PHAGOCYTIC AND SERUM KILLING OF CAPSULATE AND NON-CAPSULATE *BACTEROIDES FRAGILIS*

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SUMMARY. The relative susceptibilities of capsulate and non-capsulate variants of *Bacteroides fragilis* to serum and phagocytic killing were investigated. The capsule of *B. fragilis* did not confer resistance to serum killing. Phagocytic killing of non-capsulate *B. fragilis* occurred at bacterial concentrations of 1×10^6 and 1×10^7 cfu/ml. Capsulate *B. fragilis* organisms were also phagocytosed and killed at a concentration of 1×10^6 cfu/ml, but phagocytosis and killing were impaired at a concentration of 1×10^7 cfu/ml.

INTRODUCTION

The susceptibility of pathogenic bacteria to phagocytosis and killing by polymorphonuclear leukocytes (PMNL) and macrophages is of major importance in determining the outcome of the host-pathogen interaction. Casciato *et al.* (1975) and Bjornson, Altemeier and Bjornson (1976) demonstrated phagocytosis and killing of *B. fragilis* by human leukocytes *in vitro*. Phagocytosis of *B. fragilis* in the presence of serum occurred in aerobic and anaerobic conditions. Ingham *et al.* (1977 and 1981) investigated the effect of *Bacteroides* spp. on the phagocytic killing of facultative species. Killing of *B. fragilis* and *Proteus mirabilis* in mixtures *in vitro* was impaired when the concentration of *B. fragilis* was greater than 1×10^7 cfu/ml in the phagocytic system. Tofte *et al.* (1980) and Jones and Gemmel (1982) reported that both phagocytic uptake and killing of facultative species were impaired at high concentrations of bacteroides. None of these studies defined the ratio of capsulate to non-capsulate bacteroides used in the phagocytic system.

Considerable emphasis has been placed on the importance of the polysaccharide capsule of *B. fragilis* ATCC23745 as a virulence determinant (Kasper *et al.*, 1977; Onderdonk *et al.*, 1977). Kasper *et al.* (1980) reported that *in-vivo* passage of this strain enhanced the production of the capsule but serial subculture *in vitro* reduced the proportion of capsulate bacteria. Simon *et al.* (1982) compared phagocytosis of these strains and successfully demonstrated a reduction in uptake of the *in-vivo* passaged variant.

Recently, Percoll density gradient centrifugation was successfully used to separate capsulate and non-capsulate variants of *B. fragilis* (Patrick and Reid, 1983). In the

present study, the susceptibilities of these variants to phagocytosis, intracellular killing and the bactericidal action of serum were investigated.

MATERIALS AND METHODS

Bacterial strains. *B. fragilis* NCTC9343 was supplied by the Department of Bacteriology, University of Edinburgh Medical School; *B. fragilis* ATCC23745 by the American Type Culture Collection, Rockville, MD; and *B. fragilis* NCTC10584 was a departmental stock culture.

Bacterial culture methods. Bacteria grown in defined broth (Van Tassell and Wilkins, 1978) were used in all experiments except where otherwise stated. Cultures were incubated at 37°C in an atmosphere of H₂ 90% and CO₂ 10% in anaerobic jars. The standard anaerobic procedures of Collee *et al.* (1972) were used.

Stock cultures, grown in defined broth, were snap frozen in liquid N₂ and 1-ml portions stored at -70°C or in liquid N₂. Capsulate bacteria, recovered from the 0-20% interface of a Percoll (Pharmacia, London) discontinuous density gradient (Patrick and Reid, 1983) and grown to late log-phase in defined broth, provided a capsulate stock which was stored as above.

Bacteria were also cultured in basal broth (Deacon, Duerden and Holbrook, 1978). Total viable counts were determined by seeding lysed human blood agar with six 20- μ l drops from a standard 20-gauge steel cannula (Astell, London).

Preparation of bacteria. Bacteria were grown to late log-phase in defined broth medium. A 3-ml volume of a capsulate culture preparation was layered on to a 20% Percoll cushion. After centrifugation at 2600 *g* for 20 min a homogeneous suspension of capsulate bacteria was recovered from the 0-20% interface, suspended in quarter strength Ringer solution (Oxoid, Basingstoke, Hampshire) and centrifuged at 10 000 *g* for 30 min at 4°C. Non-capsulate bacteria obtained from the 60-80% interface of a Percoll density gradient were also washed once in Ringer solution. Standard bacterial suspensions at concentrations of 1×10^7 or 1×10^8 cfu/ml were prepared in Hanks' balanced salt solution (HBSS) containing gelatin 0.01% w/v (GEL-HBSS).

Preparation of phagocytes. Ficoll isopaque gradients were used to separate leukocytes from 25 ml of heparinised human blood. Leukocytes were washed twice in HBSS, and pure PMNL preparations were obtained by centrifugation of the leukocyte suspension on a preformed Percoll sucrose density gradient.

A stock solution of Percoll, isosmotic with physiological saline, was prepared by diluting nine volumes of Percoll in one volume of tenfold concentrated HBSS. The pH of this solution was adjusted to 7.2 with 1M HCl, and a 65% Percoll solution was then prepared by the addition of 0.25 M sucrose. Suitable volumes were centrifuged at 21 000 *g* for 20 min to produce gradients. Leukocytes suspended in 1 ml of HBSS were applied to the top of a gradient which was then centrifuged at 600 *g* for 15 min. A sharp band of PMNL formed in the lower part of the gradient just above the band of erythrocytes; the cells were removed carefully with a Pasteur pipette, washed in HBSS and resuspended to a final concentration of 1×10^7 cells/ml.

Serum. Normal human group AB serum was obtained from the Northern Ireland Blood Transfusion Service; 2-ml volumes of sera were stored at -20°C. The total haemolytic complement value (CH50) was checked routinely, and only sera with normal CH50 values were used.

Phagocytosis. Sterile siliconised screw-capped glass tubes (98 $\times$ 16 mm) containing a phagocytic system composed of 0.3 ml of PMNL suspension, 0.3 ml of AB serum, 0.3 ml of appropriate bacterial suspension and 2.1 ml of GEL-HBSS were incubated at 37°C in aerobic conditions with end-over-end rotation. GEL-HBSS was used to bring the final volume to 3.0 ml. Final PMNL and serum concentrations were 1×10^6 cells/ml and 10% respectively, and final bacterial concentrations were either 1×10^7 or 1×10^6 cfu/ml. Appropriate control mixtures were included. All experiments were performed in duplicate and repeated at least twice. Phagocytic killing at 0 and 120 min was measured by diluting 0.1 ml of the reaction mixture into 9.9 ml of distilled water containing bovine serum albumin 0.01%. After 5 min, further tenfold dilutions were performed in Ringer solution and total viable counts were determined. Phagocytic uptake after 0, 30, 60 and 120 min was measured by diluting 0.5 ml of

the reaction mixture in 1.5 ml of chilled GEL-HBSS to stop phagocytosis. The suspension was centrifuged at 110 *g* for 4 min at 4°C and the viable bacteria in the supernate were counted.

Light microscopy. Capsules were detected by light microscopy with India ink or eosin-carbol fuchsin negative staining methods (Cruickshank, Duguid and Swain, 1965).

PMNL were recovered from the phagocytic system by centrifugation at 210 *g* for 4 min and resuspended in HBSS containing 50% heat-inactivated normal human serum. Smears were prepared and stained by the M & D Diff-Quick® staining set (Merz and Dade AG, Switzerland). Preparations were examined with a × 100 oil immersion objective and the number of bacteria/50 PMNL counted.

Electronmicroscopy. PMNL for electronmicroscopy were recovered from the phagocytic system by centrifugation at 210 *g* for 4 min, resuspended in 0.1 M Sorensen's phosphate buffer pH 7.3 (SPB) containing glutaraldehyde 2.5% v/v and left at 4°C for 1 h. Cells were washed twice in SPB, resuspended in SPB containing osmic acid 1% v/v, left at 22°C for 1 h and then washed twice in SPB, dehydrated in graded ethyl alcohols and embedded in Spurr resin. Sections for electronmicroscopy were stained with uranyl acetate and lead citrate and viewed in a Philips 301 transmission electronmicroscope.

RESULTS

Bactericidal activity of human serum

Capsulate and non-capsulate variants of two of the three test strains (NCTC9343 and NCTC10584) showed no reduction in viable counts after incubation for 2 h in 10% normal human serum (table I). The non-capsulate variant of *B. fragilis* ATCC23745 was also resistant to serum killing, but the capsulate variant of this strain was susceptible to the bactericidal action of 10% normal human serum (table I). Control experiments indicated that GEL-HBSS was not toxic to capsulate *B. fragilis* ATCC23745.

TABLE I

Susceptibility of capsulate and non-capsulate variants of B. fragilis to serum and phagocytic killing

Organism	Ratio of bacteria to PMNL	Total viable count* (cfu/ml) after	
		0 min	120 min
<i>B. fragilis</i> NCTC9343 (NC)	10:1	$1.5 \pm 0.2 \times 10^7$	$3.5 \pm 2.8 \times 10^6$
	1:1	$1.4 \pm 0.2 \times 10^6$	$1.0 \pm 0.1 \times 10^5$
	Serum control	$1.3 \pm 0.1 \times 10^6$	$1.5 \pm 0.2 \times 10^6$
<i>B. fragilis</i> NCTC9343 (C)	10:1	$1.1 \pm 0.2 \times 10^7$	$1.1 \pm 0.1 \times 10^7$
	1:1	$1.1 \pm 0.1 \times 10^6$	$5.2 \pm 0.9 \times 10^4$
	Serum control	$1.2 \pm 0.1 \times 10^6$	$1.0 \pm 0.1 \times 10^6$
<i>B. fragilis</i> NCTC10584 (NC)	10:1	$1.7 \pm 0.2 \times 10^7$	$4.6 \pm 0.9 \times 10^6$
	1:1	$2.1 \pm 0.1 \times 10^6$	$0.9 \pm 0.1 \times 10^5$
	Serum control	$1.9 \pm 0.1 \times 10^6$	$2.1 \pm 0.2 \times 10^6$
<i>B. fragilis</i> NCTC10584 (C)	10:1	$1.9 \pm 0.2 \times 10^7$	$1.8 \pm 0.3 \times 10^7$
	1:1	$1.9 \pm 0.1 \times 10^6$	$5.5 \pm 0.3 \times 10^5$
	Serum control	$1.8 \pm 0.1 \times 10^6$	$1.9 \pm 0.2 \times 10^6$
<i>B. fragilis</i> ATCC23745 (NC)	10:1	$2.9 \pm 0.3 \times 10^7$	$3.0 \pm 0.2 \times 10^6$
	1:1	$2.2 \pm 0.4 \times 10^6$	$3.5 \pm 1.0 \times 10^4$
	Serum control	$2.3 \pm 0.2 \times 10^6$	$2.5 \pm 0.2 \times 10^6$
<i>B. fragilis</i> ATCC23745 (C)	10:1	$0.7 \pm 0.1 \times 10^7$	$1.0 \pm 0.9 \times 10^5$
	1:1	$1.2 \pm 0.1 \times 10^6$	$1.5 \pm 0.4 \times 10^4$
	Serum control	$1.1 \pm 0.1 \times 10^6$	$2.2 \pm 0.5 \times 10^4$

PMNL = polymorphonuclear leukocyte; C = capsulate; NC = non-capsulate.

* = mean ± SE.

FIG. 1.—A comparison of killing of capsulate (C) and non-capsulate (NC) variants of *B. fragilis* NCTC9343 and ATCC23745 by PMNL and serum. A—bacteria to PMNL ratio = 10:1; B—bacteria to PMNL ratio = 1:1; C—bacteria without PMNL; all mixtures contained serum.

Killing by polymorphonuclear leukocytes

The results in table I and fig. 1 indicate that phagocytic killing of the three non-capsulate strains of *B. fragilis* grown in defined broth occurred at bacterial concentrations of 1×10^6 and 1×10^7 cfu/ml (i.e., bacteria to PMNL ratios of 1:1 and 10:1 respectively). Non-capsulate *B. fragilis* NCTC9343 grown in basal broth was also susceptible to phagocytic killing at both bacterial concentrations (table II).

The capsulate variants of *B. fragilis* NCTC9343 and NCTC10584 were killed by

TABLE II

Susceptibility of capsulate and non-capsulate variants of B. fragilis NCTC9343 grown in basal broth to phagocytic killing by PMNL

State of test organism	Ratio of bacteria to PMNL	Total viable count* (cfu/ml) after		Percentage number of bacteria killed
		0 min	120 min	
Capsulate	10:1	$3.2 \pm 0.2 \times 10^7$	$9.1 \pm 0.5 \times 10^6$	72
	1:1	$6.1 \pm 0.4 \times 10^6$	$3.6 \pm 0.2 \times 10^5$	92
	Serum control	$6.7 \pm 0.7 \times 10^6$	$7.0 \pm 0.5 \times 10^6$	0
Non-capsulate	10:1	$2.9 \pm 0.1 \times 10^7$	$7.4 \pm 0.3 \times 10^6$	75
	1:1	$8.5 \pm 0.5 \times 10^6$	$5.2 \pm 0.4 \times 10^5$	94
	Serum control	$6.7 \pm 0.2 \times 10^6$	$6.6 \pm 0.3 \times 10^6$	0

PMNL = Polymorphonuclear leukocyte.
* = Mean $\pm$ SE.

PMNL at bacterial concentrations of 1×10^6 cfu/ml. However, when the bacterial concentration was increased to 1×10^7 cfu/ml, phagocytic killing of these strains was inhibited (table I and fig. 1). A high proportion ($> 70\%$) of capsule *B. fragilis* NCTC9343 grown in basal broth was susceptible to phagocytic killing at both bacterial concentrations (table II). Killing of capsule *B. fragilis* ATCC23745 occurred at both bacterial concentrations; however, this strain was sensitive to the bactericidal action of normal human serum (table I and fig. 1).

Phagocytosis and killing by polymorphonuclear leukocytes

The rates of phagocytosis and killing of capsule and non-capsulate variants of *B. fragilis* NCTC9343 were compared. At bacterial concentrations of 1×10^7 cfu/ml, effective phagocytosis and killing of non-capsulate *B. fragilis* NCTC9343 was evident

FIG. 2.—Phagocytosis and killing of non-capsulate *B. fragilis* NCTC9343 during incubation with 10% serum and PMNL. Phagocytic uptake (●) and killing (○) at a bacterial concentration of 1×10^7 cfu/ml; phagocytic uptake (◆) and killing (◇) at a bacterial concentration of 1×10^6 cfu/ml. Controls for uptake (....) and killing (---) were bacteria incubated in serum without PMNL. Results from experiments performed on 3 days were combined and expressed as mean $\pm$ SE.

after incubation for 60 min (fig. 2). However, phagocytosis of capsulate *B. fragilis* NCTC9343 was impaired after incubation for 60 min and no killing occurred (fig. 3). At bacterial concentrations of 1×10^6 cfu/ml, phagocytosis and killing were similar with each variant (figs 2 and 3).

Microscopy

Phagocyte-associated bacteria were difficult to detect at a low bacteria to PMNL ratio (1:1) by light microscopy with differential staining. Smears prepared from the phagocytic system containing non-capsulate *B. fragilis* NCTC9343 at a high bacteria to PMNL ratio (10:1) showed numerous PMNL-associated bacteria. When *B. fragilis* NCTC9343 was grown in defined broth the mean number of non-capsulate bacteria per PMNL was 11; growth in basal broth increased the number of

FIG. 3.—Phagocytosis and killing of capsulate *B. fragilis* NCTC9343 during incubation with 10% serum and PMNL. Phagocytic uptake (▲) and killing (△) at a bacterial concentration of 1×10^7 cfu/ml; phagocytic uptake (■) and killing (□) at a bacterial concentration of 1×10^6 cfu/ml. Controls for uptake (●) and killing (◻) were bacteria incubated in serum without PMNL. Results from experiments performed on 3 days were combined and expressed as mean $\pm$ SE.

FIG. 4.—*B. fragilis* NCTC9343 associated with PMNL after incubation for 60 min in 10% serum at 37°C. DB = defined broth; BB = basal broth; C = capsulate; NC = non-capsulate.

phagocyte-associated bacteria to 24 (fig. 4). Electronmicroscopy confirmed the intracellular location of these organisms and demonstrated that bacteria were surrounded by phagosomal membranes (fig. 5A).

Few PMNL-associated capsulate *B. fragilis* NCTC9343 were observed at a bacteria to PMNL ratio of 10:1 (figs 4 and 5B). Electronmicroscopy revealed that some capsulate organisms were associated with the outside of the PMNL membrane (fig. 6). However, high numbers of capsulate *B. fragilis* NCTC9343 grown in basal broth were observed within phagocytes at a bacteria to PMNL ratio of 10:1 (fig. 4).

DISCUSSION

These findings indicate that the capsule of *B. fragilis* is not responsible for conferring resistance to serum killing.

Non-capsulate *B. fragilis* ATCC23745 grown in defined broth was resistant to serum killing, but the capsulate variant of this strain was susceptible to the bactericidal action of 10% normal human serum. The phagocytic system used in our study contained 10% normal human serum and viable counting methods were used to detect phagocytosis and killing. Phagocytosis of serum-sensitive strains could not be investigated because phagocytic killing could not be distinguished from serum killing.

Optimum phagocytic killing of both capsulate and non-capsulate *B. fragilis* occurred at bacterial concentrations of 1×10^6 cfu/ml, but differences in the susceptibility to phagocytic killing were observed at bacterial concentrations of 1×10^7 cfu/ml. The results of our uptake and electronmicroscopy experiments suggest that ingestion of capsulate *B. fragilis* was impaired at bacterial concentrations of 1×10^7 cfu/ml. The surface of a particle must be completely coated with opsonin for successful phagocytosis (Griffin *et al.*, 1975). Incomplete opsonisation of capsulate *B. fragilis* at high bacterial concentrations with limited amounts of an essential opsonin (e.g., IgM) could result in the impaired phagocytosis observed in our experiments.

FIG. 5.—Electronmicrographs of PMNL after incubation for 60 min with (A) non-capsulate and (B) capsulate *B. fragilis* NCTC9343 in the presence of 10% serum. Scale bar = 1 μ m.

FIG. 6.—Electronmicrograph of capsulate *B. fragilis* NCTC9343 associated with the membrane of a PMNL after incubation for 60 min in the presence of 10% serum. Scale bar = 0.5 μ m.

The capsulate variant of *B. fragilis* NCTC9343 grown in basal broth produced small capsules and was successfully phagocytosed at bacterial concentrations of 1×10^6 and 1×10^7 cfu/ml; this suggests that the quantity of capsular material is important in impairing phagocytosis. A reduction in the quantity of opsonin required to completely cover the surface of organisms grown in basal broth could account for this observation.

Our results suggest that capsulate and non-capsulate *B. fragilis* have different opsonic requirements; this might explain the difference between our results and those obtained by other workers. Bjornson and Bjornson (1978) and Bjornson, Bjornson and Kitko (1980) demonstrated that the alternative complement pathway and IgM were essential for phagocytosis; however, Tofte *et al.* (1980) detected opsonisation at a reduced rate in the absence of the classical complement pathway and antibody. The proportions of capsulate and non-capsulate bacteria used in their studies were not defined. The inconsistency in these results might be explained if phagocytosis experiments were repeated with homogeneous suspensions of capsulate and non-capsulate *B. fragilis*.

Ingham *et al.* (1977 and 1981) observed that phagocytic killing was impaired when *P. mirabilis* and high concentrations of *B. fragilis* (1×10^7 cfu/ml) were mixed with serum and PMNL *in vitro*. *B. fragilis* cells with large capsules might be responsible for the inhibitory effect. Horwitz and Silverstein (1980) reported that anti-capsular antibody and complement were essential for opsonisation and phagocytosis of capsulate *Escherichia coli*. Opsonisation of capsulate *B. fragilis* might proceed in a

similar manner. In the phagocytic system of Ingham *et al.* (1977), the quantity of specific anti-capsular antibody may be insufficient to completely opsonise capsulate *B. fragilis* at a concentration of 1×10^7 cfu/ml because normal human serum contains low levels of bacteroides-specific antibodies (Hofstad, 1979). If these partially opsonised capsulate cells induced premature degranulation of PMNL lysosomes as a result of attachment to the PMNL membrane in the absence of phagocytosis, and if opsonisation of *P. mirabilis* by the alternative complement pathway proceeded at a slower rate (Tofte *et al.*, 1980), the bactericidal mechanisms of the PMNL would be depleted before phagocytosis of *P. mirabilis* occurred. This could explain the protection from phagocytic killing. Further studies are needed to investigate this hypothesis.

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