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Induction of Macrophage Procoagulant Activity by *Bacteroides fragilis*

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Fibrin deposition in the peritoneal cavity during acute peritonitis appears to predispose the host to abscess formation by providing an environment for bacterial proliferation protected from host defenses. The purpose of the present study was to determine whether the potent abscess-inducing anaerobe *Bacteroides fragilis* could promote fibrin deposition by inducing mononuclear cells to express procoagulant activity (PCA). *B. fragilis* stimulated PCA in a dose-dependent fashion, achieving a maximum at 10^7 CFU/ml. Heat-killed *B. fragilis* induced comparable levels of PCA, while a nonspecific phagocytic stimulus, latex beads, was not stimulatory. *B. fragilis* was capable of inducing PCA even when phagocytosis was blocked by preexposure of cells to latex beads. The results suggested that phagocytosis was neither necessary nor sufficient for the generation of PCA. Cell separation studies showed that PCA was solely produced by macrophages and that lymphocytes did not augment its production. These studies suggest one potential mechanism by which *B. fragilis* might initiate abscess formation.

Three major local defense mechanisms become active following bacterial soiling of the peritoneal cavity in cases of acute peritonitis (12). These include (i) mechanical clearance of bacteria via the diaphragmatic lymphatics, (ii) opsonization and phagocytosis of bacteria by neutrophils and macrophages, and (iii) bacterial sequestration within fibrinous deposits. Fibrin deposition has been shown to have a double-edged effect on the outcome of peritonitis. Bacterial trapping by fibrin reduces the magnitude of the acute bacteremia, thereby lowering the mortality rate (2, 46). However, the resultant bacterial sequestration within the fibrin matrix appears to predispose the host to abscess formation, presumably by impairing host defense mechanisms (6, 34). Ahrenholz and Simmons (2) have demonstrated that bacteria enmeshed within a fibrin clot are capable of inducing intraperitoneal abscesses, while comparable numbers of bacteria inoculated by free intraperitoneal injection are unable to do so. Further supportive evidence for the role of fibrin in abscess formation is derived from studies in which anticoagulants (4, 13) or fibrinolytic agents (33a) were able to prevent abscesses when administered at the time of the initiation of infection. Taken together, these data suggest an important role for fibrin deposition in the pathogenesis of intraabdominal abscesses.

The gram-negative anaerobe *Bacteroides fragilis* is a common isolate from intraabdominal abscesses (10), and in experimental models it is a potent abscess inducer when inoculated as a monomicrobial inoculum (28, 29, 44). Several unique properties have been documented which may account for its ability to survive in vivo. These include its oxygen tolerance (41), its ability to resist opsonophagocytosis (39), and its increased adherence to the peritoneum (30). The last two characteristics have been attributed to the unique capsular polysaccharide that is present on strains of *B. fragilis* (19). Despite the fact that we understand these survival characteristics, little is known about the critical initiating events involved in abscess induction by *B. fragilis*. Recent studies by Shapiro and colleagues (38) have demonstrated the importance of T lymphocytes in the genesis of these abscesses. Since fibrin deposition appears to be critical to the establishment of abscesses, one further hypothesis is that induction of fibrin deposition by *B. fragilis* may be one potential mechanism by which *B. fragilis* initiates abscess formation.

Several pathways have been implicated in the pathogenesis of extravascular fibrin deposition during inflammation. Increased capillary permeability observed in inflammatory lesions may expose the contact factors of plasma to activators such as collagen in the extravascular space. Alternatively, the cells of the inflammatory lesion may supply one or more procoagulants (procoagulant activity [PCA]) that are capable of activating clotting further down the cascade. Leukocytes have been known to influence the local coagulation of blood. The monocytes and macrophages that are involved in inflammatory responses have been demonstrated to express procoagulants on their cell surfaces. This activity can be generated by mononuclear cell cultures both in vitro and in vivo in response to endotoxin (5, 9, 22), mitogens (9), antigens (9), immune complexes (32), allogeneic cells (33), viruses (23), and proteolytic products of the complement cascade (26, 31). In several inflammatory processes such as delayed-type hypersensitivity as well as in disease states including glomerulonephritis and encephalomyelitis, the ability of macrophages to express PCA, thereby enhancing local fibrin deposition, has been shown to be critical to the pathogenesis of the disease (7, 11, 14, 15). Since large numbers of macrophages are present in the peritoneal cavity soon after bacterial contamination of the peritoneum (8), we hypothesized that induction of macrophage PCA by *B. fragilis* may be one of the early events contributing to the initiation of intraabdominal abscesses.

MATERIALS AND METHODS

Growth and culture of *B. fragilis*. *B. fragilis* VPI 9032 was provided by Tracy Wilkins (Virginia Polytechnic Institute and State University, Blacksburg, Va.). Stock cultures were

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maintained at −70°C in thioglycolate broth (BBL Microbiology Systems, Cockeysville, Md.). Frozen cultures were thawed, and portions of these cultures (0.2 ml) were inoculated into tubes containing 20 ml of minimal growth medium, as described previously (27). The tubes were incubated for 20 to 24 h at 35°C in an anaerobic chamber (Forma Scientific, Marietta, Ohio) in which an atmosphere of 85% nitrogen, 10% hydrogen, and 5% CO_2 was maintained.

For the addition to mononuclear cells, the *B. fragilis* culture was removed from the anaerobic chamber, pelleted by centrifugation (1,800 × *g* for 20 min), washed twice in sterile saline, and suspended in 20 ml of saline. To quantitate viable bacteria, suspended *B. fragilis* was serially diluted and surface plated onto supplemented brain heart infusion agar. Surface colonies were counted after 48 h of incubation at 35°C in the anaerobic chamber.

Isolation of peripheral blood mononuclear cells. Heparinized blood from two rats was pooled and suspended in RPMI 1640 medium (Flow Laboratories, Mississauga, Ontario, Canada) at a 5 to 6:1 dilution. Mononuclear cells were isolated over Ficoll-Hypaque gradients (density, 1.074 g/ml) by centrifugation at 22°C and 1,800 × *g* for 15 min (22). Cells at the interface were collected and were found to contain <2% neutrophils by microscopic analysis. Viability was >98% by trypan blue exclusion. Cells were washed 3 times and suspended in RPMI 1640 medium containing 10% fetal bovine serum (endotoxin free), L-glutamine 4 mM (Sigma Chemical Co., St. Louis, Mo.), penicillin (50 U/ml)-streptomycin (50 μg/ml; GIBCO Laboratories, Grand Island, N.Y.), and 20 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) (pH 7.4; Sigma) (RPMI-10).

For experiments in which we investigated the specific cellular requirement for PCA production, pure populations of lymphocytes and macrophages were obtained as follows. Peritoneal macrophages were collected by lavage by using RPMI 1640 medium containing heparin (10 U/ml) 3 days following intraperitoneal injection of 4 ml of Brewer thioglycolate (Difco Laboratories, Detroit, Mich.). Cells were washed twice in RPMI 1640 medium and were then suspended at a concentration of 2 × 10^6/ml in RPMI-10. The cell suspension contained >95% macrophages, as determined by nonspecific esterase staining and latex bead ingestion. To obtain a pure population of T lymphocytes, spleens were removed aseptically from rats and cells were suspended in RPMI 1640 medium. Mononuclear cells were obtained by centrifugation over Ficoll-Hypaque (density, 1.074 g/ml) at 22°C at 1,800 × *g* for 15 min (3). Cells at the interface were collected, washed twice, and suspended in RPMI-10. The cells were then layered in flasks and incubated for 1 h at 37°C. Nonadherent cells were rinsed and suspended to 5 × 10^7/ml. The cell suspension was then added to a nylon wool column and incubated for 45 min at 37°C. Nonadherent cells were collected by washing the column with 60 ml of medium at a rate of 1 drop per s (17). Eluted cells were spun down at 1,500 rpm for 15 min and suspended in RPMI-10 to a concentration of 2 × 10^6/ml. By the end of the isolation process, the population was >98% lymphocytes, as determined by cytospin and nonspecific esterase staining. Cell viability was >98%, as determined by trypan blue exclusion.

Culture conditions. Peripheral blood mononuclear cells (PBMs; 2 × 10^6/ml) were cultured with various concentrations of *B. fragilis* (10^2 to 10^9 CFU/ml) for 24 h at 37°C in a 5% CO_2 atmosphere. Positive control studies for the generation of PCA were performed by adding *Escherichia coli* endotoxin (lipopolysaccharide [LPS]; 10 μg/ml; Sigma) to PBMs. Negative control studies were performed by adding sterile saline without *B. fragilis* to PBMs. At the end of the incubation period, PBMs were pelleted by centrifugation at 300 × *g* for 10 min, suspended in RPMI 1640 medium, and frozen at −70°C. In some studies, viable PBMs were tested for PCA without freeze-thawing, to determine the percentage of surface-expressed PCA.

PCA. After samples of cells were freeze-thawed, the cells were assayed for their capacity to shorten the spontaneous clotting time of normal citrated human plasma in a one-stage clotting assay (35). Results of studies by Lando and Edgington (20) have indicated that LPS-induced rat monocyte PCA is only slightly less effective at shortening the clotting time of human plasma compared with its effectiveness at shortening the clotting time of rat plasma. Because of its availability, human plasma was used in these studies. To 80 μl of freeze-thawed cells at 4°C, 80 μl of citrated normal human platelet-poor plasma was added, and then 80 μl of 25 mM $CaCl_2$ was added to initiate the reaction. The time for the appearance of a fibrin gel at 37°C was recorded. Clotting times were converted to units of PCA by comparison with a rabbit brain thromboplastin standard (Dade Division, American Hospital Supply, Miami, Fla.), in which 36 mg (dry weight) per ml was assigned a value of 100,000 mU of PCA. The assay was used over the range of 1 to 10,000 mU of PCA, with this range being linear with normal plasma substrate.

Nature of PCA. The nature of the PCA induced by *B. fragilis* (10^7 CFU/ml) was analyzed by using plasma that was congenitally deficient in factors V, VII, IX, or X (Helena Laboratories, Beaumont, Tex.). These were substituted for normal plasma in the one-stage clotting assay, and the ability of the PCA to accelerate the clotting time of these deficient plasma samples was compared with its ability to accelerate the clotting time of normal plasma.

Endotoxin contamination. RPMI 1640 medium, RPMI-10, saline, sterile culture medium, and the bacterial supernatant were tested for endotoxin contamination by the standard *Limulus* amoebocyte lysate assay (Association of Cape Cod, Woods Hole, Mass.) and were found to contain <0.003 ng of endotoxin per ml, which constituted the lower limits of the test.

Statistics. Data were analyzed by a one-way analysis of variance, and differences between groups were tested by Student's *t* test. Results are expressed as means and standard errors.

RESULTS

Induction of PCA by *B. fragilis*. Following a 24-h coincubation, *B. fragilis* induced PCA by PBMs in a dose-dependent fashion, achieving a maximum at 10^6 to 10^7 bacteria per ml (Fig. 1). This increase in PCA represented a shortening of the clotting time by 25 to 30 s. The maximum levels achieved closely approximated those produced by *E. coli* endotoxin (10 μg/ml) as the positive control (167 ± 38 mU/2 × 10^6 PBMs; $n = 11$). At higher concentrations of *B. fragilis*, PCA levels progressively returned to control levels.

The time course for optimal amplification of PCA by 10^7 *B. fragilis* per ml was examined (Fig. 2). Increased PCA levels were seen as early as 4 h into the incubation period and achieved a maximum following a 12- to 16-h incubation period. PCA induction by *B. fragilis* was similar whether incubation occurred under aerobic or anaerobic conditions and was not an artifact induced by the presence of *B. fragilis* in the one-step clotting assay, since the addition of 10^7 CFU of *B. fragilis* to the clotting assay containing untreated PBMs did not hasten the clotting time.

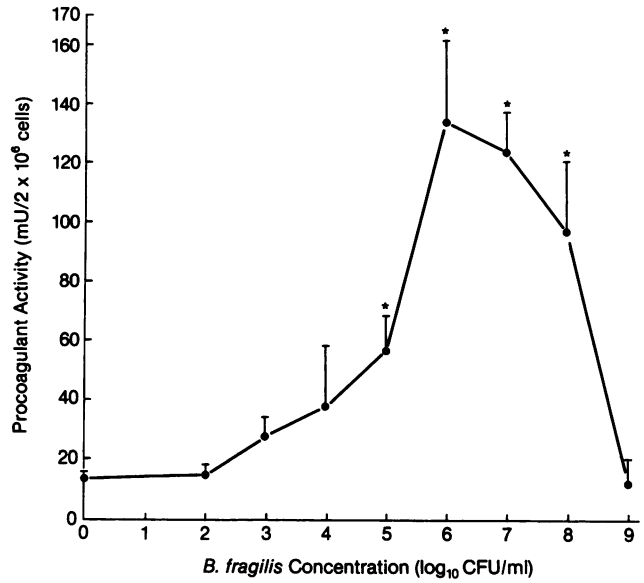

FIG. 1. Dose response of the effect of *B. fragilis* on mononuclear cell PCA. Various concentrations of *B. fragilis* were incubated with PBMs (2×10^6/ml) for 24 h at 37°C. Cells were then disrupted by freeze-thawing and tested for PCA per 2×10^6 PBMs, as described in the text. Each datum point represents the mean ± standard error of 5 to 13 experiments, each of which was performed in duplicate. The asterisk indicates $P < 0.001$, versus that in medium without *B. fragilis*.

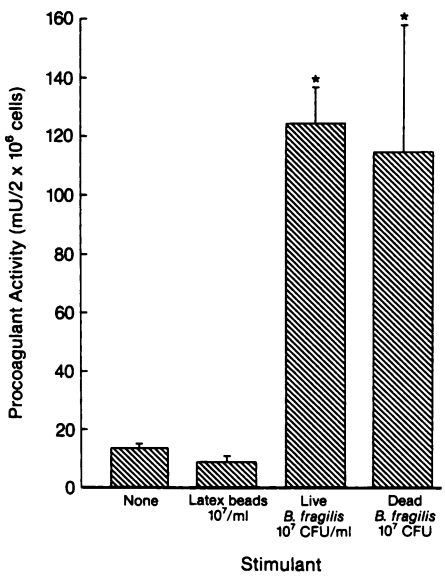

FIG. 3. Effect of latex beads or nonviable *B. fragilis* on the production of procoagulant activity by PBMs. Cells (2×10^6/ml) were disrupted by freeze-thawing following a 24-h incubation at 37°C with the designated particulate stimulus and tested for PCA. The data represent the mean ± standard error of 5 to 13 experiments per group, each of which was performed in duplicate. The asterisk indicates $P < 0.001$, versus that in cells that were incubated for 24 h in control medium.

Nature of PCA. Factor-deficient plasma samples were used to characterize PCA. Prolongation of the clotting time indicated that the deficient factor was necessary for the induced PCA to have its full effect. No clot formation occurred prior to 120 s in plasma deficient in factors V, VII, or X, indicating a PCA level of less than 10 mU. In factor IX-deficient plasma, the PCA level was comparable to that demonstrated in normal plasma. Thus, the nature of the PCA was consistent with that of tissue factor.

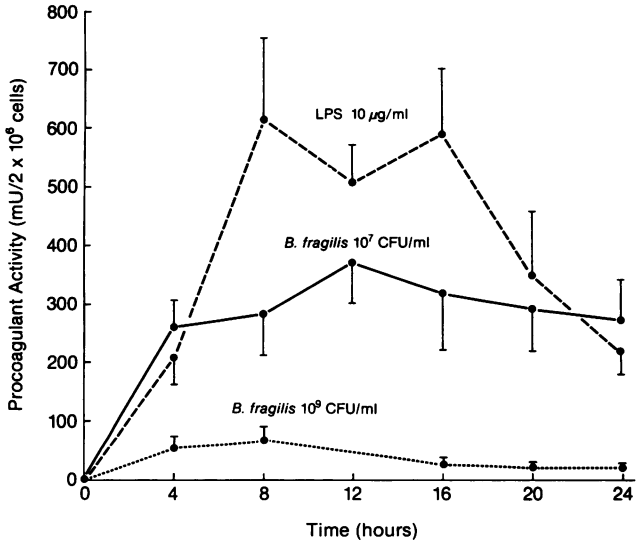

FIG. 2. Time course of induction of procoagulant activity by PBMs in response to *B. fragilis* (10^7 CFU/ml), *B. fragilis* (10^9 CFU/ml), or LPS (10 μg/ml). The results represent the mean ± standard error of four to eight experiments, each of which was performed in duplicate at each time point.

The role of phagocytosis in the induction of PCA. To determine whether phagocytosis was either sufficient or necessary for PCA expression by PBMs, nonviable particles were incubated with PBMs. Dead *B. fragilis* (10^7/ml) induced equivalent amounts of PCA, as did live *B. fragilis*, whereas latex beads (10^7/ml) did not stimulate PCA (Fig. 3). It was determined by light microscopy that both dead *B. fragilis* and latex beads were ingested in large numbers by the phagocytic cells in the PBM mixture. These results suggest that phagocytosis alone is not sufficient to induce PCA generation. To determine whether phagocytosis was necessary for PCA, PBMs were incubated with latex beads for 30 min, washed, and then incubated with *B. fragilis* for 4 h. At 4 h cells were examined by fluorescence microscopy following staining with acridine orange (8) and were tested for PCA. Preincubation of PBMs with latex beads prior to the addition of *B. fragilis* effectively prevented phagocytosis of *B. fragilis* but did not impair the ability of *B. fragilis* to induce PCA (latex alone, 22 ± 9 mU; latex beads followed by *B. fragilis*, 95 ± 17 mU; $n = 3$ per group; $P < 0.01$). Taken together, these results demonstrate that phagocytosis alone is neither necessary nor sufficient for the generation of PCA.

Cellular requirements of PCA production. Previous studies have suggested that lymphocytes may augment the response of monocytes to *E. coli* endotoxin (9, 22). Since PBMs consist of a mixture of monocytes and lymphocytes, studies were performed to determine the cell of origin of the PCA and the requirements for cell interaction (Fig. 4). Lymphocytes alone produced no PCA in response to *B. fragilis* (2 ± 0.4 mU; $n = 3$), while macrophages alone effectively did so, even in the absence of lymphocytes (502 ± 125 mU; $n = 5$). PCA measurements were generally two- to threefold higher

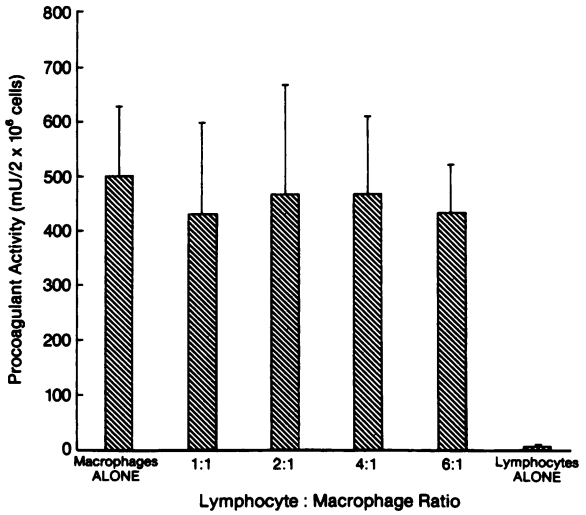

FIG. 4. Cellular requirements for the induction of procoagulant activity by *B. fragilis*. *B. fragilis* (10^7 CFU/ml) was incubated with lymphocytes alone, peritoneal macrophages alone, or a lymphocyte-macrophage coculture consisting of a 1:1 to 6:1 lymphocyte to macrophage ratio. Following a 24-h incubation at 37°C, cells were disrupted and assayed for PCA as described in the text. The results represent the mean ± standard error of three to five experiments, each of which was performed in duplicate.

for peritoneal cells than for PBMs because of the increased percentage of macrophages in the peritoneal cell population (~95 versus 30%). The addition of lymphocytes to macrophages did not further enhance PCA production.

Failure of high-dose *B. fragilis* to stimulate PCA. Several hypotheses were tested to determine why *B. fragilis* (10^9/ml) failed to induce PCA. One possibility was that an early peak of stimulation occurred that had returned to control levels by 24 h. This was unlikely, since PCA levels were only minimally elevated above that in the control at earlier times (Fig. 2). Another hypothesis was that PCA was indeed stimulated but that degradative enzymes known to be present on the surface of *Bacteroides* species rendered them inactive (40). This possibility was unlikely since *B. fragilis* (10^9 CFU/ml) that was rendered nonviable by boiling failed to induce the production of PCA by PBMs. In addition, the majority of PCA remained intracellular (96.5%; $n = 2$) and was measurable only after cellular disruption by freeze-thawing. It is unlikely that bacterial surface enzymes would have had adequate exposure to intracellular stores of PCA to effect inhibition.

Another potential explanation was that large numbers of bacteria, i.e., 10^9/ml, could inhibit the clotting mechanism, thereby rendering the one-step clotting assay inaccurate in its assessment of PCA. To test this hypothesis, PBMs were stimulated with *E. coli* endotoxin (10 μg/ml) for 24 h, and prior to performing the one-step clotting assay, 10^9 *B. fragilis* was added to the mixture. The addition of *B. fragilis* to the assay reduced the apparent PCA from 98 ± 3 mU (LPS alone; $n = 2$) to 1 ± 0.3 mU (LPS plus *B. fragilis* added to the assay; $n = 2$).

DISCUSSION

Fibrin deposition in response to bacterial peritonitis appears to play a critical role in sequestering and walling off infection (46). As a consequence of this host response, however, bacteria become enmeshed within an environment that is protected from host defense mechanisms (7, 34), thereby predisposing the host to residual infection and subsequent intraabdominal abscess formation. The ability of anticoagulants and fibrinolytic agents to prevent abscess formation in experimental infection models supports the concept that fibrin deposition may in fact be a critical initiating event in abscess formation. Results of the present studies demonstrate that *B. fragilis*, a common bacterial isolate of intraabdominal abscesses, is able to induce the generation of procoagulant activity by macrophages. This phenomenon represents a potential mechanism by which anaerobic bacteria may interact with host cells to initiate abscess formation.

B. fragilis induced PCA in a dose-dependent fashion, achieving a maximum at 10^7 bacteria per ml and returning to control levels at 10^9 bacteria per ml. PCA induction occurred early and reached a peak at 12 to 16 h. This time course of stimulation was similar to that noted for other stimuli (24). The nature of the PCA was consistent with that of tissue factor, as indicated by results of studies with factor-deficient plasma.

The biphasic nature of PCA induction by *B. fragilis*, as evidenced by the return to control levels of PCA at high bacterial concentrations, was an interesting observation. This pattern of stimulation is similar to that seen with other stimuli, including phorbol myristate acetate (24), the chemoattractant *N*-formyl-methionyl-leucyl-phenylalanine (16), gram-positive aerobic microorganisms (42), and *E. coli* LPS (22). Several hypotheses were tested to ascertain the mechanism of the inhibition observed at high concentrations of *B. fragilis*. It was unlikely to be caused by a missed peak, since PCA was reduced compared with that produced by 10^7 bacteria per ml throughout the 24-h time course examined. In addition, 10^9 heat-killed bacteria per ml failed to stimulate PCA, thereby arguing against the possibility that bacterial degradative enzymes may have contributed to the inhibition. The ability of 10^9 bacteria per ml to inhibit the LPS-induced shortening of the clotting time suggests that large numbers of *B. fragilis* in some way hindered fibrin polymerization, the endpoint of the one-stage clotting assay used to measure PCA. In this regard, *Bacteroides* species have been shown to bind fibrinogen (21). At high bacterial concentrations, this interaction may act to stearically inhibit fibrin polymerization.

Despite light microscopic evidence of bacterial phagocytosis, induction of PCA by *B. fragilis* was unlikely due to phagocytosis alone. Phagocytosis of latex beads did not stimulate PCA generation by PBMs. These findings are in agreement with those of Maier and Ulevitch (25), who used rabbit Kupffer cells, and Prydz and Allison (31), who used human monocytes. In addition, the ability of *B. fragilis* to stimulate PCA generation, despite blocking their phagocytosis by preexposure of cells to latex beads, further demonstrates that phagocytosis is not necessary for the induction of PCA. Taken together with the ability of dead *B. fragilis* to stimulate PCA, these data suggest that a surface component of *B. fragilis* is responsible for triggering PCA production. Two logical candidates for this component include (i) *B. fragilis* capsular polysaccharide, which has been shown to be able to induce abscess formation when injected intraperitoneally into mice (28, 29, 44), and (ii) *B. fragilis* endotoxin. The latter differs significantly from enterobacterial LPS, a known stimulus of PCA, in both its physical structure (18, 43) and biological activity (18). In a mouse model of intraabdominal infection, high doses of *B. fragilis* endotoxin were

able to induce abscesses (45). Further studies with purified components and different *Bacteroides* strains will help clarify the factor(s) responsible for the induction of PCA.

The present study is the first to demonstrate that the commonly isolated anaerobic bacterial species *B. fragilis* is capable of inducing macrophage PCA. Results of our studies differ significantly from those reported by van Ginkel and colleagues (42), in which opsonized gram-positive aerobic cocci were shown to induce PCA by human mononuclear cells. These investigators did not differentiate between an effect mediated by the bacteria themselves or by surface C3b, a known inducer of PCA (31). In the present studies, the use of heat-inactivated serum made it unlikely that C3b was the stimulus for PCA. In addition, based on the studies with dead *B. fragilis* as well as the phagocytosis studies, it appears that the interaction leading to the induction of PCA is not a nonspecific one resulting from the phagocytosis of a microorganism. Finally, while van Ginkel et al. (42) showed that lymphocytes are not responsible for the generation of PCA, they did not examine the role of the lymphocyte-macrophage interaction in the production of PCA. Cell separation experiments permitted us to conclude that a direct interaction between *B. fragilis* and macrophages can result in PCA production and that lymphocytes are neither necessary for nor did they augment the stimulation. In this regard, other investigators (36, 37) have reported that lymphocyte cooperation is not essential for LPS-induced monocyte PCA generation.

Fibrin deposition mediated by monocytes and macrophage PCA has been implicated as an early event in the pathogenesis of several disease processes, including glomerulonephritis (14), murine viral hepatitis (1), and encephalomyelitis (11). In the peritoneal cavity, endotoxin has been shown to cause the clumping of peritoneal macrophages by virtue of the PCA-induced fibrin deposition onto their surfaces (5). The results of the present studies indicate that *B. fragilis* can induce the expression of PCA by macrophages and suggest one possible mechanism by which these bacteria can initiate the formation of an infected fibrin nidus with the potential for subsequent abscess formation.

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