Effects of *Bacteroides asaccharolyticus* cells and *B. fragilis* surface components on serum opsonisation and phagocytosis

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Summary. The effect of *Bacteroides asaccharolyticus* on the opsonisation and phagocytosis of gram-positive and gram-negative bacteria by human polymorphonuclear leukocytes (PMNL) was investigated. Uptake of most isolates of staphylococci, streptococci and clostridia by PMNL after opsonisation with serum treated with *B. asaccharolyticus* was largely unimpaired. The same treatment of serum before opsonisation of isolates of *Pseudomonas*, *Enterobacter*, *Klebsiella* and *Gardnerella* resulted in the uptake by PMNL varying with individual isolates; a large reduction occurred with some and none with others. Treatment of serum with *B. fragilis* lipopolysaccharide before opsonisation of *Proteus mirabilis* produced a marked reduction in uptake, whereas treatment with *B. fragilis* capsular polysaccharide had little effect.

Introduction

The ability of *Bacteroides* spp. to impair phagocytosis, not only of themselves but also of accompanying bacteria, has been suggested to be an important factor in the development of mixed infections with aerobic and anaerobic bacteria (Ingham *et al.*, 1977). *Bacteroides* can activate both the classical and alternative complement pathways (Bjornson, 1984) and depletion of serum complement by bacteroides, resulting in reduced opsonic activity, has been proposed as a possible mechanism for inhibition of phagocytosis (Tofte *et al.*, 1980; Jones and Gemmell, 1982; Wade *et al.*, 1983; Vel *et al.*, 1985). The depletion by bacteroides of heat-labile serum factors necessary for intracellular killing of bacteria by polymorphonuclear leukocytes (PMNL) has also been reported (Ingham *et al.*, 1981).

There have been conflicting reports of the location of the components in the bacteroides cell that impair phagocytosis. The property has been said to be associated with: (a) the intact cell (Ingham *et al.*, 1977, 1981); (b) the extracellular products (Namasvar *et al.*, 1983); (c) the capsule (Connolly *et al.*, 1984); (d) the whole cell but *not* the capsule (Wade *et al.*, 1983).

The ability of bacteroides to inhibit phagocytosis of other bacteria has been shown to be dependent

on the particular isolate (Jones and Gemmell, 1982; Wade *et al.*, 1983; Vel *et al.*, 1985). These studies were restricted to experiments with *Proteus* spp. and *Escherichia coli*.

The aims of the present investigation were to determine the range of bacteria whose serum opsonisation and uptake by PMNL may be affected by bacteroides and to determine whether the capsular polysaccharide (CPS) and lipopolysaccharide (LPS) of *B. fragilis* inhibit phagocytosis.

Materials and methods

Bacteria

Fresh isolates from clinical material submitted to the Bacteriology Department, Royal Infirmary, Glasgow, were used except where stated otherwise. *Pr. mirabilis* strain NGH1 and *B. asaccharolyticus* strain NGH4 were kindly provided by Dr H.R. Ingham, General Hospital, Newcastle upon Tyne.

Capsular polysaccharide (CPS) and lipopolysaccharide (LPS)

Samples of CPS and LPS, extracted and purified by Sephacryl S-300 chromatography in a buffer with sodium deoxycholate 3%, from *B. fragilis* strain 23745, were kindly provided by Dr D. L. Kasper, Channing Laboratory, Harvard Medical School, Boston, MA, USA (Kasper *et al.*, 1983). It has been calculated that 90 µg of LPS and 200 µg of CPS are derived from 10⁸ organisms

(Onderdonk *et al.*, 1977). Both polysaccharides were dissolved in distilled water before use.

Cultivation of bacteroides.

B. asaccharolyticus strain NGH4 and *B. fragilis* strain 23745 were grown anaerobically for 48 h at 37°C on 5% horse-blood-agar plates (Columbia Agar; Oxoid). Growth was suspended in saline 0.85% w/v to a concentration of 5×10^8 bacteria/ml, determined spectrophotometrically.

Preparation of radiolabelled bacteria

Bacteria were radiolabelled by growth in Mueller-Hinton Broth (Oxoid) containing ^3H -adenine (specific activity 25 Ci/mmol; Radiochemical Centre Amersham) at a final concentration of 10 $\mu\text{Ci/ml}$ of broth. *Clostridium* spp. and *Gardnerella vaginalis* were grown anaerobically in Brain Heart Infusion Broth (Oxoid). After incubation, the bacterial suspensions were centrifuged, washed three times with saline 0.85% and the final suspension adjusted to contain 5×10^7 bacteria/ml before opsonisation. Counts of radioactivity/0.1 ml were in the range 10,000–20,000 cpm for the different target bacteria used in the study.

Treatment of serum with bacteroides cells, LPS and CPS

Equal volumes of normal human serum, diluted to 50% v/v with Hanks's Balanced Salts Solution containing gelatin 0.1% (gel-HBSS), and *B. asaccharolyticus* or *B. fragilis* cell suspension were mixed and placed in an orbital incubator (Gallenkamp; shaking speed 150 rpm) for 15 min at 37°C. Bacteroides cells were removed by centrifugation (3000g for 15 min) and the serum used immediately for opsonisation. With LPS and CPS the procedure was the same except that the serum was not centrifuged.

Opsonisation

Fresh human serum was obtained from the Haematology Department, Royal Infirmary, Glasgow, divided into 5-ml portions and stored at –70°C before use. For opsonisation, serum was diluted with an equal volume of gel-HBSS and this solution was mixed with an equal volume of radiolabelled bacterial suspension. The final concentration of serum in all opsonisation mixtures was 25% v/v irrespective of whether the serum had been previously treated with bacteroides cells, LPS or CPS or was untreated. Opsonisation was performed in an orbital incubator (shaking speed 150 rpm) at 37°C for 15 min, after which the suspension was centrifuged, the supernate discarded and the opsonised bacteria resuspended to the original concentration (i.e., $5 \times 10^7/\text{ml}$) in fresh gel-HBSS.

Leukocyte preparation

Venous blood donated by human volunteers was heparinised and mixed with dextran (mol.wt 150,000; 6% w/v in saline 0.85%). After sedimentation of erythrocytes, the supernate was centrifuged and the pellet resuspended in ammonium chloride 0.87% to allow lysis of erythrocytes. The leukocytes were centrifuged and resuspended to contain 5×10^6 PMNL/ml as determined with a haemocytometer.

Uptake of radiolabelled bacteria

The method of Verhoef *et al.* (1977) was followed. For each phagocytosis mixture, duplicate test vials containing 0.1 ml of radiolabelled, opsonised bacteria and 0.1 ml of the leukocyte suspension were incubated for 45 min at 37°C in an orbital incubator (shaking speed 150 rpm). Duplicate controls containing gel-HBSS in place of the leukocyte suspension were included. After incubation, 3 ml of scintillation fluid (Aqualuma, Lumac Systems AG, Basle, Switzerland) were added to one of the tests and to one of the control vials. Measurement of radioactivity in these vials provided an estimate of the total number of bacteria present in each phagocytic mixture. To the other test and control vials, 3 ml of ice-cold phosphate-buffered saline (PBS), pH 7.4, were added; the vials were centrifuged at 100g for 5 min to sediment the PMNL and the supernatant fluid was discarded. These vials were washed twice more with PBS and 3 ml of scintillation fluid was then added to each vial. Measurement of radioactivity in these vials provided an estimate of the number of ingested bacteria present in each phagocytic mixture. The counts/min of the contents of each vial were determined in a liquid scintillation counter (1210 Ultrabeta; LKB Instruments Ltd). The percentage uptake of labelled bacteria was calculated as follows:

$$\frac{\text{cpm (centrifuged mixture containing PMNL)}}{\text{cpm (uncentrifuged mixture containing PMNL)}} \times 100$$

This value was corrected by subtracting the corresponding value obtained from the counts recorded for the vials that did not contain PMNL.

Statistical analysis

Student's *t* test was used to assess significance; all experiments were performed at least twice.

Results

Effect of *B. asaccharolyticus* strain NGH4 on opsonisation and uptake of various isolates

Phagocytic uptake of *Staphylococcus aureus* isolates was mainly unaffected after opsonisation with serum treated with *B. asaccharolyticus* cells (Fig. 1); uptake of only one isolate was reduced to 70% of that of the untreated control and uptake of seven

Fig. 1. Effect of *B. asaccharolyticus* strain NGH4 cells on uptake of gram-positive bacteria by PMNL. Uptake of bacteria opsonised with serum treated with *B. asaccharolyticus* is expressed as a percentage of the control (uptake of the same strain opsonised with untreated serum). A—*S. aureus*, B—*S. epidermidis*, C—*Str. faecalis*, D—*Clostridium* sp.

Fig. 2. Effect of *B. asaccharolyticus* strain NGH4 cells on uptake of gram-negative bacteria by PMNL. Uptake of bacteria opsonised with serum treated with *B. asaccharolyticus* is expressed as a percentage of the control (uptake of the same strain opsonised with untreated serum). E—*Pseudomonas* sp., F—*Enterobacter* sp., G—*G. vaginalis*, H—*Klebsiella* sp.

isolates was unaffected. Greater variation occurred with *S. epidermidis* isolates; uptake of one isolate was reduced to 55% and that of the other isolates by smaller amounts. With *Streptococcus faecalis* isolates there was little difference in uptake after opsonisation with bacteroides-treated serum, compared with that of the untreated control. The effect on *Clostridium* isolates was similar; uptake of only two of seven isolates was affected, being reduced to 60–70% of that of the untreated controls. With

minor exceptions, it appeared that treatment of serum with bacteroides cells before opsonisation had little overall effect on uptake of the gram-positive isolates tested.

A much greater variation in the effect of bacteroides treatment of serum on opsonophagocytosis was seen with gram-negative bacteria (Fig 2). Uptake of only four (of 14) *Pseudomonas* isolates was unaffected whereas uptake of the others was reduced by 8–65% compared with that of the untreated control. *Enterobacter* isolates could be divided into two distinct groups—those whose uptake was unaffected by bacteroides treatment of the serum and those whose uptake was reduced by 40–60%. With all five *G. vaginalis* isolates, the uptake after bacteroides treatment of serum was markedly reduced by 50–85%. A wide variation was seen with *Klebsiella* isolates; the uptake of most was reduced by 50% or more after treatment of serum with bacteroides.

Effect of *B. fragilis* CPS and LPS on opsonisation and uptake of *Pr. mirabilis*

At a concentration of 20 $\mu\text{g/ml}$ of serum, LPS treatment of serum before opsonisation of *Pr. mirabilis* reduced uptake to 42% of the untreated control; the reduction was to 20% when serum was treated with 5×10^8 *B. fragilis* cells (Fig. 3). At LPS concentration of 0.2 and 0.02 $\mu\text{g/ml}$ of serum, uptake was reduced to 60% and 75% respectively.

Fig. 3. Effect on LPS on uptake of *Pr. mirabilis* strain NGH1 by PMNL. Uptake of bacteria opsonised with serum treated with LPS or *B. fragilis* cells is expressed as a percentage of the control (uptake of the same strain opsonised with untreated serum). A = *B. fragilis* 5×10^8 cells/ml; B = LPS 20 $\mu\text{g/ml}$; C = LPS 0.2 $\mu\text{g/ml}$; D = LPS 0.02 $\mu\text{g/ml}$. Means and SD (± 1) are shown. Significance values in comparison with the control are: A— <0.001 ; B— <0.001 ; C— <0.01 ; D— >0.2 .

Fig. 4. Effect of CPS on uptake of *Pr. mirabilis* strain NGH1 by PMNL. Uptake of bacteria opsonised with serum treated with CPS or *B. fragilis* cells is expressed as a percentage of the control (uptake of the same strain opsonised with untreated serum). A = *B. fragilis* 5×10^8 cells/ml; B = CPS 20 μ g/ml; C = CPS 0.2 μ g/ml; D = CPS 0.02 μ g/ml. Means and SD (± 1) are shown. Significance values in comparison with the control are: A = <0.001 ; B = <0.001 ; C = >0.1 ; D = >0.4 .

Although there was a slight reduction in uptake when serum was treated with CPS before opsonisation (Fig. 4) it was less throughout than when serum was treated with LPS, particularly when CPS was used at a concentration of 20 μ g/ml, which resulted in an uptake 70% of the control value. The results with CPS concentrations of 0.2 and 0.02 μ g/ml (72% and 82%) were not significantly different ($p > 0.1$) from those with 20 μ g/ml.

Discussion

According to our results, the ability of bacteroides to inhibit the phagocytosis of other bacteria appears to vary for different target micro-organisms. With some species, such as *S. aureus* and *Str. faecalis*, in almost all cases phagocytosis was hardly affected by treatment of the opsonising serum with bacteroides cells. In contrast, the degree of inhibition by bacteroides varied greatly with individual isolates of *Klebsiella*. Similar variation has been found amongst isolates of *E. coli* (Jones and Gemmell, 1982; Wade *et al.*, 1983; Vel *et al.*, 1985).

Surface structures are responsible for the activation and deposition of serum complement (Stendahl and Edebo, 1972; Wilkinson *et al.*, 1979). A possible explanation for the variation in impairment of phagocytosis by bacteroides may be differences in the surface structures amongst bacterial

strains. Depletion of serum complement components by bacteroides has been proposed as a mechanism for impairment of phagocytosis (Tofte *et al.*, 1980; Jones and Gemmell, 1982; Wade *et al.*, 1983; Vel *et al.*, 1985). The amounts of serum required for opsonisation of bacteria vary; for example, *S. aureus* is efficiently phagocytosed after opsonisation with serum 5% v/v, whereas *Pr. mirabilis* requires 25% for equivalent phagocytosis (Jones and Gemmell, unpublished results). Thus depletion of complement components by bacteroides may leave insufficient complement for opsonisation of isolates with a high serum requirement but sufficient for opsonisation and phagocytosis of those with a low requirement.

Both the classical and alternative complement pathways can be activated by bacteroides (Joiner *et al.*, 1981; Bjornson *et al.*, 1983). It has been reported that certain *Bacteroides* spp. can enzymatically degrade C3 and C5 components of serum (Sundqvist *et al.*, 1984, 1985), but this would appear not to be pertinent to the present investigation because heat-killed bacteroides organisms are as efficient in inhibiting phagocytosis as live cells (Ingham *et al.*, 1977; Jones and Gemmell, 1982). Although the CPS of *B. fragilis* is an important virulence factor (Onderdonk *et al.*, 1977; Kasper *et al.*, 1979) our results show that it has little activity in the inhibition of phagocytosis. This supports the findings of Wade *et al.* (1983), but not those of Connolly *et al.* (1984) who reported that CPS inhibited phagocytosis. The LPS of *B. fragilis*, as purified by Sephacryl 300 chromatography (Kasper *et al.*, 1983), impaired phagocytosis in our experiments, an effect which may be associated with the known activation of the alternative complement pathway by LPS (Nygren *et al.*, 1979) but not by CPS (Okuda *et al.*, 1978). It is of interest that the capsule of *Str. pneumoniae* is associated with resistance to phagocytosis (Wood, 1960), but that the CPS of *Str. pneumoniae* does not inhibit phagocytosis *in vitro*, whereas mucopeptide does (Dhingra *et al.*, 1977).

In conclusion, our results indicate that the LPS but not the CPS of bacteroides cells is responsible for reducing the opsonic activity of serum complement. This reduction does not appear to be sufficient to affect the phagocytosis of most gram-positive bacteria, but is sufficient to impair the phagocytosis of gram-negative bacteria to various degrees (depending upon individual isolates). It is possible that by this means bacteroides may increase the pathogenic potential of another bacterium by inhibiting its phagocytosis *in vivo*. Further investigation is necessary to establish the validity of this hypothesis.

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