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Polymorphonuclear Neutrophil Chemotaxis Induced and Inhibited by *Bacteroides* spp.

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A possible virulence factor for *Bacteroides* subcutaneous abscesses has been found. The effect of *Bacteroides* culture filtrate and outer membrane on chemotaxis of rabbit peritoneal polymorphonuclear (PMN) neutrophils was assayed in Boyden chambers and via exocytosis of *N*-acetyl-β-glucosaminidase. Both *Bacteriodes* culture filtrate and outer membrane elicited some chemotaxis, as measured in the Boyden chamber; however, they had little effect upon exocytosis in cytochalasin B-treated PMN neutrophils. In the presence of serum complement, they completely abolished PMN neutrophil movement in the Boyden chamber assay, yet they gave a definite positive response for the exocytosis assay in the presence of serum complement or activated complement fragment.

Recently, much effort has been expended toward the discovery of virulence factors associated with *Bacteroides fragilis*. This microorganism produces a lipopolysaccharide (LPS) with very low biological toxicity (9, 11, 23); it does not resist phagocytosis and killing by polymorphonuclear (PMN) leukocytes (2, 3, 6, 17, 24), and it produces a capsular polysaccharide (13) which some investigators have equated with its ability to establish intra-abdominal abscesses in rats (18). A model subcutaneous abscess infection of *B. fragilis* in mice has also been established (26). In this investigation, we report the chemotactic and antichemotactic effect of the outer membrane (OM) and culture supernatant of *B. fragilis* on rabbit peritoneal PMN neutrophils.

MATERIALS AND METHODS

Bacterial strains and cultivation. Bacteria used were obtained from the culture collection of the Anaerobe Laboratory, Virginia Polytechnic Institute and State University, Blackburg. Three species of *Bacteroides* were used: *Bacteroides fragilis* (VPI 2553 [ATCC 25285], VPI 5383 [ATCC 23745], VPI 9032), clinical isolates; *B. vulgatus* VPI 4245 (ATCC 8482), a fecal isolate; and *B. thetaiotaomicron* VPI 5482 (ATCC 29184), a fecal isolate. Stock cultures were maintained at room temperature after growth for 24 h at 37°C in prereduced chopped-meat medium (10) under an atmosphere of O_2-free CO_2.

Bacterial products and components. Some strains of *Bacteroides* were grown in a modification (20) of the defined medium of Varel and Bryant (25) under O_2-free CO_2 at 37°C for 24 h. The cultures were centrifuged at 5,000 × *g* for 30 min, and the supernatant was filter sterilized by passage through a 0.45-μm (pore diameter) membrane filter. These preparations were either used immediately or stored at −20°C.

Batches of cells were grown for extraction of LPS or OM. Bacteria were grown under an atmosphere of O_2-free CO_2 in a prereduced Trypticase-yeast extract-glucose medium containing per liter: Trypticase, 10 g; yeast extract, 10 g; glucose, 10 g; salts solution (10), 40 ml; hematin (50 mg of hemin in 1 ml of 1 N NaOH, plus 99 ml of distilled water), 10 ml; and resazurin (0.025%, wt/vol), 4 ml; adjusted to pH 7.0. The cultures were incubated at 37°C for 36 to 48 h and checked for contamination; then the cells were pelleted by centrifugation at 12,100 × *g* for 20 min. The cell pellets were washed twice by suspension in 1/10 volume of phosphate-buffered saline, pH 7, followed by centrifugation.

LPS was extracted from *B. fragilis* VPI 9032 by a modified Westphal procedure (14). The LPS was assayed (20) for the ability to gel *Limulus* amoebocyte lysate and for protein by the assay of Lowry et al. (16), with bovine serum albumin as the standard. The LPS contained 1.2% protein, and 25 ng gave a soft-gelling *Limulus* amoebocyte lysate. The OM of the *Bacteroides* was isolated by the method of Kasper and Seiler (13). The material we used was the crude OM which was isolated by centrifugation at 80,000 × *g*; this was suspended in a minimal volume of distilled water and lyophilized. The OM was assayed in the same manner as the LPS. The OM preparations contained 23 to 49% protein and gave soft gelation of the *Limulus* amoebocyte lysate at 62.5 to 125 ng.

Rabbit peritoneal exudate cells. Our procedure was similar to that of other investigators (7, 18, 27). Adult New Zealand white rabbits were injected intraperitoneally with 100 ml of 0.1% (wt/vol) sterile glycogen in isotonic saline. Four to 7 h later, 100 ml of sterile heparinized saline (5 U of heparin/ml) was injected into the peritoneum. Peritoneal fluid was withdrawn aseptically via a 16-gauge cannula by gravity drainage. Cells were not harvested from individual rabbits more often than once every 3 weeks. Contaminating erythrocytes in the peritoneal exudates were

lysed with 0.83% (wt/vol) NH_4Cl (pH 7.3), and exudates contaminated with large amounts of erythrocytes were discarded. Peritoneal exudates were centrifuged at 1,000 × *g* for 5 min at 4°C. The cell pellet was washed twice with at least 30 ml of sterile physiological saline. Wright-stained smears and wet-mount differential counts revealed that the suspensions contained at least 98% PMN neutrophils. Appropriate dilutions were prepared in Hanks balanced salt solution (HBSS) containing 0.05% (wt/vol) bovine serum albumin.

Chemotaxis assay. Modified Boyden chambers (5, 8) were used with pretested 25-mm-diameter membrane filters of 0.8-μm pore diameter. The test sample in HBSS was added to the lower compartment; then 1 ml of rabbit peritoneal PMN neutrophils was added to the upper compartment, and the chamber was incubated at 37°C for 60 min. Duplicate chambers were incubated for each sample tested. The chambers were disassembled, and the membrane filters were fixed in 100% isopropanol for 3 min, stained with hematoxylin (8), and mounted on microscope slides with permount. Chemotaxis was determined as the penetration of PMN neutrophils into the membrane filter. A high-power objective (total ×400 magnification) was used to count the cells; cells were counted from the initial monolayer in 10-μm planes to the deepest plane containing two cells. Three random fields per filter were counted. The chemotactic index (CI), the average distance that cells migrated in 1 h at 37°C, was calculated in the following manner: cells in focus were counted at each 10 μm from the original monolayer to the distant plane on the membrane filter. The number of cells counted per level was multiplied by the distance (in micrometers) of that level from the starting monolayer. The sum of the products was divided by the total number of cells migrating.

Chemotactic factor from human complement (C5fr). Purified C5a fragment (C5fr) was supplied by Donald L. Kreutzer, Pathology Department, University of Connecticut Health Center, Farmington. Chemotactically active C5fr was stored at −20°C in 0.5-ml portions. A portion was thawed before use, and 1.5 ml of 0.5 M potassium phosphate buffer (pH 7.3) was added. The quantity required to produce 50% maximal response in Boyden chambers (7) was established, and threefold this amount was used as a positive control for chemotaxis assays and enzyme release assays from one cell preparation of PMN neutrophils to another.

Induced lysosomal enzyme release. Washed neutrophils were suspended to a final concentration of 10^7/ml in HBSS containing 2 mg of bovine serum albumin and 4 μg of cytochalasin B (Sigma Chemical Co., St. Louis, Mo.). The cytochalasin B was diluted from a stock solution containing 4 mg of cytochalasin B per ml of dimethyl sulfoxide. The dimethyl sulfoxide remaining after dilution was shown not to affect enzyme release (19). In duplicate, 0.5 ml of cell suspension was added to 0.5 ml of HBSS containing 50 μl of sample in test tubes (12 by 75 mm) at 4°C. The mixtures were incubated 5 min at 37°C, and the tubes were centrifuged at 2,000 × *g* for 5 min at 4°C. The supernatants were removed, and equal portions were taken for measurement of lactate dehydrogenase and *N*-acetyl-β-D-glucosaminidase.

Enzyme assays. *N*-acetyl-β-D-glucosaminidase was assayed by an adaptation (1) of the method of Woollen et al. (27). Positive (C5fr) and negative (HBSS) controls were included with each assay. Controls using only bacterial products or bacterial components gave no *N*-acetyl-β-D-glucosaminidase activity. All tests were performed in triplicate.

Lactate dehydrogenase was assayed as the oxidation of nicotinamide adenine dinucleotide, reduced form, measured by the decrease in absorbance at 340 nm (4). The reagents cytochalasin B and *p*-nitrophenol-β-D-glucosaminide were purchased from Sigma; guinea pig complement was purchased from Flow Laboratories, Inc., McLean, Va.

Statistical analysis. The data from the Boyden chambers and the exocytosis assays were evaluated for statistical significance by a two-tailed Student *t*-test.

RESULTS

Induction of PMN neutrophil locomotion. The crude OM of *B. fragilis* (ATCC 23475) was assayed at various concentrations to determine possible chemotactic activity for rabbit peritoneal PMN neutrophils. The response of the neutrophils was positive for locomotion as assayed by increase in the number of neutrophils per high-power field at increasing depth into the micropore filter and increasing CI (Fig. 1). A proportional increase of CI was observed with increase in the amount of OM. *Bacteroides* cells were cultured in a defined minimal medium and removed from the culture by centrifugation followed by membrane filtration. CF of *B. fragilis* (VPI 9032) and *B. thetaiotaomicron* (ATCC 29184) gave chemotactic responses similar to that of the OM. Their response was also greater with increasing doses from 50 to 150 μl. Fresh sterile defined minimal medium gave no chemotactic response.

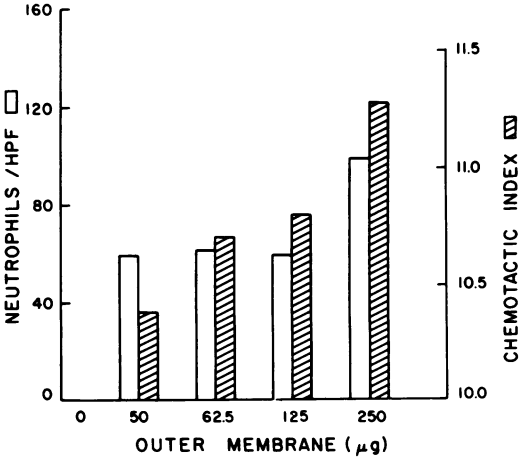

FIG. 1. *Dose response of rabbit PMN neutrophils to B. fragilis (ATCC 23745) OM.*

Chemotaxis versus chemokinesis. *B. vulgatus* (ATCC 8482) OM was definitely chemotactic (locomotion directed toward a concentration gradient) for rabbit peritoneal PMN neutrophils (Table 1). The neutrophils consistently moved toward a positive gradient of OM. When either no gradient or a negative gradient was established, neutrophils did not actively penetrate into the membrane filters.

Interaction of *Bacteroides* cellular components or products with rabbit peritoneal PMN neutrophils. LPS, CF, and OM of *B. fragilis* induced some chemotaxis of rabbit neutrophils (Table 2), not to the level of the positive control (C5fr), but definitely more than the HBSS control. Addition of guinea pig complement to the reaction mixture caused a marked increase in the CI for the LPS. The effect of guinea pig complement was even more dramatic on the CF and the OM of *B. fragilis*. Guinea pig complement reacting with the CF or OM caused all PMN locomotion to cease. Possibly an inhibitor of chemotaxis was produced or activated. When purified human C5fr of complement was reacted with CF or OM of *Bacteroides*, the effects on locomotion were additive (Table 3). The lower compartment received one half of the *Bacteroides* factor and one half of the C5fr, yielding intermediate CIs.

Exocytosis of a lysosomal enzyme. Neutrophils treated with cytochalasin B respond to chemotactic stimulus by releasing lysosomal enzymes into their surrounding medium. Table 4 gives the amount of enzyme (*N*-acetyl-β-D-glucosaminidase) released in response to the interaction of neutrophils with cellular components or products of *Bacteroides*. Slight exocytosis activity was observed with the CF of *Bacteroides*, but very little activity for the OM or LPS. In the presence of guinea pig complement, all of these components and products of *Bacteroides* were chemotaxigenic, giving an even greater response than the positive control (Cfr).

TABLE 1. *Directional locomotion[a] of rabbit PMN neutrophils induced by established gradients of B. vulgatus (ATCC 8482) OM*

Concn (μg/ml) of OM above filter	Concn (μg/ml) of OM below filter			
	0	50	100	150
0	10.0	11.61	10.92	11.89
50	10.0	10.0	12.38	10.50
100	10.0	10.0	10.0	11.32
150	10.0	10.0	10.0	10.0

[a] Measured as the CI (the average distance traveled per PMN neutrophil from the top of the membrane filter toward the bottom).

DISCUSSION

We noted that a slight to moderate amount of chemotaxis was induced by OM, LPS, and CF of *Bacteroides*. This activity was shown to be chemotaxis and not simply chemokinesis. However, the fact that this directional locomotion of PMN neutrophils was obliterated when *Bacteroides* CF or OM reacted with serum complement was even more interesting. This leads one to suggest that the OM of *Bacteroides* is a virulence factor of this microorganism which functions in a different manner from that already suggested by Onderdonk et al. (18). The OM also contains LPS, which was shown by Sveen (22) and confirmed by us to produce chemotaxins upon reaction with serum complement. The LPS alone induced only slight chemotaxis; however, in the presence of serum complement, it induced considerable chemotaxis. However, when the OM reacted with serum complement, it inhibited chemotaxis completely, in spite of the LPS-complement interaction.

In order for *Bacteroides* to induce pyogenic

TABLE 2. *Rabbit peritoneal PMN neutrophil response to B. fragilis cellular components*

Addition to lower compartment	Chemotactic activity		Chemotactic activity with complement[a]	
	PMN/4 HPF ± SEM[b]	CI	PMN/4 HPF ± SEM	CI
HBSS (control)	14.5 ± 2.1	10.0	16.3 ± 4.9	10.3
CF (50 μl), VPI 5383	257 ± 4.4[c]	12.14[c]	18.5 ± 13.1[d]	10.0[d]
LPS, (62.5 μg), VPI 9032	66 ± 4.9[c]	11.17[d]	75.8 ± 14.4[c]	13.27[c]
OM (62.5 μg), VPI 2553	62.5 ± 7[c]	10.94[c]	53.3 ± 6.4[c]	10.0[d]
OM (62.5 μg), VPI 5383	83 ± 4.2[c]	11.20[d]	24.3 ± 1.4[c]	10.0[d]
OM (62.5 μg), VPI 9032	88.5 ± 19.3[c]	12.29[c]	10 ± 7.1[d]	10.0[d]
C5fr (30 μl)	152 ± 11.5[c]	13.56[c]		

[a] One milliliter of CF or HBSS containing 62.5 μg of OM or LPS was added to 1 ml of guinea pig complement and incubated at 37°C for 30 min. Then 0.8 to 1.0 ml of one of these mixtures was added to the lower compartment of a Boyden chamber.

[b] HPF, High-power field; SEM, standard error of the mean.

[c] $P < 0.05$, significantly larger than HBSS control.

[d] $P > 0.05$; insignificant.

infections, or any infection for that matter, it must first avoid phagocytosis or intracellular killing by PMN neutrophils. Since murine peritoneal exudate PMN neutrophils readily ingest and kill *Bacteroides* (J. F. Sperry, unpublished data), another mechanism of establishing infection would be to avoid early contact with the neutrophils by inhibiting chemotaxis of neutrophils long enough to allow sufficient growth of *Bacteroides* to eventually overwhelm the phagocytes and establish infection.

Another point discovered here was that the *Bacteroides* CF or OM could separate directed locomotion of PMN neutrophils from exocytosis of granular enzymes in cytochalasin B-treated PMN neutrophils. This is contrary to earlier reports (7) which state that these two phenomena are directly related and that by observing a positive response for one reaction, the other is also positive. We may be able to further evaluate interactions during the response of PMN neutrophils to foreign stimuli because of this discovery.

The inhibitory effect upon chemotaxis produced by reacting OM or CF with serum complement was not produced by mixing OM or CF with purified C5fr of activated complement. Therefore, the effect was not directly on C5fr.

Kasper and Seiler (13) and Onderdonk et al. (18) have associated a capsular polysaccharide, only present in *B. fragilis*, as the virulence factor important for inducing intra-abdominal abscesses in rats. The antichemotactic activity was found in all the *Bacteroides* isolates we tested, including strains that were tested by Lindberg et al. (15) and shown not to contain the specific capsule demonstrated by the ruthenium red staining. Recently, Kasper et al. (12) demonstrated the appearance of a phase variation in one strain (ATCC 23745) of *B. fragilis* during passage on laboratory media. The variant lost a fair amount of the ruthenium red-staining capsular material, which was replaced by a glycan. This lends further support to our contention that the component of the OM of *Bacteroides* that reacts with serum complement to inhibit locomotion of PMN neutrophils should be a different component of the OM, perhaps a protein. This is presently under investigation.

TABLE 3. *Chemotactic response of rabbit peritoneal PMN neutrophils to B. fragilis (VPI 5383)*

Addition to lower compartment	Chemotactic activity		Chemotactic activity with C5fr	
	PMN/4 HPF ± SEM[a]	CI	PMN/4 HPF ± SEM	CI
HBSS (control)	17.3 ± 2.2	10.0	394 ± 28	17.06
CF (50 μl)	257 ± 4.4[a]	12.14[a]	313 ± 23.4	14.29
OM (62.5 μg)	237 ± 13.8[a]	12.83[a]	301 ± 49.8	14.60

[a] See footnote *b*, Table 2.
[b] $P \leq 0.05$, significantly larger than HBSS control.

TABLE 4. *N-acetyl-β-glucosaminidase activity released from cytochalasin B-treated rabbit peritoneal PMN neutrophils*

Addition to reaction mixture	nmol of *p*-nitrophenol released[a]	
	Without guinea pig complement	With guinea pig complement
HBSS (control)	66.7[a]	68.0[a]
C5fr	112.8 ± 0[b]	
CF, VPI 2553	10.6 ± 0.5[b]	118.6 ± 5.1[b]
CF, VPI 4245	13.0 ± 1.1[b]	145.7 ± 27.3[b]
CF, VPI 5383	9.1 ± 0.6[b]	142.2 ± 33[b]
LPS, VPI 9032	4.5 ± 1.1[b]	124.5 ± 8.0[b]
OM, VPI 2553	4.9 ± 13.6[c]	167.4 ± 6.8[b]
OM, VPI 5383	4.9 ± 4.5[c]	145 ± 4.5[b]
OM, VPI 9032	5.2 ± 6.8[c]	138.5 ± 4.5[b]

[a] The control value has been subtracted from the test samples.
[b] $P \leq 0.05$, significantly larger than HBSS control.
[c] $P > 0.05$, insignificant.

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