Inhibition of Macrophage Phagocytosis by *Bacteroides fragilis* In Vivo and In Vitro

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A number of investigators have provided experimental evidence for synergistic effects in mixed infections with *Escherichia coli* and *Bacteroides fragilis*. In vitro studies have suggested that competition for serum opsonins and diminished subsequent phagocytosis by polymorphonuclear leukocytes might explain these effects. In the present study we evaluated the effect of *B. fragilis* on macrophage phagocytosis. It was shown that peritoneal macrophages from mice injected intravenously 6 to 12 h earlier with 108 CFU of encapsulated *B. fragilis* were markedly suppressed in their phagocytic ability. Injections of laboratory-passaged, less-encapsulated *B. fragilis*, other bacteria, or latex particles were either not suppressive of macrophage phagocytosis or less effective. When peritoneal macrophages were treated in vitro for 24 h with the same challenge organisms prior to assessing their phagocytic capacity, encapsulated *B. fragilis* also proved significantly more suppressive than other organisms or latex particles. We conclude that suppression of macrophage phagocytosis by *B. fragilis* seems to be an important mechanism contributing to synergistic effects described for mixed aerobic and anaerobic infections.

A considerable number of clinical infections involve more than one etiologic agent. Infections after surgery often include both aerobic and anaerobic bacteria, and quite commonly, *Escherichia coli* and *Bacteroides fragilis* are found in association (6). Lately, considerable interest has focused on the pathogenic contribution of the anaerobic organisms in these mixed infections. Using a rat peritonitis model, Onderdonk et al. (14) demonstrated synergism of *Bacteroides* species and *E. coli* in abscess formation. However, encapsulated *B. fragilis* by itself or its isolated capsular material subsequently was shown to induce the same frequency of abscess formation (15). Investigating surgical incisions in guinea pigs, Kelly (13) reported on synergistic effects of *B. fragilis* and *E. coli* resulting in augmented bacterial growth and pus formation. Rodloff and Hahn (19) evaluated the effect of combined intravenous injections of *E. coli* and *B. fragilis* in murine hosts. It was found that encapsulated *B. fragilis* enhanced animal lethality due to an appropriate challenge with either *E. coli* or, in a time-dependent manner, *E. coli* lipo polysaccharide (18). The mechanisms responsible for these synergistic infections are still unclear. Kasper et al. (12, 15, 16, 21, 22) showed that the polysaccharide capsule of *B. fragilis* is an important virulence factor; however, its possible role in mixed infections is still undetermined. A number of reports have provided evidence that the addition of anaerobic bacteria to leukocyte cultures suppresses phagocytosis and killing of facultatively aerobic bacteria by polymorphonuclear leukocytes (9–11), but similar effects could not be demonstrated in vivo (5, 17). Little is known about the effects anaerobes might have on macrophages, although these cells play an eminent role in early events of host defenses. Moreover, previous studies comparing the effect of either *B. fragilis* or thioglycolate injections in experimental mixed infections showed that both could enhance animal susceptibility to subsequent challenges with *E. coli* (20). Since thioglycolate is known to alter macrophage functions (1, 23), these findings prompted us to investigate the role of *B. fragilis* in this respect. Hence, the present study was designed to evaluate the effects of *B. fragilis* on macrophage phagocytosis in vivo and in vitro.

**MATERIALS AND METHODS**

Mice. Specific-pathogen-free, female (C57BL/6 × DBA/2)F1 mice (Jackson Laboratory, Bar Harbor, Maine), approximately 8 to 10 weeks of age and weighing about 22 g each, were used throughout the study. The mice were kept in groups of three to six in pathogen-free conditions and fed commercial mouse pellets and water ad libitum.

Microorganisms and media. As in previous studies (19), *B. fragilis* ATCC 25285 was used either heavily encapsulated after at least 10 subsequent animal passages (strain BFa) or less encapsulated after repeated laboratory subculturing (strain BFl). Earlier, we have shown that these procedures result in significant differences in encapsulation as determined by electron microscopy (19). *E. coli* ATCC 25922, *Listeria monocytogenes* EGD (supplied by Emil Skamene), *Streptococcus pneumoniae* ATCC 6303, and latex particles (LP; size, 1.01 μm; Polyscience Inc., Warrington, Pa.) were used for comparison. All bacteria were grown in Schaedler broth (BBL Microbiology Systems, Cockeysville, Md.) supplemented with 0.1 mg of vitamin K1 per liter and incubated either anaerobically in GasPak jars (BBL) (*B. fragilis*) or aerobically (other bacteria) for 24 h at 36°C. These broth cultures were adjusted in optical density to yield 5 × 108 CFU/ml, and bacterial concentrations were confirmed by plating suitably diluted portions. In addition, LP were suspended in fresh Schaedler broth at 5 × 106 particles per ml as established by appropriate counts with a Petroff-Hausser...
and Helber chamber (Hauser Scientific, Blue Bell, Pa.). For
in vitro studies, heat-killed Schaedler broth cultures were
centrifuged at 4,000 \( \times \) g for 10 min, washed twice, and
suspended in Dulbecco modified Eagle medium (DMEM, GIBCO
Laboratories, Grand Island, N.Y.; supplemented with [per liter] 3.7 \( g \) of NaHCO\(_3\), 100 ml of heat-inactivated fetal bovine serum, 100,000 \( U \) of penicillin, and 0.01 \( g \) of
streptomycin) to yield a concentration of \( 10^8 \) bacteria per ml
as determined by Petroff-Hauser and Helber chamber
counts. LP suspensions were established accordingly.

Radiolabeling of yeast particles. The macrophage phago-
cytosis was assayed with radiolabeled yeast particles by the
method of Becker et al. (J. Becker, S. W. Carter III, and
R. J. Grasso, J. Immunol. Methods, in press). Saccharomy-
ces cerevisiae was originally isolated from bakers' yeast
(Aneusser Busch, St. Louis, Mo.) and grown initially for 1
day at 30°C in nutrient broth (Difco Laboratories, Detroit,
Mich.) supplemented with \( 1 g \) of glucose per liter by using a
rotary shaker (New Brunswick Scientific Co., Inc., New
Brunswick, N.J.) set at 200 rpm. The yeast was then adapted
to grow in nonsupplemented broth by subculturing it with
decreasing amounts of glucose. Radiolabel was incorporated
into yeast cells by incubating fresh cultures until first growth
was apparent, then supplementing the medium with 2.5 \( \mu C \) of \( \alpha-(5,6-\text{H}) \)-glucose solution (Du Pont Co., Boston, Mass.)
per ml and continuing the incubation for approximately 24 h.
Yeast cells were harvested from the culture by centrifuga-
tion (4,000 \( \times \) g for 10 min at 4°C), washed twice in saline
solution, and then heat killed by boiling for 60 min. The
killed cells again were washed until the supernatants had
cleared to background radioactivity. The titrated yeast
particles were suspended in DMEM at a concentration of
6 \( \times \) \( 10^7 \) cells per ml and stored at 4°C. The specific activity
of the cells was measured to be approximately 0.05 cpm per cell
and varied slightly with different batches prepared.

Experimental infections of mice. Fresh suspensions of
bacteria or(LP as described above were used immediately to
infect experimental animals. Groups of three to six mice
received intravenous injections of \( 0.2 \) ml each, equivalent to
doses of \( 10^8 \) CFU or particles, respectively. This dose was
selected since in earlier studies it was shown to be necessary
and sufficient to induce synergistic effects in mixed infec-
tions (19, 20). Control mice received 0.2 ml of Schaedler
broth only. The animals were killed by cervical dislocation at
appropriate times.

Macrophage phagocytosis assay. Murine resident leuko-
cytes were obtained by lavage of the peritoneal cavity with
5 ml of DMEM. Cells were washed twice and adjusted to a
final concentration of 1.5 \( \times \) \( 10^6 \) /ml; 100-\( \mu l \) portions were
then pipetted into 96 flat-bottomed well plates (Flow Labo-
ratories, Inc., McLean, Va.) and incubated at 36°C in 5%
CO\(_2\) containing air. Depending on the type of experiment,
macrophages were allowed to adhere for 2 (macrophages from
animals injected intravenously with different challenge
organisms or LP) or 24 (macrophages from untreated mice
for in vitro experiments) h. Nonadherent cells were removed
by intensive washings, and macrophages for in vitro tests
were incubated for another 24 h with 100 \( \mu l \) of challenge
suspensions per well. After additional washings,
phagocytosis of yeast particles was assayed by adding 100 \( \mu l \)
of the \( \alpha-(5,6-\text{H}) \)-labeled yeast particles (macrophage-to-
\( \alpha-(5,6-\text{H}) \)-labeled yeast particles (macrophage-to-
1 ratio, approximately 1/40) per well and incubating for 30 min
at 36°C. Yeast particles that were not macrophage associated
were washed off, cells were lysed with 0.1 N NaOH and
harvested with an automated cell harvester, and radioactiv-
ity of the cell lysates was counted with a liquid scintillation
spectrometer (Packard Instrument Co., Inc., Rockville,
Md.).

Statistical evaluations. Data obtained were evaluated with
Student's \( t \) test and standard tables.

RESULTS

Phagocytic activity of peritoneal macrophages of mice in-
jected with encapsulated \( B. \) fragilis at different times. Groups
of mice given intravenous injections of \( 10^8 \) encapsulated \( B.
\) fragilis (BFA), \( 10^6 \) LP, or sterile Schaedler broth only
(control) were sacrificed after different time intervals (3, 6,
12, and 24 h after inoculation). Peritoneal macrophages were
collected, pooled per experimental group, and tested in six
separate wells for uptake of radioactive yeast particles.
Macrophage-associated radioactivity was measured, and
the data obtained were combined to determine the mean values
for experimental groups. To avoid technical error, experi-
mental groups were tested simultaneously and results were
expressed as the percentage of control values, since different
batches of radiolabeled yeast particles had slightly different
specific activity and yeast uptake of control macrophages
varied from experiment to experiment. The results indicate
that neither BFA nor LP altered macrophage phagocytosis
within 3 h after injection. However, 6 h postinoculation (p.i.)
phagocytic activity of peritoneal macrophages decreased to
64% of the control in mice injected with LP and to 26% of
the control in mice given BFA (Fig. 1). At 12 h p.i. essentially
the same decrease was evident, while at 24 h p.i. macrophage
function returned to control levels. The differences between
BFA and LP were statistically significant (\( P < 0.001 \) for the
6 and 12 h p.i. values).

Comparison of the phagocytic activity of peritoneal macro-
phages of mice injected with different bacteria. In further
experiments the effects of BFI and unrelated bacterial spe-
cies, such as \( E. \) coli (gram negative, lipopolysaccharide
containing, often found in mixed infections with \( B. \) fragilis),
\( L. \) monocyctogenes (gram positive, facultatively intracellular,
containing lipopolysaccharidelike material, not associated
with \( B. \) fragilis infections), or \( S. \) pneumoniae (gram positive,
not lipopolysaccharide containing, capsule bearing, and not
associated with \( B. \) fragilis infections), were compared with
effects caused by BFA for evaluation of the specificity of
the previous observation. The animals were injected as de-
scribed earlier, and peritoneal macrophages were obtained
6 h p.i. and subjected to the phagocytosis assay. The results
are summarized in Fig. 2. Clearly, BFI and other bacteria
did not suppress yeast phagocytosis by macrophages 6 h after
their injection, while BFA again was shown to be suppressive
(\( P < 0.001 \)).

Phagocytic activity of peritoneal macrophages treated in
vitro. Additional experiments were performed to study the
direct effect of BFA, BFI, other bacteria, or LP on macro-
phage cultures in vitro. Peritoneal macrophages from normal
mice were cultured for 24 h and then exposed for 24 h to
BFA, BFI, other bacteria, LP suspended in DMEM, or
DMEM alone. Subsequently, yeast phagocytosis was as-
sayed. Figure 3 demonstrates that \( 10^6 \) CFU of BFA per well
reduced the phagocytic ability of the macrophages to about
22% of the control value and BFI reduced it to 33%, while \( E.
\) coli, \( L. \) monocyctogenes, and \( S. \) pneumoniae proved to be
significantly less active (\( P < 0.001 \)), and LP did not show any
apparent effect. A reduction in the number of bacterial
challenge organisms to \( 10^6 \) per well, however, resulted in an
almost complete loss of the suppression demonstrated by
BFA (Fig. 3).
FIG. 1. Phagocytosis of $[^3]$Hglucose-labeled yeast cells by peritoneal macrophages of mice injected intravenously with $10^8$ CFU of encapsulated _B. fragilis_ (BFa) or $10^8$ LP. Each bar represents the mean of six determinations at indicated times, and standard deviations are given as lighter bars.

DISCUSSION

In experimental mixed infections with _E. coli_ and _B. fragilis_, several investigators have observed pathogenic effects that could not be attributed to any one organism acting alone (3, 7, 8, 13, 14, 18, 19). For example, higher concentrations of bacteria were seen at infected sites (13, 19), and animals infected with both organisms displayed higher lethality than that expected from results obtained with monoinfections (3, 19). To elucidate mechanisms possibly involved in this synergy, a number of authors have investigated the influence of _Bacteroides_ species on the host immune system. Ingham and co-workers (10) showed that anaerobes may interfere with the phagocytosis and killing of aerobes by polymorphonuclear leukocytes in vitro. Furthermore, Tofte et al. (24) demonstrated that _Bacteroides melaninogenicus_ and _B. fragilis_ could compete with _E. coli_ for serum opsonins. A number of investigators have provided evidence that _Bacteroides_ sp. activates the complement system in vitro via the classical and alternative pathways (for a review see reference 2), and Dijkmans et al. (4) recently showed that _B. fragilis_ may cause complement

FIG. 2. Phagocytosis of $[^3]$Hglucose-labeled yeast cells by peritoneal macrophages of mice injected intravenously 6 h previously with $10^8$ CFU of BFa, BFI, _E. coli_ (EC), _L. monocytogenes_ (LM), or _S. pneumoniae_ (SP). Each bar represents the mean of five determinations, and standard deviations are indicated (♦).
B. fragilis might influence production of E. coli. Although these alterations contribute to the phenomenon of E. coli; the suppression is not observed to be due to mere competition for serum complement, since the test system used is opsonin independent. Thus, the actual mechanism of the suppression of phagocytosis observed here remains unclear. However, it appears that the polysaccharide capsule of B. fragilis is instrumental in causing the effects shown, since BFI was significantly less active than the animal-passaged, heavily encapsulated BFa. The suppression in independent in vivo and in vitro studies, as well as its apparent effectiveness early in the infectious process, suggests that it contributes significantly to the synergistic pathogenicity of mixed infections shown for this experimental model.

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LITERATURE CITED