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Opsonization of Four *Bacteroides* Species: Role of the Classical Complement Pathway and Immunoglobulin

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Previous investigators have suggested that opsonization of two *Bacteroides* species is mediated exclusively by the alternative complement pathway and requires immunoglobulins. In this study, the nature of the opsonic factors in nonimmune human serum for four species of *Bacteroides* was investigated by measuring uptake of [^{3}H]thymidine-labeled bacteria by human polymorphonuclear leukocytes. Normal human serum, C2-deficient serum, immunoglobulin-deficient serum, and serum chelated with ethylene glycol-bis(β-aminoethyl ether)-*N,N*-tetraacetic acid (EGTA), MgEGTA, and ethylenediaminetetraacetic acid (EDTA) were used as opsonic sources. Heat inactivation of each of these sera significantly reduced its opsonic activity for all four *Bacteroides* species, suggesting that serum complement was essential for effective opsonization. All strains were opsonized in the absence of the classical complement pathway; however, kinetics studies revealed that opsonization proceeded at a significantly faster rate when the classical complement pathway was intact. Although two strains were opsonized in immunoglobulin-deficient sera, opsonization was less efficient and appeared to occur via the alternative complement pathway. Unexpectedly, all strains were well opsonized by the classical complement pathway in 10% serum which had been effectively chelated with EGTA or EDTA. The explanation for this finding is unknown; however, it is possible that cell wall cations of *Bacteroides* species may participate in the activation of complement in chelated serum, resulting in effective opsonization. It was also found that *Bacteroides,* when incubated with an *Escherichia coli* strain in normal serum, could compete for opsonins and thereby reduce phagocytosis of *E. coli.* It is possible that competition for opsonins among bacterial species contributes to the synergistic role these organisms share in mixed floral infections.

An effective osponic source including complement or immunoglobulin is required to prepare bacteria for maximal phagocytosis by polymorphonuclear leukocytes (PMNL). Anderson et al. (1) have shown that encapsulated strains of *Haemophilus influenzae* are opsonized in immune serum by the classical complement pathway, whereas Quinn and co-workers (26) found that these same strains were opsonized by the alternative complement pathway in nonimmune serum. Other investigators (31, 34) have shown that several bacterial species can be opsonized by the complement system in the relative absence of immunoglobulin. In contrast to unencapsulated organisms, encapsulated bacteria appear to require specific antibodies for optimal opsonization (3, 24).

Casciato et al. (4) were the first to study opsonization and phagocytosis of *Bacteroides* species. They demonstrated that *B. thetaiotaomicron* could be opsonized only by heat-labile serum opsonic factors and that phagocytosis by PMNL occurred equally well under anaerobic and aerobic conditions. Recently, Bjornson and Bjornson (2) studied two species of *Bacteroides* and concluded that optimal opsonization required both the alternative complement pathway and immunoglobulin. These investigators used strictly anaerobic conditions and methods designed to block certain components of the alternative complement pathway. Methods for studying opsonization in the absence of the classical complement pathway, such as using C2-deficient serum or by blocking C1 activation by magnesium ethylene glycol-bis(β-aminoethylether)-*N,N*-tetraacetic acid (MgEGTA) (8, 31), were not used.

Several groups of investigators using different experimental systems have shown in kinetics studies that complement activation proceeds more rapidly through the classical complement pathway than by the alternative complement

pathway (9, 22, 25, 31). Ethylenediaminetetraacetic acid (EDTA) and EGTA block both the classical and alternative complement pathways, although EGTA only partially blocks alternative pathway function. Conversely, MgEGTA and C2-deficient human serum selectively block the classical complement pathway (8, 14). In the present investigation, we compared the kinetics of opsonization of various concentrations of normal human serum, of EDTA-, EGTA-, and MgEGTA-chelated normal serum, and of C2-deficient serum to determine the relative importance of the classical and alternative complement pathways for optimal opsonization of four *Bacteroides* species (5).

Ingham et al. (12) recently reported that *B. fragilis* in some way interferes with the killing of aerobic bacteria by PMNL. *Bacteroides* infections are frequently synergistic. In this study, we tested the hypothesis that *Bacteroides* can compete with an aerobic gram-negative bacillus and thereby reduce phagocytosis of this organism.

MATERIALS AND METHODS

Bacterial strains. Four species of *Bacteroides* were tested: an encapsulated *B. fragilis* (ATCC 23745; American Type Culture Collection, Rockville, Md.), *B. melaninogenicus* subsp. *melaninogenicus* (ATCC 15930), *B. thetaiotaomicron* (NCTC 10582; National Collection of Type Cultures), and a clinical isolate of *B. vulgatis*. All strains were maintained in Todd-Hewitt broth (Difco Laboratories, Detroit, Mich.) supplemented with 5 μg of menadione and 0.5 μg of hemin per ml at 25°C in an anaerobic jar (GasPak 100, BBL Microbiology Systems, Cockeysville, Md.), using palladium-coated aluminum catalyst pellets to maintain a reduced environment (26). A resazurin indicator in the broth assured anaerobic culture conditions. A 0.1-ml portion was transferred from the maintenance broth culture to 10 ml of fresh Todd-Hewitt broth every 7 days to maintain bacterial viability. *Escherichia coli* ON2 was maintained on nutrient agar at 4°C.

Radioactive labeling. Radioactive labeling of bacteria was accomplished by inoculating 0.1 ml of the broth culture into 10 ml of reduced Todd-Hewitt broth containing 0.02 mCi of [*methyl*-^{3}H]thymidine (specific activity, 35 Ci/mmol; Research Products International, Inc., Elk Grove Village, Ill.). After 18 h of incubation at 37°C, the bacteria were washed three times in phosphate-buffered (PBS), pH 7.4, and resuspended in PBS to a desired final concentration of about 10^9 colony-forming units (CFU)/ml, using a spectrophotometric method and confirming by pour plate colony counts.

PMNL. Venous blood was collected from healthy donors in preheparinized syringes (10 U of heparin/ml of blood). Pure PMNL suspensions were prepared by a previously described method (23). Leukocyte suspensions were adjusted to a final concentration of 10^7 PMNL per ml of Hanks balanced salt solution containing 0.1% gelatin (gel-HBSS).

Osponic sources. Normal serum was collected from 10 healthy adult donors, pooled, and stored in 1.0-ml portions at −70°C. Heat-inactivated serum was prepared by heating thawed serum at 56°C for 30 min. To study opsonization in the absence of an intact classical complement pathway, serum was obtained from a donor with a genetically determined complete and selective absence of C2 (C2-deficient serum) (15). Serum was diluted to specified final concentrations with gel-HBSS. In one series of experiments, undiluted and 10% concentrations of normal serum were chelated with 0.1 M EDTA or EGTA (0.1 ml of chelating agent was added to 0.9 ml of serum and incubated at room temperature for about 30 min) as previously described (8) and diluted with calcium- and magnesium-free gel-HBSS. To determine whether the serum was completely chelated, 0.55 ml of 90 and 10% concentrations of normal serum, EDTA- and EGTA-chelated normal serum, and heat-inactivated sera were individually incubated with 50 μl of sheep erythrocyte-antibody (EA) cells in gelatin Veronal buffer (37°C, 60 min), and percentage of EA cell lysis was measured. Normal serum produced complete (100%) sheep EA cell lysis, whereas chelated and heat-inactivated sera produced no EA cell lysis. These results indicated that complement activation by sheep EA cells did not occur in either 100 or 10% normal serum with a final chelator concentration of 0.01 M. The role of immunoglobulins in opsonization was investigated by using serum from a 62-year-old man with common variable immunodeficiency (immunoglobulin-deficient serum; immunoglobulin G [IgG], 73 mg/dl; IgM, 7 mg/dl; and IgA, 3 mg/dl by laser nephelometry). This serum had normal levels of all complement components except for slightly diminished C1 (96,000 hemolytic units; normal range, 126,000 to 396,000). To remove specific antibacterial antibodies from normal serum, 3 ml of ice-cold normal human serum was added to a bacterial pellet of about 10^9 CFU of *B. fragilis;* after suspension of bacteria, this mixture was incubated at 4°C for 60 min. Serum was then centrifuged (2,000 × *g*, 4°C, 15 min) and absorbed twice more in a similar fashion.

Bacterial opsonization procedure. A 0.1-ml amount of each bacterial suspension was incubated in a polypropylene vial (Bio-vials, Beckman Electronics Instruments Div., Chicago, Ill.) containing 1.0 ml of the indicated opsonic sources for specified times in an incubator shaker at 37°C (New Brunswick Scientific Co., Inc., New Brunswick, N.J.) (250 rpm) followed by centrifugation at 2,000 × *g* for 15 min (4°C). The supernatant fluid was discarded, and the bacterial pellet was vigorously resuspended in 1.0 ml of gel-HBSS.

Bacterial surface staining with fluorescein-conjugated anti-C3. To look for evidence of C3 deposition on the bacterial surface, each species was washed twice in PBS after opsonization and resuspended in 0.02 ml of PBS. Five-microliter samples were then deposited on clean glass slides and air dried at room temperature. The bacterial smears were stained with 20 μl of fluorescein-conjugated goat anti-human C3 serum and examined with a phase-contrast

immunofluorescence microscope. Surface staining intensity was quantitatively graded from 0 to 4+.

Phagocytosis mixtures and assays. Bacterial uptake by PMNL was quantitatively measured by a modification of a previously described method (32). Initial experiments were performed in an anerobic glove box (model AL 318; Coy Laboratory Products, Inc., Ann Arbor, Mich.) (28). However, it was determined that all four *Bacteroides* species studied were aerotolerant, and as described by others (4, 16), that similar results were obtained when osponization and phagocytosis were performed in an aerobic environment. Therefore, all subsequent assays were performed in an aerobic environment.

Briefly, 0.1-ml samples of the opsonized bacterial suspensions were added to each of three polypropylene vials, followed by the addition of 0.1 ml of the leukocyte suspension yielding a CFU-to-PMNL ratio of about 10:1. These vials were incubated for indicated periods in the incubator shaker at 250 rpm (37°C). Immediately after removal from the shaker, 3.0 ml of ice-cold PBS was added to two of the vials. Leukocyte-associated radioactivity was determined after washing the PMNL in one of these two vials three times by means of differential centrifugation (160 × *g* for 5 min, 4°C). The final leukocyte pellets were resuspended in 3.0 ml of scintillation liquid (Aquasol-2, New England Nuclear Corp., Boston, Mass.) and counted in a liquid scintillation counter (Beckman LS-250). The second vial was used to determine total radioactivity (representing both leukocyte-associated and non-leukocyte-associated bacteria). Vials were prepared as outlined above. After 3.0 ml of cold PBS was added, these vials were centrifuged at 2,000 × *g* for 15 min. The supernatant fluids were discarded, and the pellets were vigorously resuspended in 3.0 ml of scintillation liquid and counted. Bacterial uptake by PMNL was calculated by the following formula: percentage of uptake = (cpm in leukocyte pellet)/(total cpm) × 100. The third vial was used for morphological confirmation of phagocytosis. Immediately after incubation in the shaker for 12 min, 50-μl samples were deposited on clean glass slides by using a cytocentrifuge, stained with Wright stain, and examined with a light microscope. In every instance, there was good correlation between the quantitative phagocytosis and microscopic evaluation.

RESULTS

Opsonization by normal and heat-inactivated serum. All four *Bacteroides* species were individually incubated for 60 min in 100, 25, 10, and 1% concentrations of normal and heat-inactivated normal serum before constituting phagocytosis mixtures and determining PMNL uptake at 12 min. The opsonic capacity for each species, except *B. vulgatis,* was significantly reduced when the serum concentration was decreased from 10 to 1% (Fig. 1). Undiluted heat-inactivated serum was a relatively good opsonin for *B. melaninogenicus* (43% uptake) but was minimally opsonic for the other species (<20% uptake). At a concentration of 10%, heated serum had about 25% of the osponic activity of normal serum. In another experiment, each species was incubated for 60 min in 10% concentrations of normal and heat-inactivated serum before preparation of phagocytosis mixtures and determining PMNL uptake at 2, 6, and 12 min (Fig. 2). All four species were effectively phagocytized after incubation in normal serum; however, the rate of phagocytosis was more rapid with *B. vulgatis* than with the other species. *B. thetaiotaomicron* and *B. vulgatis* opsonized in heat-inactivated serum were more readily phagocytized than the strains of *B. fragilis* and *B. melaninogenicus* (27, 18, 6, and 5% uptake, respectively).

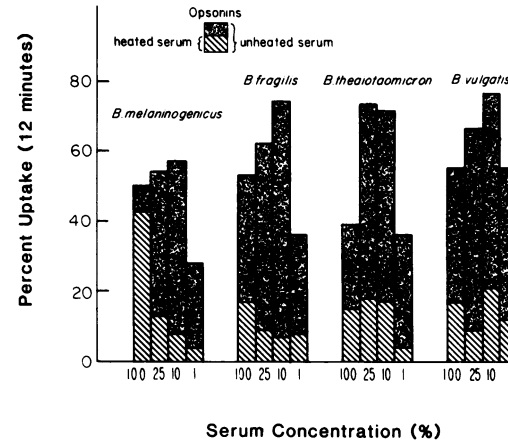

FIG. 1. *Phagocytosis of B. melaninogenicus, B. fragilis, B. thetaiotaomicron, and B. vulgatis by PMNL after incubation in human serum. Bacteria were opsonized for 60 min at 100, 25, 10, and 1% concentrations of normal and heat-inactivated serum before presentation to PMNL. Phagocytosis was quantitated by measuring leukocyte-associated radioactivity at 12 min. Slashed bars represent bacterial uptake after incubation in heat-inactivated serum.*

These results demonstrate that: (i) each *Bacteroides* species was phagocytized, albeit at different rates, after opsonization in normal human serum; (ii) *B. melaninogenicus* was more effectively opsonized by heat-stable factors in undiluted serum than other *Bacteroides* species and the heat-stable opsonic capacity of serum was progressively diminished by serial serum dilutions; and (iii) heat-labile serum factors were critically important for optimal opsonization of all four *Bacteroides* species at serum concentrations of <25%.

Osponization by C2-deficient and immunoglobulin-deficient sera. To study the importance of the classical complement pathway and immunoglobulin in the opsonization of *Bac-*

teroides species, we incubated each bacterial species for 60 min in 10% concentrations of normal serum, C2-deficient serum, heat-inactivated serum, and immunoglobulin-deficient serum before adding PMNL and determining uptake at 12 min (Fig. 3). When compared with normal serum, C2-deficient and immunoglobulin-deficient sera were effective opsonic sources for all four species. Absorbed normal serum, prepared as described above, was moderately opsonic for *B. fragilis* and *B. melaninogenicus* (30% uptake). Heat inactivation of each serum markedly reduced its opsonic activity for all four *Bacteroides* species (<10% uptake).

Studies of the kinetics of opsonization were performed to examine the potential influence of the classical complement pathway on opsonization. We incubated *B. fragilis* and *B. melaninogenicus* in 10% concentrations of normal and C2-deficient serum for 1, 5, 15, and 60 min before constituting phagocytosis mixtures and measuring PMNL uptake at 12 min. Opsonization of both species in normal serum was essentially complete within 1 min (Fig. 4). Although C2-deficient serum was an effective opsonic source at 60 min, the rate of opsonization was significantly slower than with normal serum. Additionally, when immunoglobulin-deficient serum was used (Fig. 4), opsonization proceeded more slowly than when either normal or C2-deficient serum was used as an opsonin.

These results suggest that: (i) an intact classical pathway and immunoglobulin are important for optimal opsonization of *B. fragilis* and *B. melaninogenicus;* (ii) these species can be effectively opsonized in the absence of an intact classical complement pathway (C2-deficient serum) but the rate of opsonization proceeds more slowly; and (iii) opsonization by immunoglobulin-deficient serum is less effective than opsonization by normal serum and appears to be mediated by the alternative complement pathway since the kinetics of opsonization are similar to those for C2-deficient serum.

Opsonization by chelated serum. The previous results demonstrated that there was heat-labile opsonic activity in normal serum for all four species of *Bacteroides* and that for optimal opsonization, an intact classical complement pathway was required. To further investigate the participation of the classical complement pathway in the opsonic process, normal serum was chelated with EDTA or EGTA, which have been shown to block both the classical and alternative complement pathways, and with MgEGTA-chelated serum, which selectively blocks the classical complement pathway. *B. fragilis* and *B. melaninogenicus* were individually incubated for 60 min in 10% concentrations of normal serum, MgEGTA-, EGTA-, or EDTA-chelated normal serum, and heat-inactivated sera before preparation of phagocytosis mixtures and measurement of PMNL uptake at 12 min. *E. coli* ON2 (21) was included for comparison and was treated in an indentical manner. All three chelated sera had significant heat-labile opsonic activity for both *Bacteroides* species (Fig. 5). In contrast, *E. coli* ON2, which was as well opsonized in MgEGTA-chelated normal serum as in normal serum, was not opsonized in EGTA- or EDTA-chelated serum.

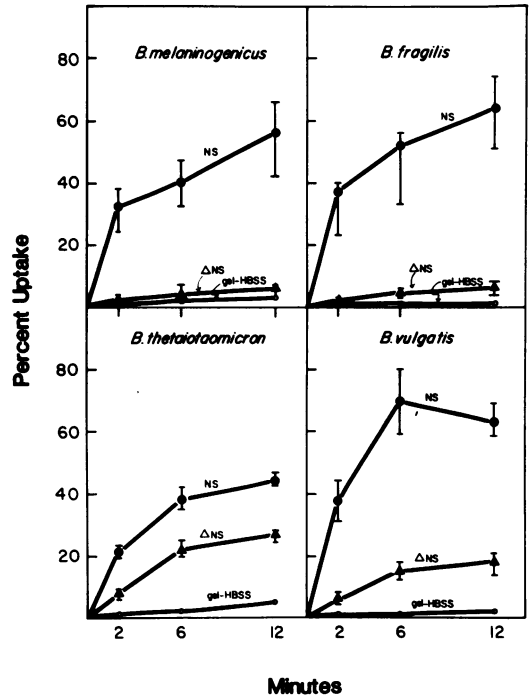

FIG. 2. *Phagocytosis of B. melaninogenicus, B. fragilis, B. thetaiotaomicron, and B. vulgatis by PMNL after incubation in human serum. Bacteria were opsonized for 60 min in 10% normal serum (NS; ●), 10% heat-inactivated serum (ΔNS, ▲), and gel-HBSS (○) before measurement of PMNL uptake at 2, 6, and 12 min. Results represent the means and ranges of three experiments.*

The finding that the *Bacteroides* species were efficiently opsonized in all three chelated sera was surprising since it had been shown that sheep EA cells were completely lysed in both 90 and 10% normal serum, but were not lysed in any of the chelated sera. To determine whether the heat-labile opsonic activity in chelated serum was indeed mediated by complement, opsonization was performed at both 4 and 37°C, and bacteria were examined for the presence of

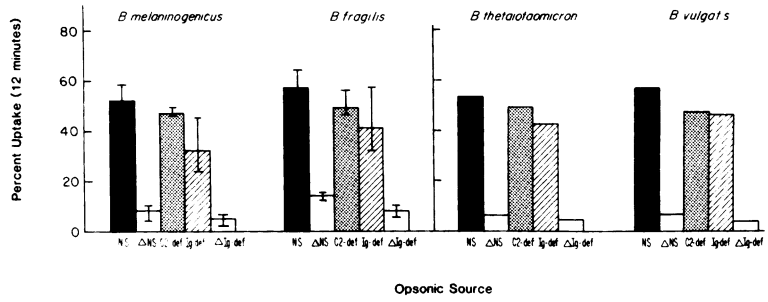

FIG. 3. *Phagocytosis of B. melaninogenicus, B. fragilis, B. thetaiotaomicron, and B. vulgatis by PMNL after incubation in human serum. Bacteria were opsonized for 60 min in 10% concentrations of normal serum (NS), heat-inactivated serum (ΔNS), C2-deficient serum (C2-def), immunoglobulin-deficient serum (Ig-def), and heat-inactivated immunoglobulin-deficient (ΔIg-def) serum. PMNL uptake was determined at 12 min. Results represent the means and ranges of three experiments.*

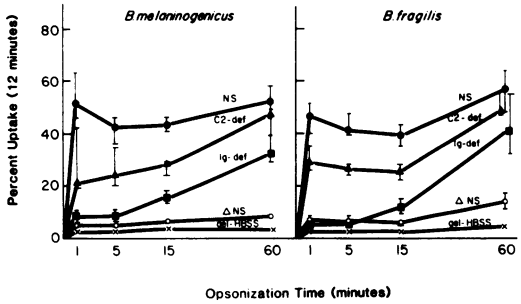

FIG. 4. *Phagocytosis of B. melaninogenicus and B. fragilis by PMNL after incubation in human serum. Bacteria were opsonized for 1, 5, 15, and 60 min in 10% concentrations of normal serum (NS; ●), C2-deficient serum (C2-def; ▲), immunoglobulin-deficient serum (Ig-def; ■), heat-inactivated normal serum (ΔNS; ○), and gel-HBSS (×). PMNL uptake was determined at 12 min. Results represent the means and ranges of three experiments.*

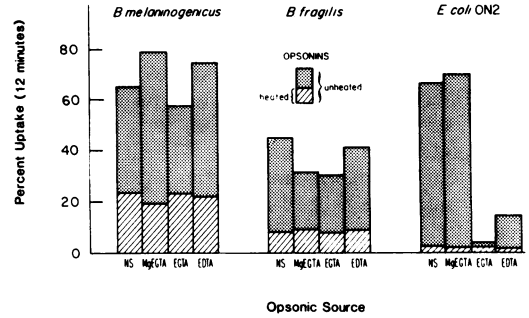

FIG. 5. *Phagocytosis of B. melaninogenicus, B. fragilis, and E. coli ON2 by PMNL after opsonization for 60 min in 10% concentrations of normal serum (NS), MgEGTA-chelated (MgEGTA), EGTA-chelated (EGTA), and EDTA-chelated (EDTA) sera, and heat-inactivated sera. PMNL uptake was determined at 12 min.*

surface C3. *B. fragilis* was incubated for 1, 5, 15, and 60 min in each of the above sera simultaneously at 4 and 37°C. Samples were taken for staining with fluorescein-conjugated anti-C3 serum, and opsonized bacteria were added to PMNL for measurement of uptake at 12 min. Bacteria were rapidly opsonized in all unheated sera at both 4 and 37°C (Table 1). There was a good correlation between the heat-labile opsonic activity in all sera and the demonstration of surface C3 by phase-contrast immunofluorescence microscopy. Bacteria incubated in heat-inactivated sera were not opsonized (<10% uptake) and did not have surface C3 deposition.

These results suggest that *B. fragilis*, unlike *E. coli* ON2, is opsonized by the classical complement pathway in 10% chelated sera as demonstrated by rapid opsonization at 37°C. Further evidence is provided by similarly rapid opsonization at 4°C, a temperature which inhibits opsonization by the alternative pathway (29).

The exact mechanism whereby complement can be activated in chelated serum is unknown. Bacterial cell walls contain variable concentrations of calcium and magnesium. It is possible that bacterium-associated cations are able to promote complement activation in chelated serum. Using atomic absorption, we found that *B. fragilis* contained 3.1 μg of calcium and 5.4 μg of magnesium per g of bacteria. In contrast, *E. coli* ON2 contained 2.3 μg of calcium and 2.5 μg of magnesium per g of dry bacteria.

Competition for opsonins. Ingham and coworkers (12) reported that in vitro incubation of three aerobic bacteria with *B. fragilis* or *B. melaninogenicus* in serum inhibited phagocytosis and killing of the aerobic strains. It was postulated that these bacteria might be acting synergistically to produce an infection which

would not occur in the presence of either bacterium alone.

Based on this observation, we postulated that there may be competition for opsonins among bacterial species, and that when opsonic factors are present in low concentrations, certain species may not be effectively opsonized and phagocytized, resulting in the development or perpetuation of a polymicrobial infection.

To test the hypothesis that *Bacteroides* might be capable of competing for opsonins with gram-negative aerobic bacilli, [^{3}H]thymidine-labeled *E. coli* ON2 was incubated in a 10% concentration of normal serum, individually and in the presence of unlabeled *B. fragilis* or *B. melaninogenicus,* for 60 min before constitution of phagocytic mixtures and measurement of uptake at 12 min. Radioactively labeled *Bacteroides* was mixed with unlabeled *E. coli* ON2 in the same manner. There was about a 50% reduction in opsonization of *E. coli* ON2 in the presence of either anaerobic species, but opsonization of the *Bacteroides* species was not diminished in the presence of *E. coli* ON2 (Fig. 6). These results were statistically significant ($P < 0.05$, Student's *t*-test for paired values). The kinetics of opsonization of these three organisms had shown that the *Bacteroides* species were more rapidly opsonized in 10% serum than *E. coli* ON2. These results demonstrate that these two *Bacteroides* species can effectively compete with *E. coli* ON2 for opsonins, thereby inhibiting phagocytosis of *E. coli* ON2.

DISCUSSION

Although nonclostridial anaerobic bacteria have been known to cause human disease since 1893 (30), the relative frequency and importance of these bacteria as a cause of serious infectious disease, particularly abscess formation (6, 10) and bacteremia (33), has only recently been recognized. Although more than 400 anaerobic species reside in the colon and about 200 species colonize the oral cavity, only six species, including *B. fragilis* and *B. melaninogenicus,* produce the majority of clinical infections (7). Interestingly, the numerically dominant *Bacteroides* species in the colon are *B. distasonis, B. vulgatis,* and *B. thetaiotaomicron,* whereas *B. fragilis* accounts for only about 0.5% of the colonic microflora (17). Its prominent role as a cause of suppuration and sepsis suggests unique virulence properties. Onderdonk and co-workers (19, 21) have described the abscess-promoting properties of the *B. fragilis* capsule as well as noting increased mesothelial adherence when compared with other *Bacteroides* strains.

To assess the opsonic requirements of clinically important *Bacteroides* species, Bjornson and Bjornson (2) studied the capacity of various sera to opsonize two species, using a killing assay.

TABLE 1. *Comparison of opsonization and C3 surface deposition on B. fragilis at 37 and 4°C*

Opsonic source[a]	% PMNL uptake at:				Intensity of surface C3 staining at:			
	1 min	5 min	15 min	60 min	1 min	5 min	15 min	60 min
37°C								
NS	52	42	45	45	+++	+++	+++	+++
ΔNS	8	10	7	8	−	−	−	−
MgEGTA	44	41	48	32	++	+	++	+++
ΔMgEGTA	7	7	9	9	−	−	−	−
EGTA	27	45	47	31	+	++	+	+
ΔEGTA	7	5	6	8	−	−	−	−
EDTA	40	40	37	51	+	++	+	++
ΔEDTA	6	8	7	9	−	−	−	−
4°C								
NS	42	43	49	38	++	++	++	++
ΔNS	17	7	8	9	−	−	−	−
MgEGTA	47	34	35	46	++	++	++	++
ΔMgEGTA	6	5	9	11	−	−	−	−
EGTA	29	32	37	38	++	+	++	++
ΔEGTA	5	5	7	7	−	−	−	−
EDTA	46	41	46	37	++	++	++	++
ΔEDTA	7	8	6	11	−	−	−	−

[a] *B. fragilis* was incubated for 1, 5, 15, and 60 min in 10% concentrations of normal serum (NS), MgEGTA-, EGTA-, and EDTA-chelated serum, and heat-inactivated sera (Δ) at 37 and 4°C. Phagocytosis was quantitated by measuring leukocyte-associated radioactivity at 12 min. Surface C3 deposition was quantitated (0 to 4+) by phase-contrast immunofluorescence microscopy.

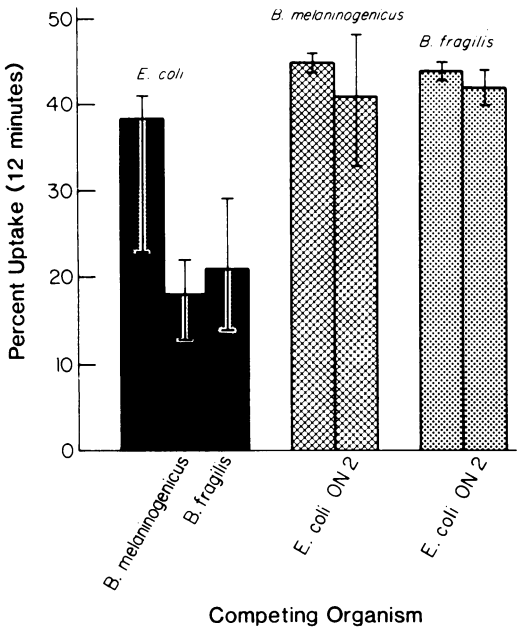

FIG. 6. *Phagocytosis of E. coli ON2, B. melaninogenicus, and B. fragilis by PMNL after opsonization for 60 min in 10% normal serum individually and in the presence of a competing organism. Phagocytosis was quantitated by measuring leukocyte-associated radioactivity at 12 min. Solid bars represent uptake of [^{3}H]thymidine-labeled E. coli ON2; hatched bars represent uptake of labeled B. melaninogenicus; and stippled bars represent uptake of labeled B. fragilis. Results represent the means and ranges of five experiments.*

They found that immunoglobulin and an intact alternative complement pathway were required for opsonization of these two species and that when either component was deleted from serum, opsonization was significantly impaired.

In this study, the nature of serum factors in nonimmune human serum which were opsonic for four species of *Bacteroides* was investigated. Undiluted normal serum contained significant heat-stable opsonic activity for *B. melaninogenicus*. When serum was diluted, there was minimal heat-stable opsonic activity for each species tested, indicating that heat-labile serum factors appeared to be of major importance for opsonization of these bacteria.

To evaluate the relative contributions of the classical and alternative complement pathways to the opsonization of the four *Bacteroides* strains, C2-deficient serum was used to study opsonization in the absence of an intact classical complement pathway. Although each *Bacteroides* species was effectively opsonized by 60 min, kinetics studies revealed that opsonization occurred at a significantly slower rate via the alternative pathway.

Although heat-stable serum factors were minimally opsonic for the *Bacteroides* species in this study, it remained possible that natural antibodies contributed to the activation of the classical or alternative complement pathway by these species. To examine the potential role of immunoglobulin in this regard, the opsonic capacities of immunoglobulin-deficient serum from a patient with hypogammaglobulinemia and of absorbed normal serum were compared with those of normal serum. Both sera contained one-half to two-thirds of the opsonic activity of normal serum for *B. fragilis* and *B. melaninogenicus*, and results of kinetics studies revealed that opsonization proceeded via the alternative complement pathway. These results suggest that *Bacteroides* species can be opsonized in immunoglobulin-deficient serum, but that for optimal opsonization, the classical complement pathway and immunoglobulin are required. The reasons for the discrepancies between our results and those of Bjornson and Bjornson (2) are uncertain, but may be related to methodological differences or to the previously described heterogeneity of opsonic requirements among strains of certain species (11, 22, 31).

To determine whether complement was the heat-labile opsonin in serum, *B. fragilis* and *B. melaninogenicus* were incubated in normal, MgEGTA-, EGTA-, or EDTA-chelated serum and in heat-inactivated sera. Surprisingly, in a 10% concentration of chelated sera, opsonization proceeded normally compared with normal serum. Opsonic activity, however, was abolished by heat inactivation. In comparison, 10% chelated sera were not opsonic for *E. coli* ON2. By immunofluorescence microscopy, C3 was seen on the surface of both *Bacteroides* species incubated in normal and chelated sera but not after incubation in heat-inactivated sera. Similar results were obtained when opsonization was performed at 4 and 37°C, indicating that opsonization was mediated by the classical complement pathway, which is preferentially activated at 4°C (29). EGTA- and EDTA-chelated sera were shown to be incapable of being activated by sheep EA cells. It is possible that the *Bacteroides* species, unlike *E. coli* ON2, supply sufficient amounts of cell wall cations to activate complement at the cell surface and promote opsonization. This results in efficient phagocytosis that may proceed in the presence of only a few surface molecules of C3b.

Ingham et al. (12) recently described several patients with polymicrobial otogenic brain abscesses who responded to an antimicrobial agent

effective primarily against anaerobic bacteria with little demonstrable in vitro activity against aerobic organisms. To investigate possible mechanisms for this clinical response, they incubated each of three aerobic organisms with either *B. fragilis* or *B. melaninogenicus* in human serum and found that the anaerobic bacteria inhibited phagocytosis and killing of the aerobic organisms. The mechanism for the decreased phagocytosis was not determined; however, it was postulated that *Bacteroides* species acted synergistically with the aerobic bacteria to produce an infection. Similarly, other investigators (18, 20) have found that mixed fecal organisms, when implanted into rat peritoneal cavities in gelatin capsules, act synergistically to produce abscesses in those animals surviving the initial period of gram-negative bacillary peritonitis and sepsis. Based on the results of these investigations, we postulated that the bacterial synergism may in part be due to competition for opsonins among bacterial species.

To test the hypothesis that competition for opsonins may occur when two or more bacterial species are present, *E. coli* ON2 was incubated in 10% normal serum individually, and with *B. fragilis* or *B. melaninogenicus*. There was a significant reduction in the opsonization of *E. coli* ON2 in the presence of either *Bacteroides* species; however, opsonization of *Bacteroides* was not influenced by the presence of *E. coli* ON2. This suggests that bacterial species which are more efficiently or rapidly opsonized than others present in a given inoculum may actually deprive the remaining organisms of sufficient opsonins. That competition for opsonins occurs in vivo is currently speculative. Since opsonins may be present in limited quantities in extravascular spaces, it is possible that in the process of competition for available opsonins, some bacterial species are poorly opsonized. Singly or in combination, these organisms are not opsonically recognized and are therefore able to escape phagocytosis while continuing the multiply unimpeded by normal host defense mechanisms.

The results of these studies suggest that optimal phagocytosis occurs when *Bacteroides* species are opsonized by the classical complement pathway in the presence of immunoglobulin. The role of the *B. fragilis* capsule with respect to opsonization remains unclear. In this study, an encapsulated *B. fragilis* strain was opsonized as well as three unencapsulated *Bacteroides* species and was no more effective in competing for opsonins with *E. coli* ON2 than was *B. melaninogenicus*. The mechanism whereby bacteria incubated in chelated sera are efficiently opsonized via the complement system requires further investigation.

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LITERATURE CITED

1. **Anderson, P., R. B. Johnston, and D. H. Smith.** 1972. Human serum activities against *Hemophilus influenzae* type B. J. Clin. Invest. **51**:31-38.
2. **Bjornson, A. B., and H. S. Bjornson.** 1978. Participation of immunoglobulin and the alternative complement pathway in opsonization of *Bacteroides fragilis* and *Bacteroides thetaiotaomicron*. J. Infect. Dis. **138**:351-357.
3. **Bjornson, A. B., and J. G. Michael.** 1973. Factors in normal human serum that promote bacterial phagocytosis. J. Infect. Dis. **128**:182-186.
4. **Casciato, D. A., J. E. Rosenblatt, L. S. Goldberg, and R. Bluestone.** 1975. In vitro interaction of *Bacteroides fragilis* with polymorphonuclear leukocytes and serum factors. Infect. Immun. **11**:337-342.
5. **Cato, E. P., and J. L. Johnson.** 1976. Reinstatement of species rank for *Bacteroides fragilis, B. ovatus, B. distasonis, B. thetaiotaomicron,* and *B. vulgatis*: designation of neotype strains for *Bacteroides fragilis* (Veillon and Zuber) Castellani and Chalmers and *Bacteroides thetaiotaomicron* (Distaso) Castellani and Chalmers. Int. J. Syst. Bacteriol. **26**:230-237.
6. **Finegold, S. M.** 1975. Anaerobic bacteria in human disease, p. 76. Academic Press Inc., New York.
7. **Finegold, S. M.** 1979. Taxonomy, enzymes, and clinical relevance of anaerobic bacteria. Rev. Infect. Dis. **1**:248-252.
8. **Forsgren, A., and P. G. Quie.** 1974. Influence of the alternate complement pathway on opsonization of several bacterial species. Infect. Immun. **10**:402-404.
9. **Gallin, J. I., R. A. Clark, and M. M. Frank.** 1975. Kinetic analysis of chemotactic factor generation in human sera via activation of the classical and alternative complement pathway. Clin. Immunol. Immunopathol. **3**:334-346.
10. **Gorbach, S. L., and J. G. Bartlett.** 1974. Medical progress: anaerobic infections. N. Engl. J. Med. **290**:1177-1184; 1237-1245; 1289-1294.
11. **Guckian, J. C., W. D. Christianson, and D. P. Fine.** 1978. Evidence for quantitative variability of bacterial opsonic requirement. Infect. Immun. **19**:822-826.
12. **Ingham, H. R., D. Tharagonnet, P. R. Sisson, J. B. Selkon, and A. A. Codd.** 1977. Inhibition of phagocytosis in vitro by obligate anaerobes. Lancet **i**:1252-1254.
13. **Jasin, H. E.** 1972. Human heat labile opsonins: evidence for their mediation via the alternate pathway of complement activation. J. Immunol. **109**:26-31.
14. **Johnson, F. R., V. Agnello, R. C. Williams, Jr., and P. G. Quie.** 1972. Opsonic activity in human sera deficient in C2. J. Immunol. **109**:141-145.
15. **Kim, Y., P. S. Friend, I. G. Dressner, E. J. Eunis, and F. S. Rosen.** 1977. Inherited deficiency of the second component of complement (C2) with membranoproliferative glomerulonephritis. Am. J. Med. **62**:765-771.
16. **Mandell, G. L.** 1974. Bactericidal activity of aerobic and anaerobic polymorphonuclear neutrophils. Infect. Immun. **9**:337-341.
17. **Moore, W. E. C.** 1977. Anaerobes as normal flora: gastrointestinal tract, p. 227-228. *In* S. M. Finegold (ed.), Proceedings of the International Metranidazole Conference. Excerpta Medica, Princeton, N. J.

18. **Nichols, R. L., J. W. Smith, E. M. Fossedal, and R. E. Condon.** 1977. Efficacy of parenteral antibiotics in the treatment of experimentally induced intraabdominal sepsis. Rev. Infect. Dis. **1**:302–309.
19. **Onderdonk, A. B., D. L. Kasper, R.L. Cisneros, and J. G. Bartlett.** 1977. The capsular polysaccharide of *Bacteroides fragilis* as a virulence factor: comparison of pathogenic potential of encapsulated and unencapsulated strains. J. Infect. Dis. **136**:82–89.
20. **Onderdonk, A. B., D. L. Kasper, B. J. Mansheim, T. J. Louie, S. L. Gorbach, and J. G. Bartlett.** 1979. Experimental animal models for anaerobic infections. Rev. Infect. Dis. **1**:291–301.
21. **Onderdonk, A. B., N. E. Moon, D. L. Kasper, and J. G. Bartlett.** 1978. Adherence of *Bacteroides fragilis* in vivo. Infect. Immun. **19**:1083–1087.
22. **Peterson, P. K., Y. Kim, D. Schmeling, M. Lindemann, J. Verhoef, and P. G. Quie.** 1978. Complement-mediated phagocytosis of *Pseudomonas aeruginosa*. J. Lab. Clin. Med. **92**:883–894.
23. **Peterson, P. K., J. Verhoef, D. Schmeling, and P. G. Quie.** 1977. Kinetics of phagocytosis and bacterial killing by human polymorphonuclear leukocytes and monocytes. J. Infect. Dis. **136**:502–509.
24. **Peterson, P. K., B. J. Wilkinson, D. Schmeling, and P. G. Quie.** 1978. Influence of encapsulation on staphylococcal opsonization and phagocytosis by human polymorphonuclear leukocytes. Infect. Immun. **19**:943–949.
25. **Polhill, R. B., Jr., K. M. Pruitt, and R. B. Johnston, Jr.** 1978. Kinetic assessment alternative complement pathway activity in a hemolytic system. J. Immunol. **121**:363–370.
26. **Quinn, P. H., F. J. Crosson, Jr., J. A. Winklestein, and E. R. Moxon.** 1977. Activation of the alternative complement pathway by *Haemophilus influenzae* type B. Infect. Immun. **16**:400–402.
27. **Rosenblatt, J. E.** 1976. Isolation and identification of anaerobic bacteria. Human Pathol. **7**:177–186.
28. **Rosenblatt, J. E., A. Fallon, and S. M. Finegold.** 1973. Comparison of methods for isolation of anaerobic bacteria from clinical specimens. Appl. Microbiol. **25**:77–85.
29. **Sandberg, A. L., and A. G. Osler.** 1971. Dual pathways of complement interaction with guinea pig immunoglobulins. J. Immunol. **107**:1268–1273.
30. **Veillon, A.** 1893. Sur un microcoque anaérobic trouvé dans des suppuration fétides. C. R. Seances Soc. Biol. Paris **45**:807–809.
31. **Verhoef, J., P. K. Peterson, Y. Kim, L. D. Sabath, and P. G. Quie.** 1977. Opsonic requirements for staphylococcal phagocytosis: heterogeneity among strains. Immunology **33**:191–197.
32. **Verhoef, J., P. K. Peterson, and P. G. Quie.** 1977. Kinetics of staphylococcal opsonization, attachment, ingestion, and killing by human polymorphonuclear leukocytes: a quantitative assay using ^{3}H-thymidine labeled bacteria. J. Immunol. Methods **14**:303–311.
33. **Washington, J. A.** 1972. Evaluation of two commercially available media for detection of bacteremia. Appl. Microbiol. **22**:604–607.
34. **Williams, R. C., Jr., and P. G. Quie.** 1971. Opsonic activity of agammaglobulinemic human sera. J. Immunol. **106**:51–55.