IMPAIRMENT BY *BACTEROIDES* SPECIES OF OPSONISATION AND PHAGOCYTOSIS OF ENTEROBACTERIA

G. R. JONES AND C. G. GEMMELL

*Department of Bacteriology, Medical School,
University of Glasgow Royal Infirmary, Glasgow G4 0SF*

SUMMARY. The ability of human polymorphonuclear leucocytes to phagocytose and kill *Proteus mirabilis* was impaired *in vitro* when the human serum, used to opsonise the target bacteria, was pretreated with cultures of various *Bacteroides* species. Live and dead, either heat-killed or clindamycin-treated, bacteroides cells elicited the same phenomenon. When bacteroides-treated serum was used to opsonise different *Proteus* species, the subsequent uptake of all strains by polymorphonuclear leucocytes was inhibited, whereas bacteroides-treated serum inhibited the uptake of some but not all of the test strains of *Escherichia coli*. The opsonic activity of untreated human serum was reduced when the classical complement pathway was inhibited by ethyleneglycol-bis-(β -aminoethyl ether)N,N'-tetra-acetic acid (EGTA); subsequent treatment with bacteroides did not further reduce the opsonic activity of the serum for *P. mirabilis*.

INTRODUCTION

The susceptibility of pathogenic micro-organisms to phagocytosis by polymorphonuclear leucocytes (PMNL) and macrophages is important in determining the outcome of the host-parasite relationship. Interference with normal phagocytic processes by bacterial products can often determine the severity and extent of infection. Individual bacteria can elaborate substances that interfere with phagocytosis (for review see Quie, Giebink and Peterson, 1981).

However, the effect of cells of one bacterial species on the phagocytosis of another was not studied experimentally until Ingham *et al.* (1977) reported that some *Bacteroides* species are capable of inhibiting the phagocytic killing of various bacteria by human PMNL *in vitro*. This inhibition depended on a low Eh and required at least 10^7 anaerobes/ml in the phagocytic system. Facultative anaerobes such as *Escherichia coli* did not impair phagocytosis of *Proteus mirabilis*.

Tofte *et al.* (1980) found that the uptake of *E. coli* by PMNL was markedly inhibited in the presence of *Bacteroides fragilis* or *B. melaninogenicus*, but conversely *E. coli* did not significantly affect the uptake of either anaerobe. These workers suggested

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that the test bacteroides were either more efficiently or more rapidly opsonised than *E. coli* and therefore deprived *E. coli* of serum opsonins needed for efficient phagocytosis by PMNL.

The present study was undertaken to determine the extent of the inhibition of phagocytosis by bacteroides. Some of the factors involved were investigated by measuring the uptake of radiolabelled bacteria and the killing of *P. mirabilis* and *E. coli* by PMNL in the presence or absence of bacteroides organisms.

MATERIALS AND METHODS

Bacterial strains. *B. melaninogenicus* strain no. 4 and *Proteus mirabilis* strain NGH were kindly provided by Dr H.R. Ingham, General Hospital, Newcastle upon Tyne, NE4 6BE. Twelve strains of bacteroides and 28 enterobacteria were randomly chosen from fresh clinical isolates obtained in the Bacteriology Department, Royal Infirmary, Glasgow G4 0SF. Identification of the *Bacteroides* species was based on the method of Duerden *et al.* (1980), and of the coliforms by the API 20E system (API Laboratory Products, Grafton Way, Basingstoke, Hants RG22 6HY).

The species included in this study were *E. coli* (14 strains), *P. vulgaris* (3 strains), *P. mirabilis* (7 strains), *P. morgani* (4 strains), *B. melaninogenicus* (6 strains), *B. fragilis* (5 strains) and *B. thetaiotaomicron* and *B. vulgatus* (1 strain each).

Culture of bacteroides. Each isolate was cultured in a medium consisting of Cooked Meat Particles (Difco) reconstituted in Brain Heart Infusion Broth (Difco) to which menadione (1 µg/ml, Sigma Chemical Company, St Louis) and haemin (5 µg/ml, Sigma) were added. Cultures were incubated for 48 h at 37°C and used in the experiments without subsequent treatment unless otherwise stated.

Culture and radiolabelling of the enterobacteria. The *Proteus* and *E. coli* isolates were cultured overnight in 20 ml of Mueller-Hinton Broth (Oxoid) containing ³H-thymidine (specific activity 25 Ci/mmol; Radiochemical Centre, Amersham, Bucks HP7 9LL) at a final concentration of 10 µCi/ml broth. After incubation, the bacteria were centrifuged at 3000 *g* for 10 min and washed three times with 0.85% saline before use.

Opsonisation. Fresh human serum obtained from the Haematology Department, Glasgow Royal Infirmary, was divided into 5-ml portions and stored at -70°C before use. For opsonisation, serum was diluted to 50% with Hanks's balanced salt solution containing 0.1% gelatin (gel-HBSS). The washed radiolabelled test bacterial suspension was diluted with saline to contain approximately 5×10^7 organisms/ml by spectrophotometric determination. Equal volumes of the standardised bacterial suspension and 50% serum in gel-HBSS were mixed, incubated at 37°C for 15 min in an orbital incubator (shaking speed 150 rpm) and then centrifuged at 3000 *g* for 10 min. The bacterial pellet was resuspended in gel-HBSS to its original volume.

In some experiments the human serum was heat treated at 56°C for 30 min to inactivate complement before opsonisation. The heated serum failed to opsonise *E. coli* or *Proteus* organisms and this was assumed to indicate that little or no antibody with opsonic activity remained. Serum complement activation via the classical pathway was inhibited by addition of 0.1 ml of 0.1M ethyleneglycol-bis-(β-aminoethyl-ether) N,N'-tetra-acetic acid (EGTA, Sigma) to 0.9 ml serum and held at room temperature for 30 min. Further dilutions of the chelated serum were then made in 0.85% saline.

Leucocyte preparation. Venous blood was obtained from individual healthy volunteers; it was heparinised (10 U heparin/ml blood) and mixed with dextran (mol. wt 150 000; 6% w/v in 0.85% saline; Fisons Limited, Loughborough, Leics LE11 0RG) in a blood:dextran solution ratio of 4:1. Sedimentation of erythrocytes was allowed for 20–30 min. The supernatant fluid and the cells at the interface were removed and centrifuged at 100 *g* for 5 min. The deposit was suspended in 0.87% ammonium chloride (BDH Chemicals Ltd, Poole, Dorset BH12 4NN) and mixed gently for 15–20 min to allow lysis of the erythrocytes. After centrifugation at 100 *g* for 5 min, the leucocytes were suspended in gel-HBSS and adjusted to contain approximately 5×10^6 PMNL/ml as determined with a haemocytometer.

Uptake of radiolabelled bacteria. The method of Verhoef, Peterson and Quie (1977) was followed. For each phagocytosis mixture, duplicate test vials containing 0.1 ml of radiolabelled, opsonised bacteria and 0.1 ml of the leucocyte 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 leucocyte suspension were included. After incubation, 3 ml of scintillation fluid (Aqualuma, Lumac Systems AG, Basle) was added to one of the test 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 vial, 3 ml of ice-cold phosphate-buffered saline (PBS), pH 7.4, was added, the vials were centrifuged at 100 *g* for 5 min to sediment the PMNL and the supernatant fluid was discarded. These vials were given two more washes 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 per minute of the contents of each vial were determined in a liquid scintillation counter (1210 Ultrobeta, LKB Instruments, 232 Addington Way, S. Croydon, Surrey CR2 8YD). 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.

Measurement of phagocytosis. Phagocytosis and killing of bacteria by leucocytes was estimated by the method of Ingham *et al.* (1977). Briefly, 0.5 ml of leucocyte suspension, 0.1 ml of serum (undiluted), 0.1 ml of coliform suspension, 0.1 ml of bacteroides suspension (containing 10^8 – 10^9 organisms/ml of Robertson's cooked meat medium) and 0.2 ml of gel-HBSS were incubated aerobically at 37°C for up to 5 h in an orbital incubator. Controls without serum, leucocytes or bacteroides culture were included, with gel-HBSS substituted in each case. After incubation, tenfold dilutions of the mixtures were made in distilled water and 20- μ l amounts of each dilution were inoculated onto agar plates in duplicate. Gentamicin blood-agar plates, containing gentamicin 50 μ g/ml, to inhibit the growth of *Proteus* and *E. coli*, were used for the anaerobes, and cystine lactose electrolyte-deficient medium (CLED, CM 301 Oxoid) was used for the coliforms. After incubation, the viable count was calculated according to the method of Miles, Misra and Irwin (1938).

Statistical analysis of the results was performed by the Mann-Whitney U test.

RESULTS

Factors affecting uptake of labelled bacteria

The mean uptake of radiolabelled opsonised cells of *P. mirabilis* strain NGH by PMNL was 81% compared with 3% for unopsonised *P. mirabilis* cells (fig. 1). The uptake of this strain was markedly reduced ($p < 0.001$) when the bacteria were opsonised with serum pretreated with *B. melaninogenicus* strain 4, and less markedly but still significantly reduced when opsonised with untreated serum in the presence of *B. melaninogenicus* cells.

In tests with several different isolates of *Proteus* and *E. coli* (see fig. 2), pretreatment of the serum with *B. melaninogenicus* strain 4 significantly inhibited ($p < 0.001$) the uptake of most (10/14) of the *Proteus* isolates. The uptake of *E. coli* isolates was more varied; the uptake of seven of the 14 isolates was inhibited by more than 50% but with five isolates there was little or no change in uptake.

Pretreatment of the serum with any of the 13 isolates of *Bacteroides* inhibited the uptake of *P. mirabilis* strain NGH by at least 50% (table I). When *E. coli* was substituted for *Bacteroides* there was little change in the uptake of the proteus by

FIG. 1.—Effect of *B. melaninogenicus* strain 4 on the uptake of *P. mirabilis* strain NGH by polymorphonuclear leucocytes (PMNL). A = opsonised; B = non-opsonised; C = opsonised with serum treated with *B. melaninogenicus* strain 4; D = opsonised in presence of *B. melaninogenicus* strain 4. Each result represents the mean and range of at least three experiments.

PMNL. Similarly, if serum treated with *E. coli* was used for opsonisation or if pre-opsonised *P. mirabilis* strain NGH cells were incubated with *E. coli*, there was no alteration in the uptake of the radio-labelled bacteria by PMNL ($p > 0.005$) (fig. 3). When simultaneous opsonisation of *P. mirabilis* strain NGH and *E. coli* was carried out, the mean uptake of the proteus was reduced from 80% to 60% but this difference was not statistically significant ($p > 0.05$).

When culture filtrates of *B. melaninogenicus* strain 4 were incubated with normal human serum before opsonisation of *P. mirabilis* strain NGH, the uptake of *P. mirabilis* NGH was significantly reduced ($p < 0.001$) indicating that a cell-free component in the culture supernate might be involved (fig. 4). Similarly, a heat-killed *B. melaninogenicus* culture and a suspension of *B. melaninogenicus* strain 4 in saline containing 1% ascorbic acid impaired the uptake of *P. mirabilis* strain NGH ($p < 0.001$). To exclude the possibility that the bacteria *per se* might be causing a direct effect on the PMNL, PMNL were preincubated with either unopsonised *B. melaninogenicus* strain 4, *P. mirabilis* strain NGH or *E. coli*, and these PMNL showed normal uptake of opsonised *P. mirabilis* (table II).

FIG. 2.—Frequency of uptake by polymorphonuclear leucocytes (PMNL) of various isolates of *Proteus* (7 strains of *P. mirabilis*, 3 of *P. vulgaris*, 4 of *P. morgani*) and 14 isolates of *E. coli* opsonised with serum pretreated with *B. melaninogenicus* strain 4. Values are expressed as a percentage of the corresponding uptake of each isolate opsonised with normal human serum. A = *Proteus*; B = *E. coli*.

TABLE I

Phagocytic uptake by PMNL of Proteus mirabilis strain NGH opsonised with serum treated with various bacteroides isolates

Species and strain no. of organism used for pretreatment of serum		Percentage uptake of <i>P. mirabilis</i> strain NGH by PMNL*
<i>B. melaninogenicus</i>	1	15
	2	14
	3	14
	4	17
	5	18
	6	21
<i>B. fragilis</i>	1	23
	2	31
	3	29
	4	30
	5	26
<i>B. thetaiotaomicron</i>		15
<i>B. vulgatus</i>		31

* Normal serum opsonisation allowed 75%–100% uptake by polymorphonuclear leucocytes (PMNL).

FIG. 3—Effect of *E. coli* on the uptake of *P. mirabilis* strain NGH by polymorphonuclear leucocytes (PMNL). A = opsonised; B = non-opsonised; C = opsonised in presence of *E. coli*; D = opsonised with serum previously treated with *E. coli*; E = opsonised and subsequently incubated with *E. coli*. Each result represents the mean and range of at least three experiments.

FIG. 4—Effect of heat-killed *B. melaninogenicus* strain 4, *B. melaninogenicus* strain 4 culture filtrate, and a suspension of *B. melaninogenicus* strain 4 in saline containing 1% ascorbic acid on the uptake by PMNL of *P. mirabilis* strain NGH opsonised with serum treated with these preparations. A = opsonised; B = opsonised with serum treated with *B. melaninogenicus* strain 4 culture; C = opsonised with serum treated with *B. melaninogenicus* strain 4 culture filtrate; D = opsonised with a heat-killed culture of *B. melaninogenicus* strain 4; E = opsonised with serum treated with *B. melaninogenicus* strain 4 suspended in saline containing 1% ascorbic acid. Each result represents the mean and range of at least three experiments.

When serum treated with EGTA to inhibit the classical complement pathway was used to opsonise *P. mirabilis* strain NGH, the mean uptake of bacteria was reduced from 88% to 30% (fig. 5). Treatment of EGTA-treated serum with *B. melaninogenicus* strain 4 before the opsonisation of *P. mirabilis* strain NGH resulted in a mean uptake of 19% by PMNL. This is comparable with a 24% uptake when normal serum pretreated with *B. melaninogenicus* strain 4 was used for opsonisation.

Effect of B. melaninogenicus on phagocytic killing

In these studies the assay system of Ingham *et al.* (1977) was used to measure the efficiency of bacterial killing by PMNL in the presence or absence of other bacteria. The susceptibility of *P. mirabilis* strain NGH was measured over a 5-h period at 37°C. Viable counts taken at this time showed that *P. mirabilis* strain NGH was markedly

TABLE II

Effect of pre-incubation of PMNL with different non-opsonised organisms on uptake of opsonised P. mirabilis

PMNL pre-incubated with	Percentage uptake of opsonised <i>P. mirabilis</i>
buffered saline control	92
<i>B. melaninogenicus</i> strain 4	90
<i>E. coli</i>	87
<i>P. mirabilis</i> strain NGH	86

protected from phagocytic killing when *B. melaninogenicus* strain 4 was present in the reaction mixture (table III) and the inhibitory index of phagocytosis (Ingham *et al.* 1977) was 4.7×10^3 . After incubation of PMNL and *P. mirabilis* strain NGH for 1 h there was a 60-fold loss of bacterial viability whereas only a 30-fold loss of viability

FIG. 5—Effect of *B. melaninogenicus* 4 on the uptake by PMNL of *P. mirabilis* strain NGH opsonised with untreated or EGTA-treated serum. A=opsonised with untreated serum; B=opsonised with EGTA-treated serum; C=opsonised with serum treated with *B. melaninogenicus* strain 4; D=opsonised with EGTA treated serum followed by treatment with *B. melaninogenicus* strain 4; E=non-opsonised. Each result represents the mean and range of at least three experiments.

TABLE III

Effect of *B. melaninogenicus* strain 4 on phagocytosis and killing of *P. mirabilis* strain NGH and *E. coli*

Organism	Viable count at 5 h* with			Inhibitory index†
	<i>B. melaninogenicus</i> present	<i>B. melaninogenicus</i> absent	control (No PMNL)	
<i>P. mirabilis</i> strain NGH	7.0×10^8	1.5×10^5	1.0×10^9	4.7×10^3
<i>E. coli</i>	3.5×10^6	6.5×10^4	1.0×10^9	5.4×10^1

* Mean of at least three experiments

† Inhibitory index is the ratio of the viable count at 5 h of *E. coli* or *P. mirabilis* in the presence of *B. melaninogenicus* to that obtained in the absence of proteus organisms.

occurred when *B. melaninogenicus* strain 4 was present. In contrast *E. coli* caused less inhibition of phagocytosis (inhibitory index: 54); most of the added bacteria were phagocytosed and killed. The number of viable *B. melaninogenicus* strain 4 remained virtually constant (5×10^7 bacteria/ml of phagocytosis mixture) throughout. Neither killing *B. melaninogenicus* strain 4 by heat (100°C for 10 min) nor clindamycin (50 µg/ml culture) affected its ability to impair phagocytosis of *P. mirabilis* by PMNL.

DISCUSSION

These results confirm and extend the findings of Ingham *et al.* (1977) who reported the inhibition of phagocytic killing of various bacteria by *Bacteroides* species. The inhibition probably results solely from impairment of the phagocytic ingestion of bacteria.

The phagocytic uptake of all the *Proteus* strains tested was markedly impaired in contrast to that of some of the *E. coli* strains. The latter could be divided into those whose uptake by PMNL was inhibited by more than 50% or by less than 50% by *Bacteroides*. This variation may be due to differences in surface structures. Tofte *et al.* (1980) investigated only one strain of *E. coli* and found that its uptake by PMNL was inhibited by *B. melaninogenicus* and by *B. fragilis*.

Ingham *et al.* (1977) suggested that complement was unlikely to be the sole serum component affected by bacteroides treatment of serum. The present studies support the theory of Tofte *et al.* (1980) that competition for serum opsonins is the basis for the observed impairment of phagocytosis. Chelation of serum with EGTA to inhibit the classical pathway of complement reduced the uptake of *P. mirabilis* by PMNL but did not alter the reduced uptake that followed opsonisation with bacteroides-treated serum. Thus it appears that the bacteroides cells do not owe their activity to the impairment of the alternative complement pathway but to an action on the classical pathway. Preliminary experiments suggest that levels of C3 are similarly reduced by 20–30% when serum is treated with representative strains of *Bacteroides* or *Proteus* or *E. coli*. However, more analysis of the biological activity of each of the initial complement components is necessary to determine the precise locus affected by the bacteroides cells.

The cell concentration and *Eh* of the bacteroides culture appeared to be important

in the impairment of opsonic activity of human serum. Bacteria harvested and washed in saline lost their biological activity, whereas resuspension in a medium with a low Eh such as saline containing 1% ascorbic acid or fresh culture medium (Robertson's cooked meat) restored their activity; these findings agree with those of Ingham *et al.* (1977). One or two batches of undiluted cooked-meat medium impaired opsonisation and phagocytosis of *Proteus* species *per se* but we were unable to determine the factor responsible; on dilution significant inhibitory activity was not detectable. At least 10^5 bacteroides/ml of human serum appeared to be necessary to cause significant impairment of opsonic activity.

Phagocytic uptake, measured over 45 min at 37°C, of *P. mirabilis* by PMNL in the presence or absence of bacteroides cells showed significant differences that were further amplified when the killing rates were monitored over 5 h. Although significant impairment of killing of *Proteus* was detectable even at 1 h, the number of viable bacteroides did not decline over the 5-h period; this suggests that little or no phagocytosis of bacteroides occurred in these conditions.

We have shown that there is competition between different bacterial species for serum opsonins and subsequent phagocytic uptake and killing by PMNL. Whether such a process might also take place *in vivo* is open to question. Reznikov, Finlay-Jones and McDonald (1981) studied the clearance of *E. coli* from the peritoneal cavity of mice in the presence or absence of *B. fragilis*. Even at an anaerobe:coliform ratio of 80:1 there was no impairment of the rate of clearing of *E. coli* from the mouse peritoneum; both organisms were cleared at the same rate. It is unlikely that serum interactions with bacteria and subsequent phagocytosis by PMNL *in vitro* and *in vivo* are directly comparable. Accordingly, we are now extending these studies in an investigation of phagocytic activity within abscesses experimentally induced by the method of Joiner *et al.* (1980).

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ADDENDUM

Since this paper was submitted for publication Ingham *et al.* (1981) have reported that interaction of bacteroides cells with human serum can inhibit intracellular killing but not ingestion of *P. mirabilis*. The results of the present study indicate that ingestion and killing are inhibited. The reasons for this difference are not clear but may be related to the times of sampling used in the two studies. Ingham *et al.* (1981) studied phagocytosis after incubation for 5 h whereas a 45-min incubation period was used in the present study. A reduced rate of uptake of radiolabelled proteus cells was evident at this time, and the killing of *Proteus* spp. became impaired within 1–2 h.

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