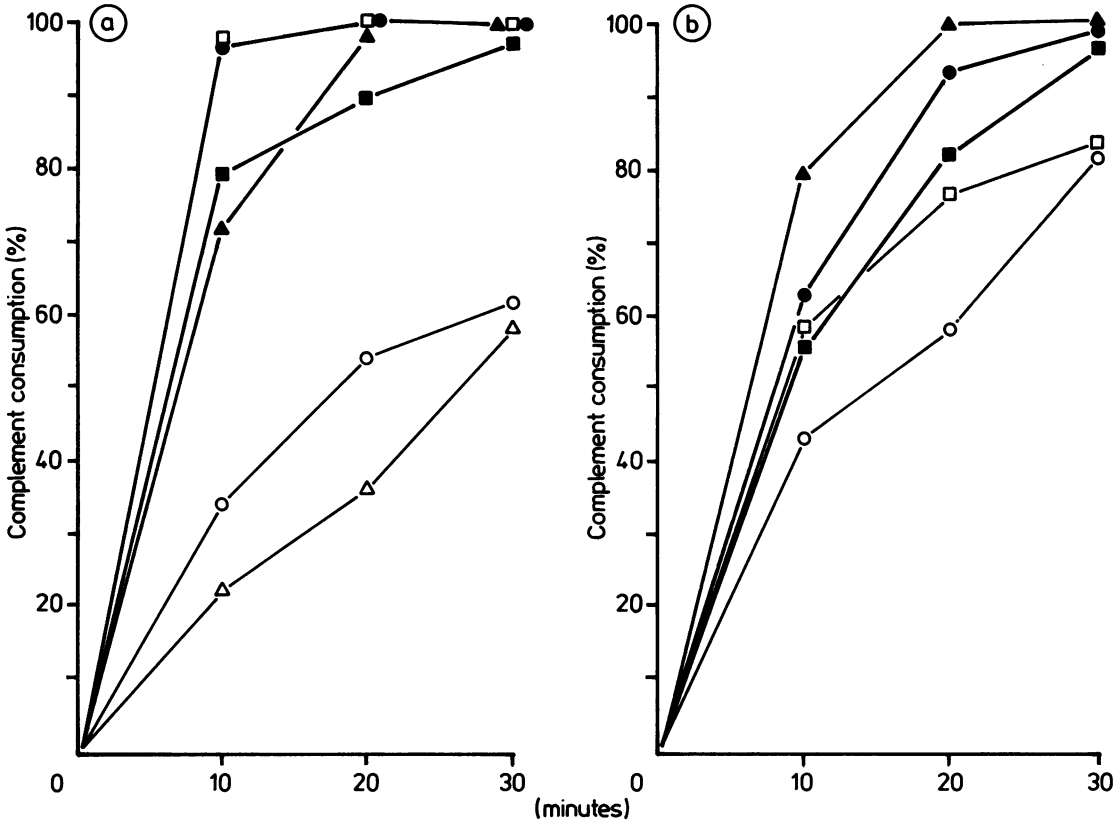

Fig. 1 *Complement consumption (% of CH50 removed by phosphate buffered saline treatment) by E coli EB1 and B fragilis strains (a), and by B vulgatus strains (b). Values represent mean of at least three experiments.*
Fig. 1a (△—△) E coli (■—■) B fragilis BE1 (□—□) BE17 (○—○) BE21 (▲—▲) BE38 (●—●) BE43.
Fig. 1b (●—●) B vulgatus BE16 (▲—▲) BE18 (■—■) BE19 (□—□) BE20 (○—○) BE53.

C3 FIXATION BY BACTEROIDES

Reaction mixtures containing 10^7 *Bacteroides* and 10% serum in 1 ml were incubated at 37°C in a shaking waterbath for 0, 5, 15, and 30 minutes. The C3 fixation was stopped by adding an equal volume of 12·5 mM cold EDTA (ethylenediamine tetraacetic acid disodiumsalt, Merck) in phosphate buffered saline. The bacteria were washed once in phosphate buffered saline and incubated with 0·5 ml of a 1/20 dilution of fluorescein labelled isothiocyanate conjugated antiserum specific for human C3 (Wellcome research laboratories, United Kingdom) in phosphate buffered saline at room temperature for 15 minutes. The cells were washed twice in phosphate buffered saline and resuspended in 2·5 ml 10 mM sodium hydroxide and kept at room temperature for 10 minutes. The bacteria were removed by centrifugation and the fluorescence of the supernatant was determined in a Kontron SFM-23 spectrofluorimeter.

The intensity of the C3 specific fluorescence was shown to be directly proportional to the absolute amount of C3 fixed on the surface of the bacteria.[15] The results were expressed as follows. The zero time values for each strain were subtracted from the values obtained at other incubation times. The values thus obtained were expressed as percentage of the t_{30}-t_0 value of *B fragilis* BE43.

Results

COMPLEMENT CONSUMPTION

Figs. 1a and b show the complement consumption by *Bacteroides* cells when they were incubated with serum. All *Bacteroides* strains tested consumed serum complement at a faster rate than did *E coli* EB1 (Fig. 1a). Differences between strains were observed within both species, and no indication was found to suggest that *B fragilis* strains were more capable of

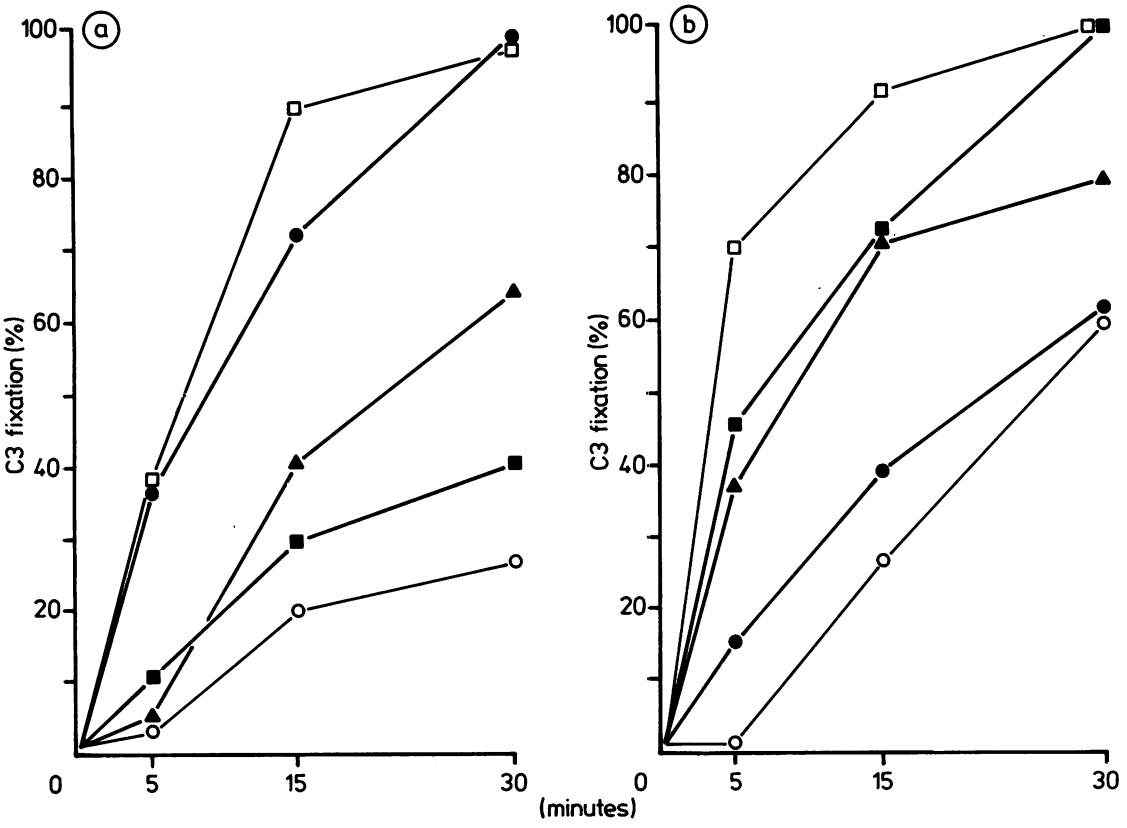

Fig. 2 *Complement C3 fixation (% of C3 fixed by B fragilis BE43 after 30 minutes) by B fragilis strains (a) and by B vulgatus strains (b). Values represent mean of at least two experiments.*
Fig. 2a (■—■) B fragilis BE1 (□—□) BE17 (○—○) BE21 (▲—▲) BE38 (●—●) BE43.
Fig. 2b (●—●) B vulgatus BE16 (▲—▲) BE18 (■—■) BE19 (□—□) BE20 (○—○) BE53.

complement consumption than *B vulgatus* strains.

To find out whether the complement consumed was fixed on the surface of the bacteroides cells or inactivated without being fixed the fixation of C3 on the surface of the bacteroides cells was measured fluorimetrically. Figs. 2a and b show the results of these experiments with *B fragilis* and *B vulgatus*, respectively. Statistical analysis of the relation between complement consumption and C3 fixation showed a highly positive relation between these variables (Spearman rank correlation test, $p < 0{\cdot}01$). We concluded that the *Bacteroides* strains had removed an equal proportion of the complement consumed by normal fixation of C3 on their surface.

BACTERICIDAL EFFECT OF SERUM AND POLYMORPHS ON BACTEROIDES

Figs. 3a and b show that there were strains which were and were not killed by serum and polymorphs in both species. The 90 minutes of aerobic incubation in these experiments did not kill the anaerobes: when serum and polymorphs were not present viable counts after 30, 60, and 90 minutes did not differ greatly from counts of the original inoculum. Incubation of the bacteria with 10% serum, but without polymorphs, showed that two *B vulgatus* strains—namely, BE18 and BE 20—were serum sensitive; all other strains were serum resistant (data not shown). No evidence has been found to suggest that *B fragilis* was more capable of surviving the bactericidal effects of serum and polymorphs than *B vulgatus*. Statistical analysis showed no relation between complement fixation and killing or serum sensitivity.

EFFECT OF BACTEROIDES ON THE KILLING OF *E COLI* EB1 K⁻ BY SERUM

E coli EB1 K⁻ was an unencapsulated mutant of *E coli* EB1 and sensitive to the bactericidal action of serum. Table 2 shows the survival of this strain after incubation with various serum concentrations for 60

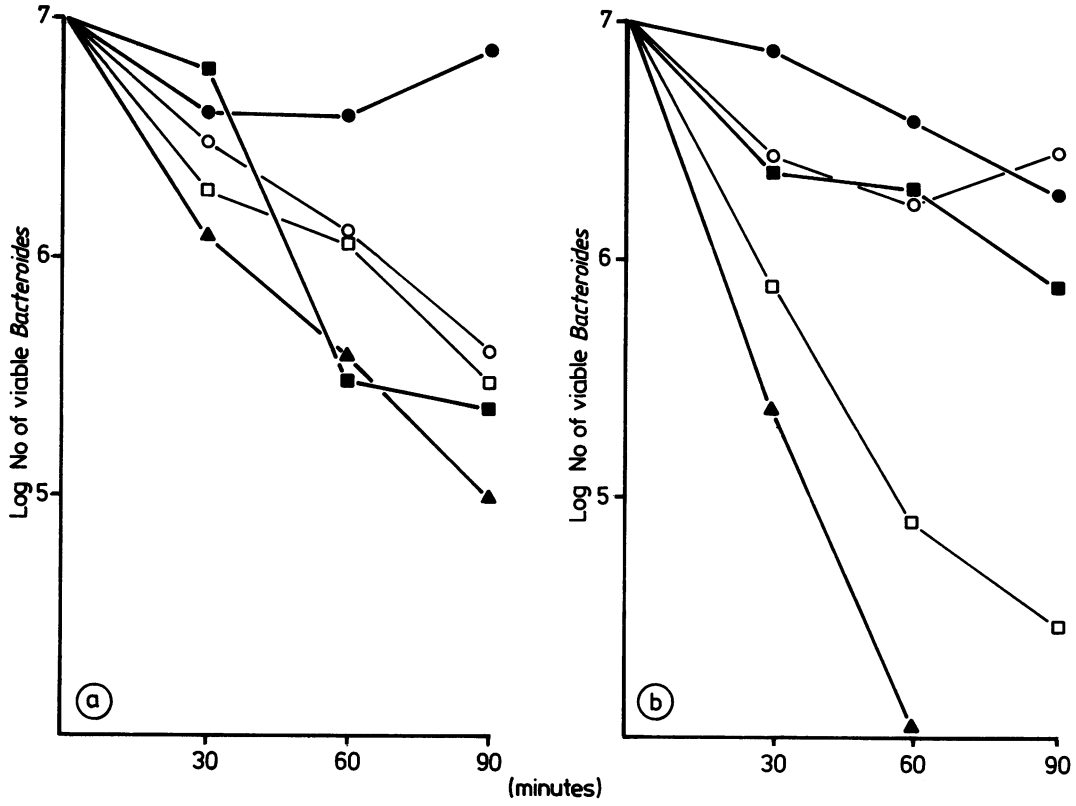

Fig. 3 *Killing of B fragilis strains (a) and of B vulgatus strains (b) by polymorphonuclear leucocytes in presence of 10% pooled normal human serum. Values represent mean of at least three experiments.*
Fig. 3a (■—■) B fragilis BE1 (□—□) BE17 (○—○) BE21 (▲—▲) BE38 (●—●) BE43.
Fig. 3b (●—●) B vulgatus BE16 (▲—▲) BE18 (■—■) BE19 (□—□) BE20 (○—○) BE53.

Table 2 *Survival of E coli EB1 K⁻ in serum pretreated with Bacteroides*

Serum pretreated with	*Log No of surviving E coli after 60 minutes of incubation in a serum concentration of:*		
	1%	*5%*	*10%*
Phosphate buffered saline	5·0	4·6	4·1
B fragilis			
BE1	7·0	5·8	5·0
BE17	7·0	6·3	7·0
BE21	7·1	7·1	5·1
BE38	nd	7·3	7·0
BE43	nd	7·2	7·3
B vulgatus			
BE16	7·0	6·3	4·7
BE18	7·0	6·9	4·7
BE19	7·0	7·0	5·0
BE20	7·0	7·0	4·7
BE53	7·0	6·5	6·3
Untreated serum	4·8	4·4	4·1

*Values represent the mean of three experiments. The log number of the inoculum and of *E coli* surviving in the absence of serum was 7·0.
nd: not determined.

Table 3 *Clearance of E coli and Bacteroides sp or Staphylococcus aureus from the skin of mice six days after injection of combinations of these bacteria*

Injected bacterial strains (log dose)	*Log No of bacteria per lesion*	
	E coli	*Bacteroides*
E coli EB1 (6·3)	1·4	
+ *B fragilis* BE1 (8·5)	4·8	5·0
+ *B fragilis* BE17 (8·3)	2·1	0
+ *B fragilis* BE21 (8·4)	5·7	4·8
+ *B fragilis* BE43 (8·1)	5·4	4·9
+ *B vulgatus* BE16 (8·3)	2·1	0
+ *B vulgatus* BE18 (8·8)	3·7	1·5
+ *B vulgatus* BE19 (8·3)	6·7	6·7
+ *B vulgatus* BE53 (8·3)	2·8	0·3
		S aureus
E coli EB1 (6·7)	2·4	
+ *S aureus* BE50 (8·6)	7·6	5·6

minutes. Preincubation of the serum with phosphate buffered saline for 30 minutes at 37°C did not change the outcome of viable counts, unlike preincubation of the serum with *Bacteroides*. Whereas 60 minutes of incubation with 1% untreated, or phosphate buffered saline treated serum reduced the viable count by ca. 2 log, the whole inoculum survived incubation with bacteroides treated serum. Differences in inhibition of the bactericidal effect of serum between strains of *Bacteroides* were seen at a serum concentration of 5% or 10%: at 5% all strains had some effect, but of varying degrees, and at 10% only *B fragilis* BE17, BE38, and BE43 gave complete inhibition.

EFFECT OF BACTEROIDES ON THE KILLING OF *E COLI* EB1 BY SERUM AND POLYMORPHS

In the presence of 10% serum *E coli* EB1 was killed by polymorphs: the viable count after 90 minutes was reduced by 1·85 log. Ten per cent serum was the minimal concentration required for efficient killing of this strain. When the serum had been pretreated with *Bacteroides*, or when *Bacteroides* was present in the reaction mixture, all strains gave complete inhibition of the killing of *E coli* EB1. In the presence of 15% untreated serum the number of *E coli* was reduced by 1·5 log by polymorphs, but when *B vulgatus* BE53 or *B fragilis* BE43 were present in the reaction mixture 1·3 log and 0·9 log of *E coli* were killed, respectively. Neither BE53 nor BE43 had any inhibiting effect on the killing of *E coli* in 20% serum, indicating that enough serum factors remained for the efficient killing of *E coli* EB1 in this case. Thus differences in inhibiting effect between strains of *Bacteroides* could be seen only at a serum concentration of 15%: BE43 had a greater inhibiting effect than BE53, but the difference was small.

When the results of the experiments with *E coli* EB1 and *E coli* EB1 K^- were compared with those of the complement fixation experiments it was obvious that the largest inhibiting effect was produced by the best complement fixing strains. Differences in inhibiting effect between *Bacteroides* strains could, however, be seen only at critical serum concentrations.

Discussion

The higher incidence of *B fragilis* in mixed infections with *E coli* in comparison with other related species like *B vulgatus* may be explained by a greater virulence of *B fragilis*, or a greater ability to act in synergy with *E coli*. Recently, Verweij-van Vught *et al*[12] compared four *B fragilis* strains with four *B vulgatus* strains with regard to their virulence in monoinfections and mixed infections with *E coli*. Table 3 shows that three of four *B fragilis* strains had a greater inhibiting effect on the clearance of *E coli* than had three of four *B vulgatus* strains. *Staphylococcus aureus* also had an inhibiting effect on the clearance of *E coli*. The authors' study in monoinfections showed that *B vulgatus* was more rapidly cleared than were *B fragilis* and *S aureus*, and, therefore, it was concluded that the inhibiting effect on the clearance of *E coli* was not specific for *B fragilis* but could be achieved with organisms which are capable of maintaining themselves in numbers large enough for at least a few days: thus it is a reflection of the virulence in monoinfections.

In this study we compared five *B fragilis* strains with five *B vulgatus* strains with regard to their interaction with serum, polymorphs, and *E coli* in vitro. Among the strains tested were those which had been used by Verweij-van Vught *et al.*[12] We compared the complement consumption and C3 fixation using the two species, their inhibition of the bactericidal effect of serum and polymorphs on *E coli*, and the killing of the anaerobes themselves by serum and polymorphs. Inhibition of the killing of *E coli* EB1 K^- by serum and that of *E coli* EB1 by polymorphs was best achieved with the *Bacteroides* strains which most readily consumed complement. These strains were found within both species.

Differences in inhibition between *Bacteroides* strains could be observed only at serum concentrations within a narrow range. We found a positive rank correlation between complement consumption and C3 fixation on the surface of the bacteria but not between these variables and the killing of the anaerobes. Thus it seemed that an equal proportion of the consumed complement was fixed on the surface of the bacteria, by all strains without equally effectively opsonising them. Strains which fixed complement, however, but were not killed subsequently, were found within both species and were not necessarily virulent in vivo.

To summarise, *B fragilis* was not more able to resist killing by polymorphs or to protect *E coli* from killing by serum and polymorphs in vitro by competition for complement than was *B vulgatus*.

A study of Sundqvist *et al*[16] showed that a local shortage of complement can occur in vivo: a pathogenic strain of *B gingivalis* was injected in subcutaneously implanted teflon cages in guinea pigs. Immunoelectrophoresis of the fluid from the cages showed that complement C3 was degraded and completely removed by the proteolytic activity of this strain. This study shows that, at least in subcutaneous abscesses, the supply of complement is limited and can be exhausted. Perhaps other organisms can achieve this effect by complement fixation.

Our conclusion, therefore, is that factors other than the ability to act in synergy with *E coli* by inhibiting its killing, determine the greater pathogenic potential of *B fragilis* in mixed infections with *E coli*. Inhibition of the killing of *E coli* can also be achieved by other complement fixing organisms and may have a role at sites where a local shortage of complement can occur. The ability of these organisms to maintain themselves in numbers sufficient to compete for complement successfully is, however, a prerequisite which *B fragilis* meets more adequately than does *B vulgatus*.

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