THE BACTERICIDAL ACTION OF HUMAN SERUM ON *BACTEROIDES* SPECIES

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SUMMARY. Sera obtained from 60 healthy normal subjects were tested for their inhibitory action against four clinical isolates of the *Bacteroides fragilis* group. All the sera inhibited clinical strains of *B. vulgatus* and *B. ovatus*, 92% of 60 sera inhibited *B. thetaiotaomicron* and 62% inhibited *B. fragilis*. Bacterial inhibition required components of uninactivated serum and was related to the serum concentrations and species of the bacteria. The viable counts of all the strains, except *B. fragilis*, were significantly reduced in pooled sera from patients with anaerobic systemic infections and from non-infected patients. The bactericidal activity of the sera from infected patients was more pronounced than that of the 'normal' sera, on all strains except *B. fragilis*. These observations may explain in part the predominance of *B. fragilis* in serious clinical infections involving anaerobes.

INTRODUCTION

Anaerobic bacteria form the bulk of the normal bacterial flora of the oral cavity (particularly in the gingival crevice, dental plaque and interdental spaces), the gastrointestinal, female genital and respiratory tracts (Drasar, Shiner and McLeod, 1969; Duerden, 1980a; Rotimi and Duerden, 1981a; Masfari, Kinghorn and Duerden, 1983). In the last two decades *Bacteroides* spp. and anaerobic cocci have been increasingly implicated in a number of clinical infections, some of which were life-threatening (Sinkovics and Smith, 1970; Felner and Dowell, 1971; Bartlett and Finegold, 1972; Gorbach and Bartlett, 1974; Rotimi and Abudu, in press). *B. fragilis*, the type species of the *B. fragilis* group which includes *B. thetaiotaomicron*, *B. vulgatus*, *B. fragilis*, *B. ovatus* and *B. distasonis*, is the species most commonly isolated from human infections (Holland, Hill and Altemeier, 1977; Polk and Kasper, 1977; Duerden, 1980b). These anaerobes have often been regarded as opportunistic pathogens which express their pathogenic potential when the integrity of the host defence is breached.

However, little information is available on the specific host defence mechanisms. Roberts (1970) and Williams (1972) showed that the activities of the phagocytes, macrophages and polymorphonuclear leukocytes (PMN) were of paramount importance in the killing of facultative and aerobic bacteria. Some *Bacteroides* spp. are

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known to interfere with the phagocytosis of some facultative bacteria *in vitro* (Ingham *et al.*, 1977). Faecal isolates of members of the *B. fragilis* group have been shown to be sensitive to the bactericidal action of normal human serum (Casciato *et al.*, 1979) which is also enhanced by the presence of active PMN (Casciato *et al.*, 1975). The work reported here was undertaken to study the susceptibility of clinical isolates of the *B. fragilis* group to normal human serum and to compare the viability of these clinical isolates in serum from patients with proven anaerobic infections with that in serum from non-infected controls.

MATERIALS AND METHODS

Bacterial strains

Four clinical isolates were used in the study: *B. fragilis*, isolated from a positive blood culture; *B. vulgatus* and *B. thetaiotaomicron*, isolated from post-surgical wound infections; and *B. ovatus* isolated from an infected ulcer in a patient with an underlying malignant lesion. All isolates were identified by the methods of Duerden *et al.* (1980) and Rotimi, Faulkner and Duerden (1980) and kept frozen in 1-ml portions, as stock cultures, at -70°C . When needed they were grown on freshly prepared BM-Kanamycin agar (Holbrook, Ogston and Ross, 1978) and also inoculated on to blood agar incubated aerobically to check for purity. They were harvested, washed and suspended in sterile phosphate buffered saline (PBS, pH 7.3) at a concentration of $c.10^5$ bacteria/ml; this was used to provide the standard inoculum.

Serum

Blood (30 ml) was drawn from 60 healthy normal adult volunteers, 20 patients with anaerobic infections and 10 patients admitted to the medical ward for reasons other than infected lesions. The blood was centrifuged at 3000g for 10 min. Serum (c. 16–20 ml from each blood sample) was sterilised by filtration through 0.45- μm membrane filters (Millipore). The serum from each normal healthy subject was divided aseptically into three parts and stored at -20°C when not used immediately. Sera from infected patients (infected sera) and sera from non-infected controls ('normal' sera) were also stored at -20°C until used. Only five sera that satisfied the definition of an infected serum were used, i.e., they were obtained before administration of any antibiotics from patients from whom an anaerobic organism was isolated in pure culture (two had positive blood cultures and three post-surgical wound infections).

Serum bactericidal test

Tests to demonstrate the bactericidal activity of normal human serum were performed in three stages:

(a) *Unheated serum test*. Either fresh serum or one of the three parts of the frozen serum from each subject thawed only once was diluted in BM broth (Holbrook *et al.*, 1978) containing L-cysteine hydrochloride (BDH) 0.075%, to give final serum concentrations of 10%, 50% and 80% (v/v) in 4-ml amounts in sterile plastic screw-capped bottles (Sterilin). Three drops of each of the bacterial suspensions were then seeded into each set of bottles and into another bottle containing plain BM broth.

(b) *Inactivated serum test*. A second part of the serum was inactivated in a waterbath at 56°C for 30 min and then diluted to give 10%, 50% and 80% v/v final concentrations of inactivated serum in broth. Four sets of bottles, each containing a range of the serum concentrations and plain BM broth without serum, were also seeded with corresponding bacterial suspensions as in (a).

(c) *Control test with complement*. The third part of the serum was inactivated as in (b) and dilutions made up in 3-ml amounts in four separate sets of bottles, each containing 10%, 50% and 80% v/v serum in BM broth. One ml of commercially prepared preserved guinea-pig serum complement (Wellcome Reagents Ltd, Beckenham, England) was added to each bottle. The

haemolytic dose of complement for each of the clinical isolates was initially determined, in a pilot study, by a standard method (Mayer, 1961). Each bacterial strain was seeded into corresponding sets of bottles, into plain BM broth and into BM broths to which one haemolytic dose of complement was added.

The bottles were all incubated anaerobically (Gas-generating kit system, Oxoid) for 24 h at 37°C. After incubation, the bottles were inspected for evidence of bacterial inhibition. Contents of bottles with no visible signs of growth were subcultured on to fresh blood-agar plates and incubated anaerobically.

Experiments for survival of bacterial strains in serum

The effects of serum from infected patients and 'normal' serum on the growth rate of each test strain was tested by performing viable counts after 0, 4, 12 and 24 h in duplicate experiments. A slight modification of the procedure of Miles, Misra and Irwin (1938) was used for the tests. The sera from the infected and non-infected patients were pooled separately. Each serum pool was then divided into equal parts and each part diluted to give final concentrations of 10% and 50% (v/v) serum in broth; 0.1 ml of each of the bacterial suspensions was then seeded into bottles containing each concentration so that the viable count of each of the test strains was performed in two concentrations of serum after 0, 4, 12 and 24 h. One anaerobic jar was used for each period to ensure continuous anaerobic incubation for the required time. At the end of the specified periods the viable count of the test strain was performed on solid medium in duplicate and the plates were incubated for a continuous period of 48 h. Plain broth seeded with each strain was included in each jar as a control.

RESULTS

Serum sensitivity and concentrations

Table I summarises the numbers of sera from healthy subjects with bactericidal activity against each test strain at three concentrations. *B. ovatus* and *B. vulgatus* were the most susceptible strains and *B. fragilis* was the least susceptible. Each test strain grew luxuriantly in the BM broth with or without guinea-pig complement. Analysis of the overall effect of each serum on all four strains shows that the five sera that did not have any bactericidal activity against *B. thetaiotaomicron* were among the 23 that did not inhibit the *B. fragilis* strain. The remaining 18 of the 23 sera that did not inhibit *B. fragilis* inhibited the other three test strains. In no case was there bactericidal activity against any of the test strains with heat-inactivated serum at any concentration. The addition of one haemolytic dose of complement to each concentration produced results identical with those obtained in the tests with unheated sera (table II).

TABLE I
Inhibition of Bacteroides spp. by sera from healthy normal subjects

<i>Bacteroides</i> species	Number out of 60 sera that were bactericidal at concentrations of		
	10%	50%	80%
<i>B. fragilis</i>	5	14	37
<i>B. vulgatus</i>	46	60	...
<i>B. ovatus</i>	56	60	...
<i>B. thetaiotaomicron</i>	16	44	55

TABLE II

The bactericidal effects of normal serum, inactivated serum and complement on *Bacteroides* spp.

<i>Bacteroides</i> species	Number out of 60 sera with bactericidal activity		
	Normal serum	Inactivated serum	Inactivated serum + C*
<i>B. fragilis</i>	37	0	37
<i>B. vulgatus</i>	60	0	60
<i>B. ovatus</i>	60	0	60
<i>B. thetaiotaomicron</i>	55	0	55

*C = complement

Viable counts in serum from infected patients and 'normal' serum

The effect of serum from infected patients and 'normal' serum on the viable count of *B. fragilis* is shown in fig. 1. After incubation for 24 h, there was a smaller number of viable cells of *B. fragilis* in 10% and 50% 'normal' serum (2.4×10^6 and 1×10^6 cfu/ml) than in the control broth (1.5×10^7 cfu/ml). Conversely *B. fragilis* thrived in the sera from infected patients; after 24 h, the viable counts were 3×10^7 and 2.1×10^7 in 10% and 50% serum respectively.

There was a noticeably lower viable count of *B. thetaiotaomicron* in serum from infected patients than in the control broth; the reduction was less with normal serum

FIG. 1.—Effects of 'normal' serum and serum from infected patients on *B. fragilis*: ○—○ = 10% 'normal' serum; ●—● = 50% 'normal' serum; △---△ = 10% serum from infected patients; ▲---▲ = 50% serum from infected patients; ×—× = control culture in BM broth without serum.

FIG. 2.— Effects of two concentrations of sera from 'normal' individuals and from patients with anaerobic infection on *B. thetaiotaomicron*; for key to symbols, see caption to fig. 1.

(fig. 2). After 24 h, the counts were 4×10^7 and 1.5×10^6 cfu/ml in 10% and 50% 'normal' serum, and 4.5×10^6 and 7.6×10^5 cfu/ml in 10% and 50% serum from infected patients respectively; the count in the broth was 1.1×10^7 cfu/ml.

The markedly lower viable counts of *B. vulgatus* in 'normal' serum and in serum from infected patients are shown in fig. 3; the effect of the sera was noticeable after incubation for only 4 h. The rate of killing increased by 24 h when the count in broth was 1×10^8 cfu/ml compared to 1.5×10^3 cfu/ml in 50% 'normal' serum. There was a very marked bactericidal effect within 24 h with the serum from infected patients; the number of surviving bacteria had fallen to 4.5×10^1 cfu/ml in 10% serum and 2.1×10^1 cfu/ml in 50% serum.

With *B. ovatus* the bactericidal effect increased progressively from the start to 24 h in both sera and this is shown in fig. 4. After incubation for 24 h, while the count in the broth remained high (5.5×10^7 cfu/ml), the counts in both concentrations of the 'normal' serum and the serum from infected patients were only 5×10^3 and 1.6×10^3 cfu/ml and 7.6×10^2 and 1×10^2 cfu/ml in 10% and 50% serum respectively.

DISCUSSION

The *B. fragilis* group forms the bulk of the anaerobic normal bacterial flora of the faeces; *B. vulgatus* and *B. thetaiotaomicron* are the predominant species (Duerden,

FIG. 3.—Effects of two concentrations of sera from 'normal' and infected patients on *B. vulgatus*; for key to symbols, see caption to fig. 1.

1980a; Rotimi and Duerden, 1981b). *B. fragilis*, the type-species of the group, is a less common member of the normal anaerobic flora of the faeces but is the commonest species isolated from clinical infections after abdominal or pelvic surgery (Holland *et al.*, 1977; Duerden, 1980b).

Several reasons have been given for this. They include the possession of a polysaccharide capsule which resists phagocytosis and interferes with the phagocytosis of other bacteria (Kasper 1976; Ingham *et al.*, 1977; Onderdonk *et al.*, 1977), and resistance to the bactericidal action of normal human serum (Casciato *et al.*, 1979). The results of the present study confirm and extend the findings of Casciato *et al.* (1979) that some clinical isolates of *B. fragilis* are not killed by human serum. Out of 60 sera tested, 23 did not inhibit the growth of the clinical isolate of *B. fragilis*. Five of these 23 sera failed to inhibit the growth of the clinical isolate of *B. thetaiotaomicron*, but all 60 sera inhibited *B. vulgatus* and *B. ovatus* at relatively low serum concentrations. Other investigations have also demonstrated bactericidal activity in normal serum against facultative gram-negative pathogens isolated from local infections but not from bacteraemic or septicaemic patients (Roantree and Rantz, 1960; Vosti and Randall, 1970; Young and Armstrong, 1972). The strains of *B. thetaiotaomicron*, *B. vulgatus* and *B. ovatus* that we studied were isolated from localised infections, post-surgical sepsis and an infected malignant ulcer and the *B. fragilis* was from a blood culture.

In many cases, the concentrations of the sera that inhibited the *B. fragilis* strain were higher than those that inhibited the other species. Thus, the bactericidal activity

FIG. 4.—Effects of two concentrations of sera from 'normal' patients and infected patients on *B. ovatus*; for key to symbols, see caption to fig. 1.

of serum depended upon the species of *Bacteroides*; the pattern of activity was nearly always the same in the sera from healthy normal individuals. The results of this study also showed that inhibitory activity depended upon heat-labile factor(s) in normal serum, and was restored to heated serum by complement. This agrees with the observation of other workers on the role of complement in lysis of gram-negative bacteria (Meshulam, Verbrugh and Werhoef, 1982).

The viable counts of the four clinical isolates in pooled serum from infected patients and in 'normal' serum were noteworthy. After incubation for 24 h there were significantly fewer viable bacteria of *B. vulgatus*, *B. ovatus* and, to a less extent, *B. thetaiotaomicron* in both sera than in control broths; bactericidal activity was clearly demonstrated against *B. vulgatus* and *B. ovatus*. In all cases except with *B. fragilis*, the serum from infected patients was more inhibitory than the 'normal' serum at both concentrations. *B. fragilis* was relatively unaffected; its count was higher in the serum from infected patients than in the broth after incubation for 24 h. This indicates that the susceptibility of individual *Bacteroides* spp. to the bactericidal activity of human serum is related to the source of the serum.

Most normal human sera from all age groups contain circulating humoral antibody of the IgM and IgG classes to *Bacteroides* spp. of the normal bacterial flora (Quick, Goldberg and Sonnenwirth, 1972; Hofstad, 1974; Kasper, 1976). Although

the present study did not provide any evidence that these types of antibodies may be involved in the bactericidal action of normal serum and serum from infected patients, it showed that heat-labile components of human serum (Muschel, 1960), and perhaps other factors, are essential for the inhibition of all serum-sensitive *Bacteroides* spp.

Serum bactericidal capacity is probably an important host-resistance factor against systemic invasion by organisms of the *B. fragilis* group other than its type species, *B. fragilis*, which possesses the ability to multiply in serum from infected patients and other virulence factors, such as a capsule (Kasper, 1976; Onderdonk *et al.*, 1977; Polk and Kasper, 1977). The relative resistance and viability of *B. fragilis* in serum may partly explain its pathogenic potential and predominance over other species in serious clinical infections despite its minor contribution to the normal anaerobic bacterial flora.

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