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Polymorphonuclear Neutrophil Chemotaxis Modulated by *Bacteroides fragilis* Peptidoglycan

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Peptidoglycan was isolated from *Bacteroides fragilis* with boiling sodium dodecyl sulfate, and some was treated with pronase to eliminate contaminating protein. This peptidoglycan was chemotactic for rabbit polymorphonuclear neutrophils and had even greater chemotactic activity along with some chemokinetic activity after it was partially hydrolyzed with lysozyme. Significant chemotaxis-inhibitory activity was observed for an acid-precipitable component of the lysozyme-treated crude peptidoglycan of *B. fragilis*.

Previously, we demonstrated that crude outer membranes (OM) extracted from *Bacteroides fragilis* are chemotactic for rabbit polymorphonuclear (PMN) neutrophils (1); we also noted chemotactic activity in the culture supernatant that was membrane filtered (pore diameter, 0.45 μ m). We have also demonstrated that certain components of *B. fragilis* are either antichemotactic or react with serum to inhibit PMN neutrophil chemotaxis to the partially hydrolyzed peptidoglycan (PG) of *B. fragilis* and direct chemotaxis inhibition to protein-associated PG fragments of *B. fragilis*. Since *B. fragilis* readily lyses and releases cell envelope fragments (some of which modulate PMN neutrophils) these fragments might be acting as a smoke screen to interfere with the normal inflammatory response long enough for the bacteria to initiate an abscess.

We noticed considerable variation in chemotaxis induced by different OM preparations from *B. fragilis*. All crude OM and mixed membranes (pelleted from culture supernatant at 40,000 $\times g$ for 45 min) showed a definite increase in chemotactic activity following treatment with lysozyme, even OM isolated from a mucoid colony phase variant (Table 1). Therefore, we isolated the PG from *B. fragilis* VPI 9032 which had been grown in Trypticase (BBL Microbiology Systems, Cockeysville, Md.)-yeast extract-glucose broth under O₂-free CO₂ for 24 h at 37°C (11). Bacteria (8 liters of culture) were harvested by centrifugation at 12,100 $\times g$ for 20 min at 4°C and washed twice in phosphate-buffered saline (pH 7.2). Either whole bacteria or the first pellet from the OM extraction procedure of Kasper and Seiler (7) was suspended in distilled water (approximately 100 ml) and added dropwise to 300 ml of boiling 4% (wt/vol) sodium dodecyl sulfate with constant stirring (10). After being boiled for an additional 10 min, the solution was cooled to lukewarm and centrifuged at 36,000 $\times g$ for 1 h at 20°C. The pellet was suspended in distilled water and recentrifuged; this step was repeated five times. The final pellet was resuspended in 60 ml of distilled water, sonicated for 30 s, and lyophilized. This material contained 11% protein as assayed by the procedure of Lowry et al. (8). Another batch (150 mg) of crude PG was suspended in 60 ml of 0.01 M Tris hydrochloride buffer (pH 7.4), sonicated for 30 s, and treated with 3 mg of pronase (Sigma Chemical Co., St. Louis, Mo.) at 37°C for 30 min. The suspension was centrifuged at 36,000 $\times g$ for 1 h at 20°C, suspended in 60 ml of distilled water, and lyophilized. The pronase-treated PG contained 1% protein.

The PG were suspended (0.5 mg/ml) in 0.01 M Tris hydrochloride buffer (pH 7.4), and 1 mg of lysozyme (Sigma) was added per 50 mg of PG. The suspensions were incubated at 37°C for 15 h and then centrifuged at 36,000 $\times g$ for 1 h at 20°C. The pellet was designated pellet A. The supernatant was adjusted to pH 3.7 with acetic acid and was centrifuged at 36,000 $\times g$ for 1 h to pellet muramyl dipeptides and muramyl dipeptide-linked lipoprotein (3). This pellet was designated pellet B.

Adult New Zealand White rabbit peritoneal exudate cells were elicited with 0.1% (wt/vol) glycogen in sterile saline and harvested in sterile heparinized saline (5 U/ml) as described

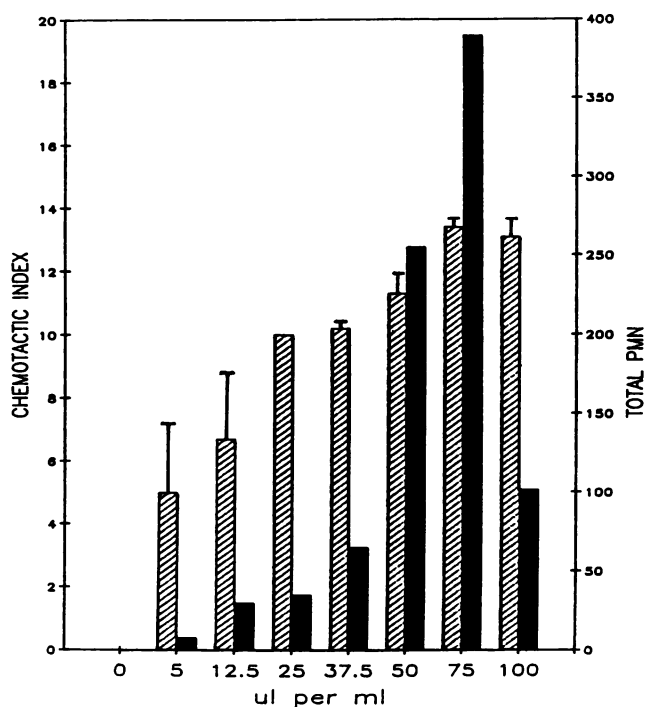


FIG. 1. Dose-dependent chemotaxis of rabbit peritoneal PMN neutrophils induced by the lysozyme-treated *B. fragilis* PG pellet. The pellet from 21.5 mg (dry weight) of *B. fragilis* PG treated with 430 μ g of lysozyme at 37°C for 15 h and then centrifuged 36,000 $\times g$ for 1 h was suspended in 1 ml of distilled water. \square , Chemotactic index \pm standard error of the mean; \blacksquare , total number of PMN neutrophils penetrating $\geq 10 \mu$ m into six high-power fields (three random fields per filter).

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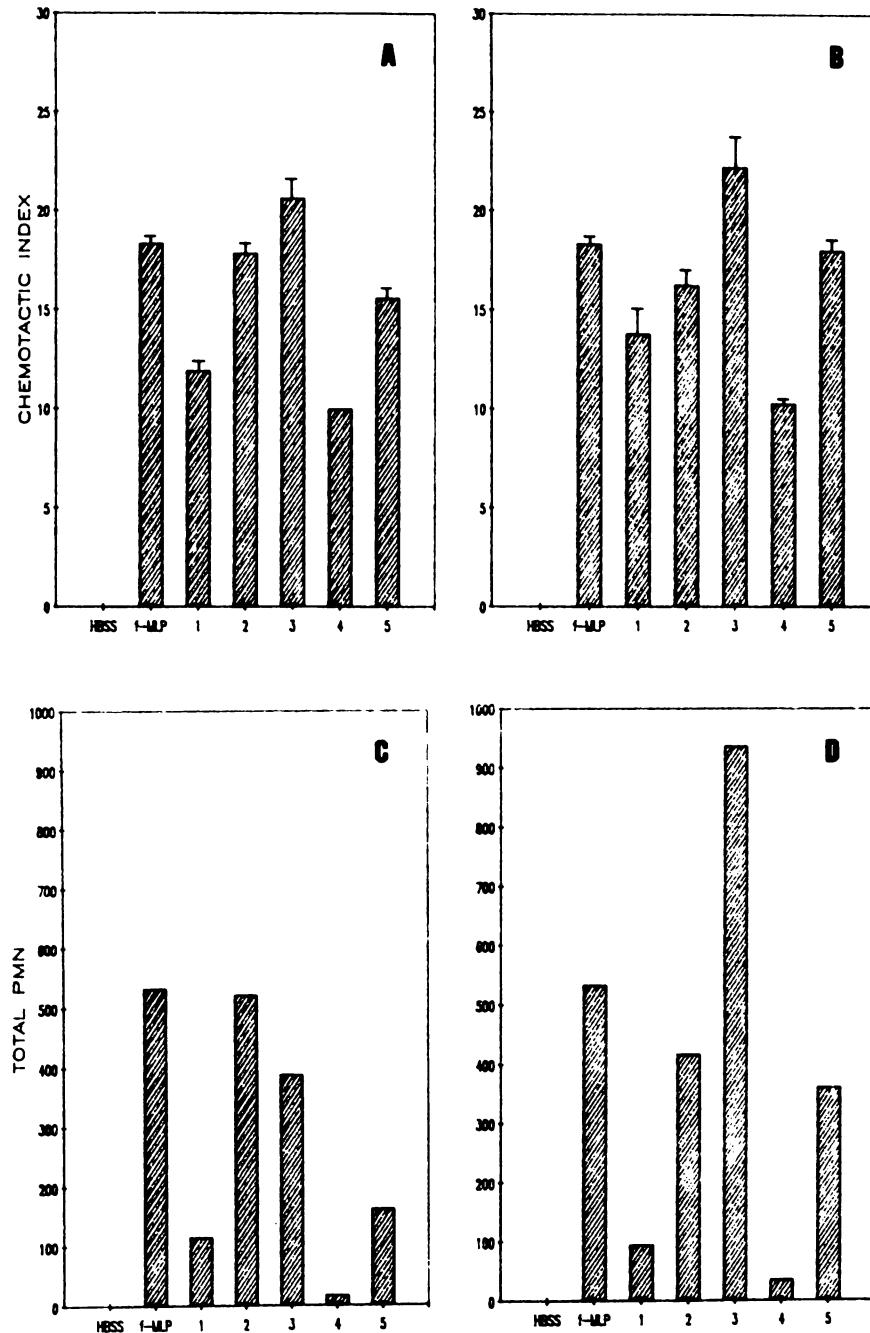


FIG. 2. Effects of lysozyme treatment on crude and pronase-treated *B. fragilis* PG components. HBSS, Negative control; f-MLP, positive control. (A and C) Crude PG; (B and D) pronase-treated PG. Bars: 1, untreated PG; 2, pellet A of lysozyme-treated PG; 3, pellet A plus f-MLP; 4, pellet B of lysozyme-treated PG; 5, pellet B plus f-MLP. In panels A and B the chemotactic index \pm standard error of the mean is given, and in panels C and D the total number of PMN neutrophils penetrating $\geq 10 \mu\text{m}$ into six high-power fields (three random fields per filter) is given.

before (1). Cells were washed twice with sterile physiological saline. Wright-stained smears and wet-mount differential counts revealed that the suspensions contained at least 98% PMN neutrophils. Appropriate dilutions were made in Hanks balanced salt solution (HBSS) containing 0.5% (wt/vol) bovine serum albumin. Chemotaxis was measured by a modified Boyden chamber assay (2) by using pretested membrane filters (diameter, 25 mm; pore diameter, 0.8 μm). HBSS (1 ml) containing 2.5×10^6 neutrophils was added to the upper compartment, and 1 ml of HBSS containing

chemoattractant was added to the lower compartment. Chambers were incubated at 37°C for 1 h. Duplicate chambers were incubated for each sample tested. Chambers were disassembled, and filters were fixed in 100% isopropanol, stained with hematoxylin (1), and mounted on microscope slides with immersion oil. The chemotactic index was calculated as described before (6). Previously, we demonstrated that crude OM is chemotactic for rabbit PMN neutrophils by showing that neutrophils move toward an OM gradient and that the effect is dose-dependent chemotaxis (1). Lysozyme-

TABLE 1. Rabbit PMN neutrophil chemotaxis produced by *B. fragilis* crude OM

Chemotaxin	Chemotactic index ^a	Total cells/six high-power fields
HBSS	2.22 ± 1.75	0
OM VPI 2553	1.67 ± 1.67 ^b	2
OM VPI 2553 (lysozyme-treated)	17.61 ± 1.31 ^c	129
OM VPI 9032	1.67 ± 1.67 ^b	2
OM VPI 9032 (lysozyme-treated)	14.11 ± 0.90 ^c	80
OM VPI 9032m ^d	13.38 ± 0.94 ^c	38
OM VPI 9032m ^d (lysozyme-treated)	16.51 ± 1.51 ^c	42

^a Chemotactic index reported as average penetration of membrane filters in micrometers ± standard error of the mean.

^b No significant ($P > 0.05$) chemotaxis compared with HBSS control.

^c Significant ($P < 0.01$) chemotaxis compared with HBSS control.

^d Mucoid colony phase variant (VPI 9032m) was isolated from a mouse subcutaneous abscess produced by inoculating the mouse with translucent VPI 9032.

treated PG pellet A showed similar dose-dependent chemotaxis (Fig. 1); at 12.5 μ/ml the chemotactic index was significantly ($P < 0.05$) greater than that for the HBSS control, and the higher concentrations were significantly ($P < 0.01$) more chemotactic. There was also positive directional locomotion of PMN neutrophils toward an increasing gradient of the lysozyme-treated PG (Table 2). Chemokinetic activity was also observed in increasing penetration of filters by neutrophils as the concentration of the reactant was increased in the absence of a gradient (values in boldface type). Expected values due to chemokinesis alone were calculated by the method of Zigmond and Hirsch (15).

The effects of lysozyme treatment on the crude and pronase-treated PG of *B. fragilis* are shown in Fig. 2. Partially hydrolyzed PG (A2 and B2) was significantly ($P < 0.01$) more chemotactic than unhydrolyzed PG was (A1 and B1). There appeared to be some chemotactic inhibitory activity in the protein associated with the crude PG; i.e., there was no significant increase of total chemotactic activity when formylmethionyl-leucyl-phenylalanine (f-MLP) was added to the partially hydrolyzed crude PG (A2; compare A3 and A2). However, there was a significant increase in total chemotactic activity when f-MLP was added to the partially hydrolyzed pronase-treated PG (B2; compare B3 and B2). Pellet B (A4, B4, C4, and D4; possible lipoprotein-containing pieces of PG) appeared to have little chemotactic activity.

TABLE 2. Directed locomotion of rabbit PMN neutrophils induced by established gradients of *B. fragilis* VPI 9032 lysozyme-treated PG pellet

Concn (μ/ml) of PG below filter	Directed locomotion with concn (μ/ml) of PG above filter ^a :			
	0	10	20	30
0	0	10.3 (9.8)	10.6 (12.3)	10.2 (13.1)
10	12.0 (0.2)	10	10.0 (12.4)	11.4 (13.1)
20	13.1 (0.4)	12.7 (10.1)	12.5	11.4 (13.2)
30	13.9 (0.6)	12.0 (10.2)	11.4 (12.5)	13.2

^a Directed locomotion was measured as the chemotactic index (average distance traveled per PMN neutrophil from the top of the membrane filter toward the bottom). The numbers in parentheses are theoretical values expected on the basis of chemokinetic activity. The table field is divided into the negative gradient (upper right) and the positive gradient (lower left) of chemotaxin by homologous values (boldface).

However, when f-MLP was added to pellet B from crude PG (A5 and C5) and to pellet B from the pronase-treated PG (B5 and D5), the results further suggested that the protein associated with the PG has chemotaxis-inhibitory activity.

Muramyl dipeptides from *Mycobacterium tuberculosis* have adjuvant activity (13), inhibit macrophage migration (9), induce epithelioid granuloma formation (5, 12), and cause uveitis in rabbits (14). Adjuvant activity has also been demonstrated for muramyl dipeptides of *Escherichia coli*, *Bacillus cereus*, *Micrococcus roseus*, and *Staphylococcus epidermidis* (4). Therefore, the possibility that the effects of lysozyme-treated PG components of *B. fragilis* on phagocytes are involved in the initiation of subcutaneous abscesses does have precedent. The presence of a protein component that inhibits chemotaxis (Fig. 2) might help initiate production of subcutaneous abscesses by interfering with phagocytic clearing of the infection site long enough to allow formation of the abscess.

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