THE EFFECT OF CAPSULAR POLYSACCHARIDE AND LIPOPOLYSACCHARIDE OF \textit{Bacteroides fragilis} ON POLYMORPH FUNCTION AND SERUM KILLING

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SUMMARY. The determinant responsible for the ability of \textit{Bacteroides} spp. to inhibit polymorph phagocytic killing of aerobic organisms has not yet been identified. Therefore, the roles of lipopolysaccharide and capsular polysaccharide of \textit{B. fragilis} were investigated. Serum-resistant and serum-sensitive strains of \textit{Proteus mirabilis} were used to indicate inhibition of phagocytic killing and serum killing of aerobes. Whole organisms of \textit{B. fragilis}, purified lipopolysaccharide and capsular polysaccharide were added to an in-vitro phagocytosis system. Results showed that >10^7 bacteroides/ml inhibited both serum and phagocytic killing. Concentrations below 10^7/ml had little effect on either process. Purified capsular polysaccharide (10 or 100 µg/ml), either alone in the system or in combination with sub-inhibitory concentrations of \textit{B. fragilis} also markedly inhibited serum and phagocytic killing. Lipopolysaccharide (9 µg/ml) appeared relatively inert. \textit{B. ovatus}, reputedly non-capsulated, produced identical results to those obtained with \textit{B. fragilis}, but an encapsulated strain of \textit{Streptococcus pneumoniae} did not inhibit serum or phagocytic killing.

INTRODUCTION

The predominance of \textit{Bacteroides fragilis} in exudate and blood from patients with sepsis associated with gram-negative anaerobic bacteria suggests that this organism possesses unique virulence properties. Onderdonk \textit{et al.} (1976) demonstrated a synergistic relationship between \textit{B. fragilis} and \textit{Escherichia coli} in intra-abdominal infections in rats and Ingham \textit{et al.} (1977) showed that \textit{Bacteroides} spp. inhibit killing of aerobic organisms by polymorphonuclear leukocytes. When present in concentrations >10^7/ml, these anaerobes inhibit killing of themselves as well as that of concomitant facultative aerobes (Ingham \textit{et al.}, 1977). Jones and Gemmell (1982) and Tofte \textit{et al.} (1980) suggest that serum opsonins, essential for phagocytosis, are preferentially removed by the \textit{Bacteroides} spp., but Ingham \textit{et al.} (1981) demonstrated that the reaction of high concentrations of anaerobes with serum does not interfere with uptake by neutrophils but with the later stage of phagocytic killing.
The polysaccharide capsule of *B. fragilis* has been implicated in the promotion of abscess formation (Onderdonk et al., 1977). Having isolated and purified the capsular polysaccharide and lipopolysaccharide (LPS) from the outer membrane of one strain of *B. fragilis*, we have examined the role of these components of the cell wall in the inhibition of polymorph function.

**Materials and Methods**

**Bacterial strains.** *B. fragilis* strain ATCC23745 was grown anaerobically in PYG broth (Holdeman, Cato and Moore, 1977), washed twice in 0-15 m NaCl solution and resuspended to a concentration of 5 x 10^7 cfu/ml in Hank's Balanced Salt Solution (HBSS). *B. ovatus* strain ATCC8483 was grown under identical conditions and adjusted to the same concentration. Two strains of *P. mirabilis* were used as indicator organisms in these experiments. *P. mirabilis* strain SBH was a non-motile hospital isolate found to be sensitive to serum lysis. *P. mirabilis* strain 665 was a motile and serum-resistant organism kindly supplied by Dr H.R. Ingham, Regional Public Health Laboratory, Newcastle upon Tyne. An overnight broth culture of the organism was subcultured into nutrient broth, prewarmed to 37°C, and incubated for 3 h to an optical density corresponding to c. 2.5 x 10^8 cfu/ml. An encapsulated hospital strain of *Streptococcus pneumoniae* isolated from sputum was grown in Todd Hewitt broth to optical densities equivalent to 2.5 x 10^7/ml and 2.5 x 10^8/ml.

**Isolation and purification of capsular polysaccharide and lipopolysaccharide.** *B. fragilis* strain ATCC23745 was grown in 6-L volumes of culture and the outer membrane extracted by a method based on that of Kasper and Seiler (1975) and Kasper (1976). After thorough washing, the organisms were suspended in 0.01 M buffered EDTA and incubated at 60°C for 30 min. They were then subjected to two 15-s bursts in a Waring blender to remove the outer membrane, which was separated from whole cells by differential centrifugation. Loosely-bound lipid was removed and treatment with 0.5% sodium deoxycholate allowed separation of the LPS by G-100 sephadex column chromatography.

Void volume fractions containing capsular polysaccharide were pooled and attached protein was degraded by treatment with pronase at 37°C for 24 h. The pool was then concentrated and chromatography was performed on a sepharose 4-B column. Polysaccharide-containing fractions were collected in the void volume and separated protein, together with the pronase enzyme, were detected spectrophotometrically at 280 nm. Sugar content was estimated finally by the anthrone-sulphuric acid method (Dreywood, 1946) after a further treatment with pronase.

The presence of sodium deoxycholate allowed chromatographic separation of LPS from the capsular polysaccharide by temporarily disaggregating the LPS into fragments of mol. wt 12 000. An elution profile of known mol. wt standards run through the same G-100 column established the appropriate fractions containing LPS. These were pooled and the LPS was precipitated by 100% ethanol and washed extensively in HBSS. Both the purified polysaccharide capsular material and the LPS were reconstituted to 1000 µg/ml and 900 µg/ml respectively in HBSS containing gelatin 0·1%, these were divided into small portions and stored at −20°C.

**Phagocytosis**

**Preparation of leukocytes** Heparinised blood was collected from healthy volunteers and erythrocytes were separated with dextran from the leukocyte-rich plasma. White blood cells and any residual erythrocytes were deposited by gentle centrifugation at 578 g for 10 min and the cells were resuspended in ammonium chloride 0·87% to lyse the erythrocytes. Leukocytes were washed twice in HBSS with gelatin 0·1% and resuspended to give a concentration of c. 10^9/ml.

**Measurement of phagocytosis.** Control procedures in each experiment included monitoring the growth of *P. mirabilis* indicators from an inoculum of c. 5 x 10^7/ml in HBSS alone, in the presence of 10% pooled human serum, and with 10% serum plus polymorphonuclear leukocytes 8 x 10^9/ml. The latter constituted the uninhibited phagocytosis control system to which test inhibitors were added.

**Inhibitors of phagocytosis and killing of *P. mirabilis.*** In some systems, *B. fragilis* strain ATCC23745 was added in PYG broth to give a final concentration of c. 5 x 10^9/ml or 5 x 10^7/ml.
Other tests included a combination of $5 \times 10^6$ whole organisms of \textit{B. fragilis} with either purified capsular polysaccharide 100 \(\mu\)g/ml (0.1 ml of the stock preparation) or lipopolysaccharide 90 \(\mu\)g/ml (0.1 ml of the stock preparation), both extracted from \textit{B. fragilis} strain ATCC23745. These amounts were equivalent to those present in $10^9$ organisms (Onderdonk \textit{et al.}, 1977). Controls also included the addition of PYG broth without \textit{B. fragilis}. Capsular polysaccharide suspended in HBSS was tested alone at concentrations of 100 \(\mu\)g/ml and 10 \(\mu\)g/ml as an inhibitor of \textit{P. mirabilis} uptake and killing. Similar tests were also performed using an encapsulated strain of \textit{S. pneumoniae} or a reputedly non-capsulated strain of \textit{B. ovatus} (strain ATCC8483) in place of \textit{B. fragilis}.

Stopped tubes containing test and control systems of constant volume were incubated at 37°C for 4 h. The tubes were rotated manually every 30 min. Samples were removed initially and at hourly intervals. Suitable dilutions were made and 20-\(\mu\)l volumes were plated out in triplicate on nutrient-agar plates to monitor the growth of \textit{P. mirabilis}. Viable counts were made according to the method of Miles, Misra and Irwin (1938). Viable counts of \textit{B. fragilis} and \textit{B. ovatus} were made on blood-agar plates containing neomycin 100 \(\mu\)g/ml incubated anaerobically. \textit{S. pneumoniae} was plated on blood-agar plates and incubated in 5\% CO$_2$. After incubation for 4 h, sample tubes were centrifuged at 578 g for 3 min. The supernate was removed leaving the cellular deposit in c. 0.1 ml. A small volume of this resuspension was transferred to a glass slide and allowed to dry at room temperature, before being fixed in methanol and stained with a May Grunwald-Giemsa stain.

Preparation of specimens for electronmicroscopy. A buffer containing 0.1 M sodium cacodylate, 700 ppm ruthenium red and glutaraldehyde 2.5\% was added to the remainder of the cells after preparing the slide for light microscopy. The cells were left to fix in this buffer overnight. Deposits were washed twice in distilled water before being resuspended in uranyl acetate and left for 1 h. Samples were then dehydrated sequentially in 30\%, 50\%, 70\%, 90\%, and 100\% acetone and were finally embedded in resin.

\section*{RESULTS}

\textit{Serum-resistant P. mirabilis strain 665}

All results obtained with strain 665 as an indicator of phagocytosis are expressed as an average of three experiments. Controls showed approximately one log$_{10}$ increase in the viable count during the incubation period in HBSS with or without 10\% serum. With the addition of polymorphs to the system, numbers of \textit{P. mirabilis} had dropped by 0.5 log$_{10}$ after 2 h, after which an increase in the viable count was noted (fig. 1). The addition of PYG broth to this system had no effect on the numbers of organisms. Whole \textit{B. fragilis} organisms added to a control phagocytosis system at a concentration of $> 10^7$/ml allowed numbers of \textit{P. mirabilis} to increase to those in the growth control (fig. 1). At a concentration of $5 \times 10^6$/ml, \textit{B. fragilis} inhibited phagocytosis to a smaller extent, resulting in a 0.25 log$_{10}$ reduction after 2 h. The addition of LPS to this lower concentration of \textit{B. fragilis} produced no significant further inhibition of phagocytosis of \textit{P. mirabilis}. However, marked inhibition resulted from the inclusion of capsular polysaccharide in combination with the lower concentration of \textit{B. fragilis} (fig. 1).

\textit{Serum-sensitive P. mirabilis strain SBH}

These results are expressed as an average of five experiments, and show a wider variation between test systems. Control growth of the indicator strain again increased by approximately one log$_{10}$ during the observed incubation period (fig. 2). Sensitivity to serum was demonstrated by the addition of 10\% serum, after which viable counts fell by an average of 2.5 log$_{10}$ during the first 2-h period, but during the remainder of the
4-h incubation period, indicator organisms were found to multiply. Polymorphs in the presence of 10% serum produced a similar reduction and it was only after the first 2 h that a further reduction in cell numbers could be attributed to phagocytosis (fig. 2). PYG broth, added to the system, had no effect on killing of the serum-sensitive indicator strain. The increase in the bacterial cell count demonstrated in the third and fourth hours of incubation in fig. 2 was shown to be due to limiting heat-labile serum components because heat-inactivated serum had no effect and the addition of a further 0.1 ml of fresh serum significantly reduced the cell numbers (fig. 3). Pre-absorption of serum for 2 h at 37°C with $5 \times 10^7$ cfu/ml of washed *B. fragilis* whole organisms prevented killing of *P. mirabilis*.

The addition of *B. fragilis* in concentrations of $<10^7$/ml caused little inhibition of killing when compared with the phagocytosis control system (fig. 4). At a
concentration of $5 \times 10^7$/ml, *B. fragilis* considerably inhibited killing of the indicator strain, numbers of which remained similar to initial counts throughout each experiment. The effect of different concentrations of *B. fragilis* whole cells was, therefore, more marked when *P. mirabilis* strain SBH was substituted for the serum-resistant strain 665.

Capsular polysaccharide or LPS was added to the control phagocytosis system in combination with low concentrations of *B. fragilis* (fig. 5). LPS made little difference to the viable counts of *P. mirabilis* strain SBH compared with those when *B. fragilis* alone was added. Capsular polysaccharide, however, markedly inhibited serum killing in the system (fig. 5A). After incubation for 4 h, there were approximately ten
times as many viable *P. mirabilis* organisms as were present when LPS and sub-inhibitory concentrations of *B. fragilis* were incubated in the system, demonstrating a further inhibition of phagocytosis (fig. 5B). Tests in which 10 or 100 μg/ml of capsular material was added without *B. fragilis* whole organisms indicate a similar degree of inhibition of serum killing and phagocytosis (fig. 6).
Average initial count $5.7 \times 10^7$/ml

Fig. 4. — The effect of whole organisms of *B. fragilis* on the viable counts of a serum-sensitive strain of *P. mirabilis*:
- - - control growth of indicator *P. mirabilis* strain SBH;
- - - control phagocytosis;
- - - phagocytosis with low numbers of *B. fragilis* (< $10^7$/ml);
- - - phagocytosis with high numbers of *B. fragilis* (> $10^7$/ml).

Identical tests were performed with intact cells of *S. pneumoniae* or *B. ovatus* at similar concentrations in place of *B. fragilis* strain ATCC 23745. *S. pneumoniae* did not inhibit killing of either strain of *P. mirabilis*, even when present in concentrations up to $4.0 \times 10^7$/ml. *B. fragilis* capsular material, when added to the test system with $3.5 \times 10^6$ *S. pneumoniae* organisms/ml produced results identical to those from experiments performed with whole *B. fragilis* organisms. The inhibitory effect of the capsule preparation, therefore, appeared to be independent of whole organisms. Intact cells of *B. ovatus*, however, behaved similarly to *B. fragilis*.

**Microscopical observations**

Examination of polymorphs made by light microscopy after incubation for 4 h,
Fig. 5.—The effect of *B. fragilis* and its cellular components on serum and phagocytic killing of a serum-sensitive strain of *P. mirabilis*: ■—■ control growth of *P. mirabilis* strain SBH; ○—○ control + 0.1 ml of serum (10%) only; □—□ control phagocytosis; △—△ phagocytosis with low numbers of *B. fragilis* (<10⁷/ml); ▲—▲ phagocytosis with low numbers of *B. fragilis* (<10⁷/ml) + LPS; ▼—▼ phagocytosis with low numbers of *B. fragilis* (<10⁷/ml) + capsule; ◇—◇ phagocytosis with high number *B. fragilis* (>10⁷/ml).

Fig. 6.—The effect of capsular polysaccharide alone on the killing of a serum-sensitive strain of *P. mirabilis*: ■—■ control growth of *P. mirabilis* strain SBH; ■—■ control phagocytosis; △—△ phagocytosis with low numbers of *B. fragilis* (<10⁷/ml) + capsule 100 μg/ml; ○—○ phagocytosis with capsule 100 μg/ml; □—□ phagocytosis with capsule 10 μg/ml.
showed that neutrophils in all test systems contained many organisms, but there were marked variations in the numbers of extracellular organisms remaining.

Bacteria were intimately associated with the polymorphs in all control systems, as well as those containing low numbers of *B. fragilis* with or without the addition of LPS (fig. 7). In tests in which killing was inhibited by either high concentrations of anaerobes or by low concentrations plus capsular material, large numbers of bacteria
FIG. 9.—Electronmicrograph of a polymorph in the presence of *P. mirabilis* $5 \times 10^7$/ml and *B. fragilis* $5 \times 10^6$/ml; arrows indicate degraded organism in phagosome. → 1 μm.

FIG. 10.—Electronmicrograph of polymorph in the presence of *P. mirabilis* $5 \times 10^7$/ml, *B. fragilis* $5 \times 10^6$/ml and capsular polysaccharide 100 μg; arrows indicate whole organisms in phagosomes. → 1 μm.
were again seen within the cytoplasm, but many organisms extracellular to and not associated with the polymorphs were always visible (fig. 8).

Examination of phagocytes by electronmicroscopy indicated that there was less degeneration of intracellular vacuolated organisms in the inhibited systems. Control tests and those with low numbers of *B. fragilis* (fig. 9) typically showed polymorphs with empty vacuoles and some that contained partially digested bacteria. Fig. 10 shows a neutrophil incubated with a low concentration of *B. fragilis* plus capsular material. Bacteria within the phagosomes appear not to be successfully degraded.

**DISCUSSION**

This series of experiments corroborates work by Ingham *et al.* (1981) by demonstrating inhibition of killing of aerobic bacteria in an in-vitro phagocytosis model by high concentrations of *B. fragilis*. However, we have now identified the capsular polysaccharide as the determinant responsible to some degree for this phenomenon; inhibition of killing is mediated by capsular material either with or without the addition of anaerobic organisms. The lipopolysaccharide appears to have little effect. Our results also indicate that this inhibition is mediated by a complex series of interactions, some specific and some probably non-specific.

Our experiments with a serum-resistant indicator strain of *P. mirabilis* (strain 665) demonstrated inhibition of phagocytosis by high concentrations of *B. fragilis* whole organisms alone and by low concentrations together with capsular material. However, this organism repeatedly showed only a low degree of killing by phagocytes, even in control systems, and reductions in numbers were only observed during the first 2-h incubation period. This apparent low degree of killing by phagocytes may be, in part, related to the high input of organisms. Because it is resistant to serum, we postulated that *P. mirabilis* strain 665 may also be partially resistant to opsonisation. Joiner, Hawiger and Gelfand (1981) reported that serum-resistant anaerobes are less effective at activating the alternative complement pathway. This may also apply to serum-resistant aerobes, thereby reducing opsonisation. Other workers have found that opsonisation of a serum-resistant strain of *P. mirabilis* is mediated almost entirely via the classical complement pathway (Jones and Gemmell, 1982). The reduction in phagocytic killing by either the capsule or whole organisms of *B. fragilis* may be due to competitive depletion of serum complement components. However, inhibition still occurred after the addition of a further 10% serum after initial incubation for 2 h, which suggests that the inhibition was not due to limiting serum factors (results not shown). These phenomena have, therefore, yet to be explained.

Studies with the serum-sensitive strain of *P. mirabilis* (strain SBH) as the indicator organism showed that killing by 10% serum in this system is also restricted to the first 2 h of the incubation period. The addition of a further 10% of fresh serum at this time lead to a rapid drop in viable counts, indicating a limited supply of heat labile serum components, possibly complement (fig. 3). Fig. 5 might, therefore, be analysed in two parts to demonstrate the means by which inhibition of killing by anaerobes is mediated. During the first 2-h period in this model, when viable counts in the control phagocytosis model closely followed those obtained in the presence of 10% serum alone, killing of bacteria might be attributed solely to serum components. No inhibition in serum killing of strain SBH was, therefore, demonstrated by either low
numbers of *B. fragilis* alone or in combination with LPS. Marked inhibition in complement-mediated lysis was apparent in tests including capsular material with or without low numbers of anaerobes, and killing was prevented to an even greater degree by high concentrations of *B. fragilis* alone (fig. 5A).

After the initial 2-h period, when serum factors were found to be limiting (fig. 5B), phagocytosis by polymorphs was considered responsible for any further reductions in indicator numbers. Counts of *P. mirabilis* in inhibited test systems tended to stabilise. The wide variations in viable counts from test to test had been established by serum lysis, and during the last 2 h of the experiment there was minimal further inhibition of phagocytosis demonstrated by high concentrations of *B. fragilis* or by the capsule in the presence of sub-inhibitory levels of the anaerobe. Capsular material alone, at concentrations of either 100 μg/ml or 10 μg/ml, inhibited the killing of *P. mirabilis* strain SBH to the same extent as that demonstrated by a combination of capsule 100 μg/ml plus low concentrations of whole *B. fragilis* (fig. 6). Similar results were also obtained with the serum-resistant strain 665 (results not shown).

With both serum-sensitive and serum-resistant indicator strains, not only does the presence of whole anaerobic organisms appear not to be essential, but inhibition was also apparent without the inclusion of a reducing agent. Previous work (Ingham et al., 1977; Jones and Gemmell, 1982) demonstrates that inhibition of phagocytosis by anaerobes depends upon a low redox potential in the system. Inhibition of serum killing by anaerobes may be due to a non-specific depletion of complement factors that would normally be responsible for the bacterial lysis of serum-sensitive strains, triggered either by the classical complement pathway or by the alternative complement pathway. Namavar et al. (1983) have demonstrated a heat labile component of *B. gingivalis*, also present in culture supernates, that is responsible for the inactivation of complement. Tofte et al. (1980) suggest from their results that competition for serum opsonins is responsible for the inhibition of killing of *E. coli* in a similar model.

Inhibition of killing by phagocytes would seem to be a more specific process. Jones and Gemmell (1982) have reported that only a proportion of *Proteus* organisms, and even fewer strains of *E. coli* were susceptible to inhibition by anaerobes. This inhibition of killing may, however, be mediated by reduced opsonisation of certain strains in the presence of anaerobes. It is also possible that immune complexes, activated by the anaerobic organisms, block specific receptor sites on the polymorphonuclear leukocyte membrane, thereby impairing the capacity of the phagocyte to kill ingested bacteria. Other workers have suggested that cytophilic IgG antibodies act as receptors for bacteria-antibody-complement complexes on human polymorphs (Bjornson and Michael, 1974) and competition for these sites by *Bacteroides* organisms may result in apparent inhibition of polymorph function.

The results of light and electron microscopy showed that, in all cases, polymorphs were able to engulf bacteria. In inhibited systems, however, light-microscopy preparations showed that the presence of *B. fragilis* capsular polysaccharide prevented complete clearance of extra-cellular organisms compared with controls. Electronmicrographs indicate a reduction in degeneration of bacteria within phagosomes in inhibited systems. These observations accord with those reported by Ingham et al. (1981), but also show that it is the capsular polysaccharide that was responsible. The capsule of *S. pneumoniae*, which consists of a different array of sugar molecules, did not inhibit the phagocytosis and killing of aerobic bacteria. *B. ovatus*, reputedly
non-capsulated, behaved similarly to \textit{B. fragilis}; however, we have as yet to verify that this organism does not possess a capsule. Okuda and Takazoe (1973) report that a capsulated strain of \textit{B. melaninogenicus} inhibits the opsonisation and killing of \textit{Staphylococcus aureus}.

A heat stable component of \textit{B. gingivalis} with a mol. wt < 3500 has also been implicated as being responsible for inhibition of polymorph killing (Namavar \textit{et al.} 1983). It is not known, however, whether this component could be fragments of capsular materials. In our experience, the LPS of \textit{B. fragilis} appears to take little part in the inhibition. The extent of the role played specifically by the polysaccharide capsule remains unclear, although these experiments have demonstrated that it is responsible for a significant amount of inhibition.

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\textbf{REFERENCES}


