Effects of *Escherichia coli* and *Bacteroides fragilis* on Peritoneal Host Defenses

DAVID L. DUNN, RODERICK A. BARKE, DAVID C. EWALD, AND RICHARD L. SIMMONS

Departments of Surgery and Microbiology, University of Minnesota, Minneapolis, Minnesota 55455

Received 1 October 1984/Accepted 24 January 1985

*Escherichia coli* and *Bacteroides fragilis* are common copathogens in clinical intra-abdominal sepsis, yet it is unclear how they interact synergistically in vivo. We sought to determine whether *E. coli* and *B. fragilis*, in combination but not alone, could exert a detrimental effect on the peritoneal host defenses of translymphatic absorption and bacterial phagocytosis. Our data indicated that nonviable *E. coli* (O18ab:K56/K7:- and O111:B4), *Klebsiella pneumoniae*, *B. fragilis*, and *Bacteroides thetaiotaomicron* were handled in a similar fashion by both host defenses of the peritoneal cavity. The use of $2 \times 10^2$ nonviable radiolabeled *E. coli* as a tracer and either $2 \times 10^2$ *B. fragilis* or $2 \times 10^2$ *E. coli* (either viable or nonviable) as a competing agent to inhibit host defenses demonstrated that although clearance and phagocytosis could be inhibited, the inhibition occurred to a similar degree with either *E. coli* or *B. fragilis*. Thus, *B. fragilis* did not compete to any greater extent than *E. coli* did for peritoneal clearance or opsonization and phagocytosis in vivo. These data indicate that bacterial synergy probably does not occur on the basis of reduced peritoneal clearance or by a reduction in the opsonization and phagocytosis of either organism by the copathogen. These results provide indirect support for the hypothesis that in bacterial synergy, one organism directly stimulates the growth of the other, perhaps by providing a growth factor.

*Escherichia coli* and *Bacteroides fragilis* are common copathogens in clinical intra-abdominal sepsis (2, 9). On the basis of several experimental studies, these two organisms have been postulated to act synergistically, leading to either enhanced mortality (7) or increased abscess formation (7, 17, 18). Although several hypotheses have been put forth to explain this phenomenon, the mechanism by which it occurs remains obscure. Only a single previous study by Reznikov et al. (19) has attempted to assess the in vivo effect of these organisms on intraperitoneal host defense. These authors found no alteration in bacterial counts when both organisms were injected intraperitoneally (i.p.) compared with either organism alone. Several publications, however, have provided in vitro data that support the hypothesis that anaerobic bacteria exert a detrimental effect on the opsonization, phagocytosis, and killing of aerobic bacteria and have implied that this may be operative in vivo (11, 12, 16, 20). The purpose of the present study was to reassess the interaction of *E. coli* and *B. fragilis* with peritoneal host defense in an in vivo model. It is well known that bacteria are readily removed from the peritoneal cavity into the bloodstream by host peritoneal translymphatic absorption and that concomitant uptake into autophagocytic cells takes place during peritoneal contamination. We therefore sought to compare and contrast the effects of *E. coli* and *B. fragilis* alone and in combination on in vivo rat peritoneal host defenses to determine whether impairment of these defenses occurs, thus providing an explanation for the synergistic interaction of these organisms which has been described in some models.

**MATERIALS AND METHODS**

**Bacterial growth.** A clinical isolate of *E. coli* that was initially obtained from a human peritoneal infection at the University of Minnesota Hospitals was used for all studies. The serotype of this strain was O18ab:K56/K7:-. This strain has been repeatedly used in previous studies from this laboratory (1, 5–8). *E. coli* O111:B4 was kindly provided by Henri Verbrugh. An isolate of *Klebsiella pneumoniae* was obtained from the American Type Culture Collection (ATCC 13883). A loop sample from a stock plate culture was inoculated into 20 ml of brain heart infusion broth (Difco Laboratories, Detroit, Mich.) and incubated in a shaker bath for 18 h at 37°C. Bacteria were then washed three times in 0.9% saline. Bacterial numbers were estimated by spectrophotometry with a Klett-Summerson colorimeter, and exact quantitation was established by enumeration of the developed colonies. Clinical isolates of *B. fragilis* (VPI 9032) and *Bacteroides thetaiotaomicron* were kindly provided by Carol Wells and Ann Bjornson, respectively. Stock cultures of each were maintained in chopped meat medium (Gibco Diagnostics, Madison, Wis.) in an anaerobic chamber (type B; Coy Laboratory Products, Ann Arbor, Mich.), and an inoculum was obtained from this source for each experiment. Under these growth conditions, small, dense encapsulation of *B. fragilis* was noted by India ink staining. Brain heart infusion medium was supplemented with 0.5 mg of hemin (Alrdrich Chemical Co., Inc., Milwaukee, Wis.) per dl, 0.05 g of cystine hydrochloride (Sigma Chemical Co., St. Louis, Mo.) per dl, and 5 g of yeast extract (Gibco) per liter. A platinum loop was used to inoculate supplemented reduced brain heart infusion medium from the stock culture. The culture was then grown anaerobically at 35°C for 18 h. The initial approximation of bacterial numbers was performed by direct visual counting with a Petroff-Hauser chamber. Serial dilutions were performed with dilution blanks prepared as described by Holdeman et al. (10), and surface plating took place on preduced 5% sheep blood agar plates. Exact quantitation was established by enumeration of the developed colonies. Each anaerobic culture was then removed from the anaerobic chamber and centrifuged three times. After each centrifugation step, supernatant fluid was removed and resuspended in preduced 0.9% sterile saline in the anaerobic chamber. Bacterial numbers enumerated by the plating tech-
nique before and after centrifugation were identical, demonstrating no loss of viability by this procedure.

**Preparation of $^{125}$I-labeled nonviable microorganisms.** A loop sample of each strain of *E. coli* or *K. pneumoniae* from a stock culture was inoculated into 20 ml of diluted Mueller-Hinton broth (Difco). The broth was prepared by adding 25 ml of phosphate-buffered saline to 10 ml of full-strength Mueller-Hinton broth. Glucose (6.50 g) was then dissolved in 65 ml of sterile water, to which the partially diluted Mueller-Hinton broth was added for a total volume of 100 ml. $[^{125}]$iododeoxyuridine (1 mCi/ml; New England Nuclear Corp., Boston, Mass.) was added in a quantity of 0.5 ml per 19.5 ml of diluted Mueller-Hinton broth. Incubation took place in a shaker bath for 24 h at 37°C. Radiolabeled *B. fragilis* or *B. thetaiotaomicron* were similarly prepared with the exception that growth took place in the anaerobic chamber in supplemented brain heart infusion medium (0.5 ml of iododeoxyuridine per 19.5 ml of brain heart infusion medium). A sample of each bacterial suspension was obtained, serial dilutions were performed, pour or spread plates were made, and exact quantitation was established by enumerating the developed colonies. The remainder of the culture was subjected to centrifugation, after which resuspension in a volume of 10% Formalin equal to the volume of the initial culture took place. Formalin killing of the radiolabeled bacteria was allowed to proceed for 20 min, after which the bacterial suspension was subjected to two further cycles of centrifugation. The first cycle was followed by resuspension in 10% Formalin, and the next cycle was followed by resuspension in a volume of 0.9% saline equal to that of the initial bacterial suspension. The bacterial suspensions were then stored at 4°C. Cultures were also heat killed by being placed in a 100°C water bath for 2.5 h. Complete killing was established by examining pour or spread plates made at the time of use for sterility. The following day, after enumeration had established the exact bacterial concentration, the suspension was adjusted to $2 \times 10^8$ bacteria per ml. Petroff-Hauser chamber counts of the killed bacterial suspensions demonstrated good agreement with the viable colony counts.

Serial dilutions of the killed, radiolabeled bacterial suspensions were then made. 1-ml samples were counted in a 200-sample gamma counter (Biogamma II; Beckman Instruments, Inc., Fullerton, Calif.), and a calibration curve relating gamma counts per minute to the number of radiolabeled bacteria was established. The stability of this label was assessed by the incubation of $2 \times 10^8$ radiolabeled nonviable *E. coli* or *B. fragilis* with $5 \times 10^7$ rat peritoneal leukocytes. Samples were rotated, centrifuged at 1,600 × g for 10 min, and radioactivity in the supernatant was determined at various times. At 4 h, 7.8 ± 0.12% and 4.9 ± 2.3% release of the radiolabel had occurred for radiolabeled *E. coli* and *B. fragilis*, respectively, and by 24 h only 14.8 ± 2.8% and 12.2 ± 5.4% release had taken place. These results indicated that label degradation and release after phagocytosis did not significantly affect the clearance studies.

**Clearance of radiolabeled microorganisms from the peritoneal cavity.** To study the clearance of radiolabeled bacterial particulates from the peritoneal cavity, male Sprague-Dawley rats (200 to 250 g) were injected i.p. with $2 \times 10^8$ radiolabeled nonviable bacteria in 1 ml of 0.9% saline. Competition studies were performed in identical fashion, except that $2 \times 10^6$ viable or nonviable *E. coli* or *B. fragilis* were simultaneously injected in 1 ml of saline. Nonviable bacteria were chosen as markers so that bacterial proliferation would not interfere with the precise measurement of each host defense component. Immediately after injection, at 0.3 and 0.6 h, and at 1-h intervals up to 8 h after bacterial challenge animals were rapidly asphyxiated in a CO$_2$ chamber, and 10 ml of sterile saline was injected i.p. The animals were agitated for 1 min to ensure adequate mixing of the peritoneal contents, and a midline laparotomy was performed. Four 1-ml samples of peritoneal fluid were obtained, and the amount of radioactivity was directly determined. Immediately after injection, >95% of the radiolabel was recoverable in all cases from the peritoneal fluid sample. The remaining two samples were collected in glass tubes containing EDTA at 4°C and were subjected to three cycles of low-speed centrifugation (160 × g for 10 min), and after each cycle the samples were resuspended in 0.9% saline. This procedure has been demonstrated to be effective for separating leukocytes from other contaminants and bacteria that remain in the supernatant (22). The amount of leukocyte-associated radioactivity in each sample was then determined. Control experiments in which rat peritoneal leukocytes and *E. coli* or *B. fragilis* were combined in vitro in glass tubes containing EDTA at 4°C demonstrated no significant radioactivity in the leukocyte pellet, and bacterial radiolabel did not sediment during the low-speed centrifugation steps. Punch biopsy specimens (3 mm in diameter) of the rat parietal peritoneum were obtained and washed with saline three times before gamma counting. No significant adherence of either radiolabeled *E. coli* or *B. fragilis* (<10$^7$) occurred at any time up to 8 h after bacterial challenge.

**Statistics.** Clearance and phagocytosis data were assessed by a two-tailed Student t test and standard tables.

**RESULTS**

**Removal of $2 \times 10^6$ nonviable *E. coli* from the rat peritoneal cavity.** By 1 h, $1.38 \pm 0.18 \times 10^6$ *E. coli* were removed from the peritoneal cavity (Fig. 1). Peritoneal leukocyte association with *E. coli* was rapid, with over $6.30 \pm 1.70 \times 10^7$ bacteria being phagocytosed by 20 min. After 20 min, the number of both total bacteria and leukocyte-associated bacteria steadily declined, and by 8 h, $1.20 \pm 0.48 \times 10^6$ *E. coli* remained. No significant difference was noted in the clearance or leukocyte uptake of *E. coli* that had been either Formalin or heat killed ($P > 0.1$, data not shown).

**Removal of $2 \times 10^8$ radiolabeled nonviable *B. fragilis* from the peritoneal cavity.** *B. fragilis* was removed from the peritoneal cavity in a fashion similar to that of nonviable *E. coli* (Fig. 2). $1.44 \pm 0.70 \times 10^8$ *B. fragilis* were removed by 1 h, and peritoneal leukocyte association with *B. fragilis* was not statistically different from that with *E. coli* at any time ($P > 0.1$). After an initial increase in the uptake of radiolabel by leukocytes, the number of both total and leukocyte-associated bacteria steadily declined over the 8-h period in a fashion similar to *E. coli* clearance. No significant difference was noted in the clearance or leukocyte association of *B. fragilis* when Formalin- or heat-killed organisms were compared ($P > 0.1$, data not shown).

**Removal of $2 \times 10^8$ radiolabeled nonviable *E. coli* O111:B4, *K. pneumoniae*, or *B. thetaiotaomicron* from the peritoneal cavity.** These experiments were performed to determine whether different strains and types of organisms interact with host peritoneal defenses in a similar fashion. *E. coli* O111:B4, *K. pneumoniae*, and *B. thetaiotaomicron* were removed from the rat peritoneal cavity in a fashion nearly identical to that of either nonviable *E. coli* O118ab:K56/K7- or *B. fragilis* ($P > 0.1$, data not shown). The leukocyte uptake of these organisms was not different from that which
occurred with either nonviable *E. coli* O18ab:K56/K7:- or *B. fragilis* (*P > 0.1, data not shown).

**Effect of** $2 \times 10^8$ nonviable or viable *B. fragilis* or *E. coli* on clearance of $2 \times 10^8$ radiolabeled nonviable *E. coli* from the peritoneal cavity. The clearance of $2 \times 10^8$ nonviable *E. coli* was minimally inhibited within the first 3 h and was significantly inhibited from 4 to 6 h after injection by the simultaneous injection of $2 \times 10^8$ nonviable *B. fragilis* (*p < 0.01, Fig. 3).* Peritoneal leukocyte association with *E. coli* also was significantly inhibited between 1 and 3 h after bacterial challenge, compared with control animals that received only $2 \times 10^8$ radiolabeled nonviable *E. coli* and saline (*p < 0.05).* Nonviable *E. coli* ($2 \times 10^8$) injected simultaneously with the radiolabeled tracer ($2 \times 10^8$ nonviable *E. coli*) demonstrated a similar degree of inhibition of the clearance of radiolabel from the peritoneal cavity at similar times as those animals that received $2 \times 10^8$ nonviable *B. fragilis*, both in comparison with animals that received $2 \times 10^8$ radiolabeled nonviable *E. coli* and saline (*P < 0.01, Fig. 3).* The peritoneal leukocyte association of *E. coli* was also inhibited between 1 and 3 h after bacterial challenge in this experiment (*P < 0.01).* Thus, clearance and host phagocytosis of $2 \times 10^8$ *E. coli* were inhibited by $2 \times 10^8$ *E. coli* or *B. fragilis* but to a similar degree. Use of $2 \times 10^8$ live *E. coli* or *B. fragilis* as the competing factor led to similar competition when the total clearance and the leukocyte uptake of radiolabel were compared with those from the experiments described above, in which nonviable bacteria were utilized (*P > 0.1).*

**DISCUSSION**

Although bacterial synergism has been defined as the ability of two or more microbes to act in concert to produce a more severe infection than that which occurs with either pathogen alone, the phenomenon is poorly understood clinically or experimentally. Melenev et al. (15) and Altemeier (4) noted synergism among aerobic anaerobic microbial participants in intra-abdominal sepsis and were able to reproduce this synergism in experimental models. Weinstein and colleagues (23) have described an animal model in which gelatin capsules containing barium sulfate and the pooled colonic contents of rats were implanted in the peritoneal cavities of rats; this demonstrated a synergistic interaction between aerobes and anaerobes in terms of abscess formation. Onderdonk et al. (18) showed that *E. coli*, enterococci, and *B. fragilis* predominated during an early stage of peritonitis in this model and that blood cultures were uniformly positive (usually for *E. coli*) during that stage. The abscesses that formed later in surviving animals contained primarily *B. fragilis* and *Fusobacterium* sp. These same investigators (17)
implanted pure and mixed cultures of *E. coli*, enterococci, *B. fragilis*, or *F. varium* into the peritoneal cavities of rats. They noted that significant mortality was present only in those animals receiving *E. coli*, but the combination of an anaerobe with a facultative organism was required for increased abscess formation. Kelly (13) demonstrated that *E. coli* and *B. fragilis* injected subcutaneously into guinea pigs would produce a more severe soft tissue infection than either organism alone does. This work demonstrated that a critical minimal inoculum size was required for either organism to synergize with the other; it also provided evidence that synergy was correlated with the growth of both microorganisms in the subcutaneous tissue. We have previously demonstrated that *E. coli* and *B. fragilis* act synergistically in the rat peritoneal cavity, leading to both enhanced abscess formation and mortality when implanted in a fibrin clot matrix, and that the effect is critically dependent on the initial inoculum size (7).

A number of mechanisms by which synergy between aerobic and anaerobic organisms occurs have been demonstrated in experimental models. One organism may: (i) provide necessary nutrients for another (14); (ii) create a suitable environment for another (e.g., *E. coli* lowering the redox potential of the media, thereby permitting *B. fragilis* to grow) (14); or (iii) protect another organism from natural host defenses (11, 12, 16, 20). For example, *B. fragilis* has been shown to reduce the opsonization and phagocytosis of *E. coli* in vitro, presumably because of competition for opsonins (20). Evidence has also been provided indicating that *B. fragilis* may diminish the in vitro phagocytosis (12), chemiluminescence (16), or intracellular killing (11, 12, 16) of facultative aerobes such as *P. mirabilis*, although this also has not been demonstrated in vivo. Despite the plethora of in vitro studies in which a mechanism has been sought to explain how *E. coli* and *B. fragilis* interact, only one previous study has directly addressed this problem in vivo (19). These investigators noted that simultaneous i.p. infection with *E. coli* and *B. fragilis*, compared with either organism alone, did not lead to the enhanced growth of either organism on the basis of viability counts.

The present study sought to determine whether both *E. coli* and *B. fragilis*, in combination but not alone, could exert a detrimental effect on the peritoneal host defenses of translymphatic absorption and bacterial phagocytosis, thus accounting for the synergistic interaction demonstrated in some models. To examine the interactions of host peritoneal defenses with invading microorganisms, the clearance from the peritoneal cavity and the host phagocyte uptake of either *E. coli* or *B. fragilis* were first examined. A 10-fold larger number of either *E. coli* or *B. fragilis* without radiolabel was then injected simultaneously with 2 × 10⁹ radiolabeled *E. coli*, and the clearance and host phagocyte uptake of radiolabeled tracer were measured to test the hypothesis that *B. fragilis* significantly inhibits the peritoneal host removal mechanisms for *E. coli*. Previous anatomic studies have demonstrated that particulate material in the peritoneal cavity is rapidly removed via specialized diaphragmatic pores (stomata) that drain into lymphatic lacunae (3, 21). We have previously demonstrated the rapidity by which *E. coli* particulates are removed into the bloodstream from the peritoneal cavity of the rat (5).

Our data indicated that nonviable *E. coli* and *B. fragilis* bacterial particulates were handled in a similar fashion by the host defenses of the peritoneal cavity. The translymphatic absorption and phagocytosis of the two organisms were similar. The use of 2 × 10⁹ nonviable radiolabeled *E. coli* as a tracer and either 2 × 10⁸ *B. fragilis* or 2 × 10⁸ *E. coli* (either viable or nonviable) as a competing agent to inhibit either clearance or opsonization and phagocytosis demonstrated that although clearance and phagocytosis could be inhibited, the inhibition occurred to a similar degree with either *E. coli* or *B. fragilis*. These latter studies indicated that *B. fragilis* does not compete to any greater extent than *E. coli* does for peritoneal clearance or opsonization and phagocytosis in vivo.

These data indicate that bacterial synergy probably does not occur on the basis of reduced peritoneal clearance or a reduction in the opsonization and phagocytosis of either organism caused by its copathogen. It should be noted, however, that phagocytic killing was not directly assessed, since nonviable bacteria were utilized as tracers. Although these studies do not establish a mechanism for bacterial synergy, the results do appear to exclude an effect of either copathogen on the in vivo translymphatic clearance or phagocytosis of other organism, contrary to the conclusions drawn from the results of several in vitro studies. These results provide indirect support for the hypothesis that in bacterial synergy, one organism directly stimulates the growth of the other, perhaps by providing a growth factor. Further examination of the interactions of copathogens with each other and with host defenses in this or similar models.
should allow better definition of the mechanisms by which bacterial synergy occurs in intra-abdominal sepsis. This will be critical to determining the pathophysiology of such infections.

ACKNOWLEDGMENT

This work was supported by grant AI 14032 and Postdoctoral Fellowship grant AM 0635301 from the National Institutes of Health and was written in partial fulfillment of the degree of Ph.D. in microbiology.

LITERATURE CITED