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Evidence of the Proinflammatory Role of *Enterococcus faecalis* in Polymicrobial Peritonitis in Rats

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Although the role of members of the *Enterobacteriaceae* and anaerobes in the pathogenesis of intra-abdominal infections has been extensively demonstrated, the role played by enterococci in these infections remains controversial. The pathophysiological mechanisms induced by enterococci in intra-abdominal infection were studied in a nonfatal model of peritonitis in rats by implanting a gelatin capsule containing *Escherichia coli* and *Bacteroides fragilis* with or without increasing concentrations of *Enterococcus faecalis* or heat-inactivated enterococci. The ability of the rat peritoneal cavity to sterilize itself after bacterial challenge was evaluated by quantifying the inflammatory response in the peritoneal cavity, reflected by both phagocyte and cytokine responses. Effects were evaluated 6, 12, and 24 h and 3 and 6 days after inoculation. On day 6 after inoculation, the highest enterococcal concentration (10^8 CFU/ml) was accompanied by significantly increased concentrations of *E. coli* in peritoneal fluid and peritoneal phagocytes when compared to other groups. In the first 12 h after inoculation, tumor necrosis factor and interleukin-6 concentrations were significantly increased in the peritoneal fluid of the animals that had received the highest inoculum of enterococci or heat-inactivated enterococci. In the late period of the study (3 and 6 days), significantly increased leukocyte counts were observed in the peritoneal fluid of these animals. These results suggest that *E. faecalis* somehow inhibited phagocytosis and intracellular killing of the other pathogens and also played an inflammatory role, which might account for the bacterial synergy observed in this model.

The frequencies of isolation of enterococci from surgical patients and those with nosocomial infections have increased dramatically during the past decade. The role of enterococci as potential pathogens in intra-abdominal infection has become a source of increasing concern, especially since the emergence of vancomycin-resistant strains (19). Although the role of the *Enterobacteriaceae* and anaerobes in the pathogenesis of intra-abdominal infections has been extensively demonstrated (16, 24, 28, 29), the part played by enterococci in these infections remains controversial (22, 23), especially because enterococci are frequently isolated from the polymicrobial flora (21, 22).

Synergy between enterococci and other pathogens has been previously suspected in experimental models. Matlow et al. (17) reported an increased incidence of intra-abdominal abscesses and higher mortality when *Enterococcus faecalis* was part of the inoculum. In an experimental model of polymicrobial peritonitis, we observed that enterococci, when inoculated at a high concentration, induced an increased frequency of bacteremia due to the other pathogens and an increase in the bacterial counts of the other pathogens in the peritoneal exudate (20).

To elucidate which of the mechanisms of bacterial synergy observed in our model of experimental intra-abdominal infection are attributable to enterococci, we evaluated the effects of *E. faecalis* and heat-inactivated enterococci on the ability of the rat peritoneal cavity to sterilize itself after mixed bacterial challenge. In addition, we quantified the inflammatory response in the peritoneal cavity, reflected by both phagocyte and cytokine responses.

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MATERIALS AND METHODS

Microorganisms. Three strains of bacteria previously described were used for this study: an encapsulated strain of *Bacteroides fragilis*, AIP5-86; a strain of *Escherichia coli*, CB1496 (6); and a strain of *E. faecalis*, UA174 (13).

The strains of bacteria used in this study were obtained from the collection of the Laboratoire des Anaérobies (*B. fragilis* AIP5-86) and the Unité des Agents Anti-bactériens, Institut Pasteur, Paris, France (*E. coli* CB1496 and *E. faecalis* UA174). These strains, specifically chosen because of their susceptibility to antibiotics, were easily identified. The resistance of *B. fragilis* to amoxicillin was due to penicillinase production. In the *E. coli* strain, plasmid pIP55 which encodes the production of β-lactamase (OXA-3 type) and the production an adenylyltransferase enzyme (AAD-2″ type) was inserted, leading to a strain resistant to amoxicillin and gentamicin. The *E. faecalis* strain had an intrinsic low-level resistance to aminoglycosides.

Animals. Male Sprague-Dawley pathogen-free rats (Charles River France, St-Aubin-les-Elbeuf, France), weighing 250 to 300 g and housed five per cage, were used for all the experiments. All animals had access to chow and water ad libitum throughout the experiment.

Preparation of the inoculum. *B. fragilis* was grown and diluted anaerobically in prereduced thioglycolate broth, while *E. coli* and *E. faecalis* were grown in brain heart infusion broth. The final mixtures of strains were made when they were in the log phase of growth; bacteria were diluted to obtain the numbers of microorganisms for the bacterial challenge that are shown in Table 1. Purity was assessed and counts of each strain were validated immediately before mixing. In each group, semisolid agar medium was prepared by adding 2% (wt/vol) agar to the diluted broth cultures mixed with barium sulfate (10% [wt/vol]). Aliquots (0.5 ml) of the final mixture were placed in double gelatin capsules for intraperitoneal implantation.

Suspensions of heat-inactivated *E. faecalis* were prepared by growing the strain in brain heart infusion broth. The cultures were interrupted when they were in the log phase of growth, and the suspensions were adjusted to a concentration of 10^8 CFU/ml by using a spectrophotometer (Sequoia-Turner Corp., Mountain View, Calif.). Bacterial inactivation was achieved by heating the suspensions at 220°C under an atmospheric pressure of 2×10^5 Pa for 1 h. Subsequent cultures of these heat-inactivated suspensions remained sterile.

Six groups of 25 animals were studied (Table 1). A group of animals (group I, Table 1) underwent a sham procedure consisting of implantation of a gelatin capsule containing barium sulfate, semisolid agar medium, and sterile broth. In group III animals, an enterococcus-free inoculum was used in which the total number of bacteria remained constant relative to the animals that had received the highest enterococcus inocula (group V). In addition, a group of animals (group VI, Table 1) received a capsule containing heat-inactivated *E. faecalis* associated with *B. fragilis* and *E. coli*.

TABLE 1. Contents of the gelatin capsule implanted intraperitoneally in the rats[a]

Group	Inoculum (CFU/ml)
I	Control (agar + barium sulfate + sterile broth)
II	*E. coli* (10^8) + *B. fragilis* (5×10^7)
III	*E. coli* (2×10^8) + *B. fragilis* (10^8)
IV	*E. coli* (10^8) + *B. fragilis* (5×10^7) + *E. faecalis* (10^7)
V	*E. coli* (10^8) + *B. fragilis* (5×10^7) + *E. faecalis* (10^8)
VI	*E. coli* (10^8) + *B. fragilis* (5×10^7) + heat-inactivated *E. faecalis* (10^8)

[a] In each experiment, 25 rats were studied in each group; evaluation was performed at 6, 12, and 24 h and 3 and 6 days after challenge in subgroups of five animals. In each group, the gelatin capsule contained agar, barium sulfate, and one of the inocula.

Implantation of inoculum. The rats were anesthetized with an intramuscular injection of ketamine (30 mg/kg of body weight), and the gelatin capsule was inserted intraperitoneally into the pelvic cavity through a midline abdominal incision (28). The wound was closed with a musculoperitoneal layer and a skin layer by using interrupted nylon sutures.

Assessment of spontaneous outcome. After implantation of the inoculum, the animals were returned to separate cages. No death was observed within 6 h of capsule implantation. Microbiological parameters, peritoneal cellular responses, and inflammatory cytokine responses, as described below, were used to evaluate bacterial interactions. The animals were sacrificed by being given chloroform 6, 12, and 24 h and 3 and 6 days after bacterial challenge. For each experiment, five animals were studied in each group.

Microbiological study. Blood samples were obtained by aseptic percutaneous transthoracic cardiac puncture. In all animals but group I rats, semiquantitative blood cultures were performed by pouring 0.1 ml of fresh blood onto agar for bacterial counts.

Then, 10 ml of cold phosphate-buffered saline (PBS; Gibco BRL, Cergy Pontoise, France) was injected intraperitoneally. A midline laparotomy was performed, and peritoneal fluid samples were recovered from all regions of the peritoneal cavity. Abscesses usually observed on day 6 (20) and disseminated in the abdominal cavity were deliberately disregarded. A dilution factor, taking into account the fluid present in the peritoneal cavity prior to the injection of the PBS, was applied in all calculations. Therefore, we used urea as an endogenous marker of peritoneal dilution. Since urea diffuses readily throughout the body, the urea concentrations in peritoneal fluid and plasma are the same (14). In this setting, if the concentrations of urea in plasma and a lavage sample are known, the dilution of the initial volume of peritoneal fluid obtained can be obtained as follows: dilution factor = (concentration of urea in peritoneal fluid)/(concentration of urea in plasma). Once the dilution factor is known, the concentration of any component in the peritoneal fluid (e.g., cytokines, cells, or bacteria) can be assessed. The urea content of peritoneal fluid and plasma was determined by using a commercially available kit (Boehringer Mannheim, Meylan, France).

A 0.5-ml aliquot of the peritoneal fluid sample was serially diluted, and 0.1 ml of each dilution was spread on agar plates for bacterial enumeration. The remaining peritoneal fluid was then centrifuged at low speed ($300 \times g$ for 5 min), and cell-free supernatants were diluted and plated as described above. To determine the number of host cell-associated viable bacteria, peritoneal cells pelleted by low-speed centrifugation were washed three times with PBS and incubated for 30 min at 4°C with 0.1% Triton X-100 (Sigma Chemical Co., L'Isle d'Abeau, France). Serial dilutions were made, and 0.1 ml of each dilution was spread on agar to count intracellular bacteria and cell-related bacteria. These procedures did not alter the viability of the bacteria. The reproducibility of the cell-pelleting technique was evaluated at the beginning of the investigation. Peritoneal samples were assayed in triplicate, and the standard deviation was ≤10% of the mean.

The limit of detection for every microbiological test was $<1 \log_{10}$ CFU/ml. In every case, plates were incubated in the appropriate environments (i.e., jars or air) for 2 to 5 days. The selective medium used for detection of *B. fragilis* was Columbia agar base (BioMérieux, Charbonnières-les-Bains, France) with 5% sheep blood containing 75 μg of kanamycin per ml, 7.5 μg of vancomycin per ml, and 4 μg of pefloxacin per ml. Selective media used for culturing aerobes were Drigalski agar (Diagnostics Pasteur, Marnes-la-Coquette, France) and bile-esculin-azide agar (Diagnostics Pasteur).

Quantitation of host peritoneal leukocyte populations. Total cell counts were made with an aliquot of the original peritoneal fluid by using a Malassez counting chamber. Aliquots of original fluid, adjusted to 5×10^4 cells, were cytocentrifuged in a Cytospin 2 (Shandon Southern Products, Cheshire, England), and individual preparations from every rat were stained with May-Grünwald-Giemsa or Gram stain. Differential cell counts were made by examining at least 300 cells. The mean total number of intraperitoneal leukocytes was calculated.

Cytokine assays. Blood samples were drawn by cardiac puncture and placed into sterile glass tubes. After coagulation, the sera were divided into aliquots and kept at −70°C until assayed. Four 1-ml samples of the original peritoneal fluid recovered from all regions of the peritoneal cavity were centrifuged ($300 \times g$ for 15 min), divided into aliquots, and stored at −70°C until assayed. The serum and peritoneal fluid samples were assayed in triplicate, and the standard deviation was ±10% of the mean.

Tumor necrosis factor (TNF) activity was determined by a modified version (15) of previously described cytotoxicity assays (11, 18, 30). The murine connective-tissue cell line LM was diluted to 4.0×10^5 cells/ml in E199 culture medium (Gibco BRL), and 150 μl was deposited into all the wells of 96-well flat-bottomed microtiter plates (Falcon, Polylabo S.A., Paris, France). After 4 h of incubation (37°C under 5% CO_2), 50 μl of human recombinant TNF-α (hrTNF-α) (Genzyme, Tebu S.A., St.-Quentin-en-Yvelines, France), which was used as the standard, and 50 μl of heat-inactivated samples (30 min at 56°C), previously diluted fivefold to avoid nonspecific effects, were serially diluted twofold in culture medium, and the mixture was added to each well. Actinomycin D (Sigma) was also added to each well at a final concentration of 1 μg/ml.

After 48 h of incubation (37°C under 5% CO_2), the amount of lysis was quantified by a colorimetric assay with a tetrazolium salt (MTT; Sigma), and the results were read spectrophotometrically at 550 nm with an automatic plate reader (Molecular Devices, Medi Sciences, Paris, France) hooked up to an IBM-PC computer. For each serum sample, the TNF concentration that resulted in 50% cell lysis was determined by interpolation of the standard curve obtained simultaneously with hrTNFα (10 pg/ml to 5 ng/ml). The percent lysis was calculated as follows: [(optical density of the control well − optical density of the sample well)/optical density of control well] × 100; control cells were incubated with culture medium alone. The limit of detection in serum and peritoneal fluid was 0.12 ng/ml. The specificity of the response for TNF was assessed by using an anti-mouse TNF antibody immunoglobulin fraction (Immugenex, Los Angeles, Calif.).

Interleukin-6 (IL-6) activity was measured by a bioassay with the murine hybridoma cell line B9 (1). The cells were grown in RPMI 1640 medium (Gibco BRL) containing 5% fetal calf serum, 5×10^{-5} M 2-mercaptoethanol (Gibco BRL), 100 U of penicillin per ml, 100 μg of streptomycin (Gibco BRL) per ml, and 50 U of hrIL-6 (Immugenex) per ml (5). The cells were harvested by centrifugation and, after one wash in IL-6-free medium, distributed at 5×10^3 cells/200 μl into flat-bottomed wells and then cultured for 68 to 72 h in the presence of heat-inactivated (30 min at 56°C) samples.

The proliferation of B9 cells was quantified by means of a colorimetric assay with MTT. The results were read spectrophotometrically as described above. Values were compared to a dose-response curve obtained with hrIL-6 expressed in nanograms per milliliter. The IL-6 concentration in each serum sample was defined as the reciprocal dilution corresponding to 50% proliferation of the cell line. The limit of detection in serum and peritoneal fluid was 0.2 ng/ml. The specificity of the response for IL-6 was assessed with polyclonal rabbit anti-murine IL-6 antibodies (Genzyme).

Statistical analysis. Results are expressed as the mean ± standard error of the mean (SEM). Since the design of the study was essentially to evaluate the effect of an enterococcus on bacterial and inflammatory response, we were not interested in all possible comparisons and multiple comparison tests were overly conservative. All parameters were compared by analysis of variance, followed, in the case of significance, by limited comparisons of means by Fisher's least-significant-difference procedure (26) between enterococcus-free animals (group II) and enterococcal groups (groups IV, V, and VI) on one hand and comparisons between groups IV and V and heat-inactivated enterococci (group VI) on the other hand, according to an a priori decision. In addition, the possible effect of gross numbers of bacteria was evaluated by comparisons between the animals receiving a high bacterial inoculum without enterococci (group III) and enterococcal groups (groups IV, V, and VI). Statistical significance was inferred for comparisons for which $P < 0.05$ (27).

RESULTS

Effects of inoculum on survival. All control animals survived. No mortality was observed in the other groups prior to the end of the study.

Effects of inoculum on blood cultures. The results of semiquantitative blood culture and the concentration of pathogens cultured are shown in Fig. 1. Positive blood cultures were observed in all the animals within 6 h after inoculation, and thereafter the bacterial concentrations were progressively lower. At 6 h postinfection, the concentrations of *B. fragilis* isolates in group IV animals, which had been given the smallest number of enterococci, were significantly lower than in the other groups. The high enterococcal concentrations in the inoculum of group V animals was associated at 24 h with high concentrations of *E. faecalis* isolates.

Effects of inoculum on total peritoneal isolates. The highest concentrations of pathogens were recorded at 6 and 12 h post-

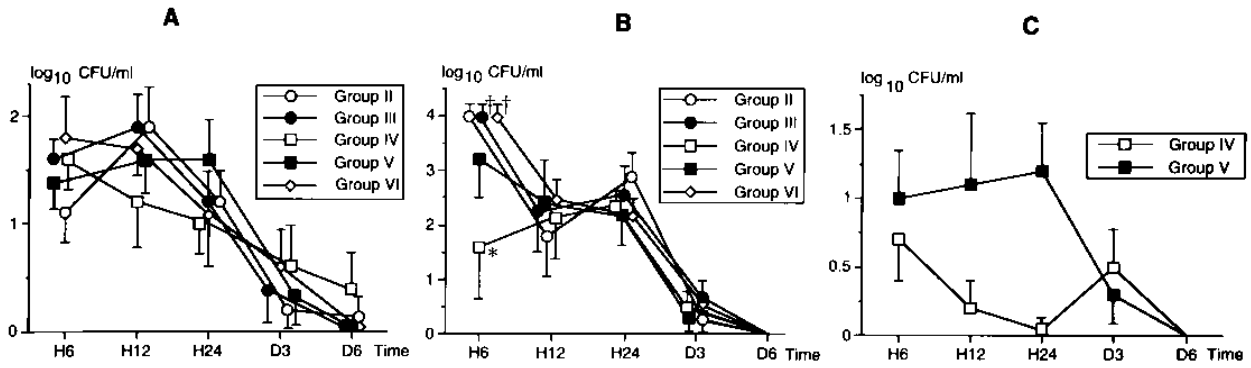

FIG. 1. Bacterial counts ($\log_{10}$ CFU per milliliter; mean ± SEM) of *E. coli* (A), *B. fragilis* (B), and *E. faecalis* (C) isolated from semiquantitative blood cultures obtained at the time of sacrifice from infected animals receiving a combination of *B. fragilis* and *E. coli* at low dose (group II) or high dose (group III) alone or associated with *E. faecalis* at 10^7 (group IV) or 10^8 (group V) CFU/ml or heat-inactivated enterococci (group VI). *, $P < 0.05$ compared to group II; †, $P < 0.05$ compared to group IV.

inoculation, followed by progressive tapering (Table 2). No significant difference between groups was observed at 24 h and 3 days (data not shown). Significantly larger numbers of *E. coli* were observed on day 6 in group V animals than in enterococcus-free animals (low-inoculum group II and high-inoculum group III) and in group IV animals. This discrepancy was not observed in the group of animals that had been given heat-inactivated enterococci (group VI).

Effects of inoculum on intracellular and cell-related bacteria within the peritoneal cavity. The highest concentrations of pathogens were recorded at 6 and 12 h postinoculation, followed by progressively tapering values (Table 3). No significant difference between groups was observed at 24 h and 3 days (data not shown). Higher *E. coli* counts were recorded in the two groups of animals that had received live enterococci than in enterococcus-free animals at 12 h after bacterial challenge. On day 6, significantly higher *E. coli* counts were noted in group V animals than in enterococcus-free animals (low-inoculum group II and high-inoculum group III) and group IV animals. This discrepancy was not observed in the group of animals that had been given heat-inactivated enterococci (group VI).

Effects of inoculum on total leukocyte counts in the peritoneal cavity. Significantly higher leukocyte counts were noted on days 3 and 6 in the animals that had received the high concentration of enterococci (group V) and heat-inactivated enterococci (group VI) than in those that had been given the enterococcus-free inoculum (Fig. 2). Polymorphonuclear neutrophil counts remained elevated for a prolonged period in the animals that had received the septic inocula, without any difference within these groups (data not shown).

Effects of inoculum on cytokine release into the circulation. Although TNF and IL-6 were detected in serum, the concentrations measured were far below the peritoneal concentrations of these mediators. The TNF concentrations measured in serum reached the limit of detection at 6 h postinoculation and then were always below this threshold: 0.13 ± 0.01, 0.12 ± 0.01, 0.14 ± 0.02, 0.16 ± 0.02, 0.13 ± 0.02, and 0.18 ± 0.04 ng/ml at 6 h for groups I, II, III, IV, V, and VI, respectively.

Interleukin-6 was detected in serum from the beginning of the study, followed by progressive tapering: 0.9 ± 0.2, 1.3 ± 0.2, 1.7 ± 0.5, 2.2 ± 0.5, 2.3 ± 0.1, and 2.7 ± 0.5 ng/ml at 6 h for groups I, II, III, IV, V, and VI, respectively. From 24 h onward, the concentrations of IL-6 in serum remained below the limit of detection. A significantly higher IL-6 concentration was noted at 6 h in group V than in group II animals ($P < 0.05$). In addition, significantly higher IL-6 concentrations were recorded at 12 h in groups V and VI compared to groups II and III (1.4 ± 0.6 and 1.5 ± 0.5 ng/ml for groups V and VI versus 0.2 ± 0.07 and 0.4 ± 0.14 for groups II and III; $P < 0.01$ and $P < 0.01$, respectively).

Effects of inoculum on cytokine release into the peritoneum. Significantly higher TNF concentrations were observed at 6 h postinoculation in the animals that had received enterococci than in those in groups II and III (Fig. 3). IL-6 concentrations were significantly higher in the animals that had been given enterococci compared to those in enterococcus-free animals (Fig. 3).

TABLE 2. Organisms enumerated from the whole peritoneal fluid collected within the peritoneal cavity in infected animals at the time of sacrifice

Time post-infection	No. of organisms ($\log_{10}$ CFU/ml) (mean ± SEM, $n = 5$) in[a]:											
	Group II		Group III		Group IV			Group V			Group VI	
	E. coli	*B. fragilis*	*E. coli*	*B. fragilis*	*E. coli*	*B. fragilis*	*E. faecalis*	*E. coli*	*B. fragilis*	*E. faecalis*	*E. coli*	*B. fragilis*
6 h	7 ± 0.1	7.4 ± 0.6	7.2 ± 0.3	7.7 ± 0.1	7 ± 0.1	8.1 ± 0.6	6.3 ± 0.2	7 ± 0.1	8.1 ± 0.4	6.4 ± 0.1	7.1 ± 0.2	7.6 ± 0.1
12 h	6.8 ± 0.2	7.6 ± 0.2	7.1 ± 0.6	7.4 ± 0.2	7.3 ± 0.1	7.5 ± 0.2	5.3 ± 0.3	7.1 ± 0.2	7.5 ± 0.2	5.8 ± 0.2	7.1 ± 0.2	7 ± 0.1
6 days	2.5 ± 0.6	2.8 ± 0.9	2.3 ± 0.6	3.1 ± 0.9	2.2 ± 0.5	3.1 ± 0.9	1.6 ± 0.4	4.7 ± 0.4[b,c,d]	4 ± 1.3	4 ± 0.3[d]	2.4 ± 0.5	3.7 ± 1.1

[a] For a definition of the experimental groups, see Table 1.
[b] $P < 0.01$ compared to group II.
[c] $P < 0.01$ compared to group III.
[d] $P < 0.01$ compared to group IV.

TABLE 3. Numbers of intracellular and cell-related bacteria within the peritoneal cavity in infected animals at the time of sacrifice

Time post-infection	No. of organisms ($\log_{10}$ CFU/ml) (mean ± SEM, $n = 5$) in[a]:											
	Group II		Group III		Group IV			Group V			Group VI	
	E. coli	*B. fragilis*	*E. coli*	*B. fragilis*	*E. coli*	*B. fragilis*	*E. faecalis*	*E. coli*	*B. fragilis*	*E. faecalis*	*E. coli*	*B. fragilis*
6 h	6.5 ± 0.3	3.7 ± 1	6.9 ± 0.1	5.1 ± 0.2	6.8 ± 0.3	4.6 ± 0.7	6 ± 0.2	6.5 ± 0.2	5.3 ± 0.4	6.2 ± 0.3	6.9 ± 0.1	5.1 ± 0.2
12 h	6.2 ± 0.2	3.2 ± 1	6.4 ± 0.2	4.6 ± 0.6	7 ± 0.1[b]	5.1 ± 0.4	5.1 ± 0.3	6.8 ± 0.2[b]	4.8 ± 0.2	5.7 ± 0.2	6.3 ± 0.2	4.9 ± 0.3
6 days	2.3 ± 0.6	1.7 ± 0.4	2.2 ± 0.6	1.8 ± 0.3	2 ± 0.4	1.6 ± 0.3	1 ± 0.6	4.2 ± 0.4[b,c,d]	2.6 ± 1.1	3.8 ± 0.4[d]	2 ± 0.4	1.8 ± 0.3

[a] For a definition of the experimental groups, see Table 1.
[b] $P < 0.01$ compared to group II.
[c] $P < 0.01$ compared to group III.
[d] $P < 0.01$ compared to group IV.

DISCUSSION

Several attempts have been made to demonstrate a pathophysiological role of enterococci in intra-abdominal infections. Our present results suggest that enterococci play a role in sustaining the peritoneal infection, by inhibiting phagocytosis and intracellular killing of the other pathogens, in conjunction with the prolongation of the peritoneal inflammatory response.

Initial data from experimental studies suggested that enterococci acted as synergistic organisms and were not primary pathogens (28, 29). An experimental study conducted by Onderdonk et al. (24) with a rat model employed various bacterial species alone and in combination to induce intra-abdominal sepsis. Neither early death due to septicemia nor late abscess formation occurred when enterococci were inoculated alone. A mixed inoculum of *E. coli* plus enterococci also failed to result in abscess formation, although a combination of *B. fragilis* or *Fusobacterium varium* with enterococci resulted in a high rate of peritoneal abscess formation. These investigators concluded that the pathogenicity of enterococci is mainly due to a synergism with anaerobes that results in the formation of abscesses.

Despite these initial observations, we have only limited insight into peritoneal defenses against polymicrobial infections. Most investigations evaluating these processes were carried out with artificial substrates (8), killed bacteria (7), or monomicrobial infection (7). In addition, spontaneous recovery is one of the common characteristics of intra-abdominal infections in rats, at least those with infections below a threshold specific to each model (7). Several mechanisms are involved in this recovery, including lymphatic absorption, opsonization, phagocytosis, and killing of the pathogens (7, 8, 25).

The peritoneal absorptive mechanism, achieved by lymphatic absorption of the pathogens, is one of the earliest means of defense against intra-abdominal infection (7). After injection of 2×10^8 bacteria into the peritoneal cavity of rats, the bacterial levels of 10^7 organisms reported by Dunn et al. (7) 30 min after inoculation decreased progressively in the following 8 h. Our results confirm the very high concentration of pathogens cultured from blood in the first 24 h of peritoneal infection. The concentration of *E. faecalis* in the inoculum might influence the concentrations of other pathogens such as *B. fragilis* in blood culture as demonstrated in group IV and V animals. However, such concentrations of pathogens in blood cultures have rarely been determined in the course of mixed intra-abdominal infection. Thus, it seems difficult to draw any conclusion on the role of enterococci from such preliminary observations.

Two studies have demonstrated opsonization of enterococci via the alternative complement pathway (2, 10). However, the enhancement of neutrophil killing of enterococci in the presence of complement and specific anti-enterococcal immune globulin remains controversial (2, 10). The ease with which enterococci are opsonized is consistent with their apparent lack of capsular polysaccharide. Such enterococcal properties could account for the relatively high concentrations of intracellular and cell-related enterococci observed in our study compared with those of *B. fragilis*.

A variety of species of obligate anaerobes has been shown to interfere with the phagocytosis and killing of aerobic bacteria in vitro. The effect was strongest with strains of *Bacteroides melaninogenicus* and *B. fragilis* (12, 25). By contrast, aerobes tested in vitro did not have any significant effect on phagocytosis inhibition and killing (12). The relative concentrations of each pathogen might also play a role in the appearance of the mechanisms of inhibition of intracellular killing. Ingham et al. (12) observed maximum inhibition of intracellular killing when at least 10^7 CFU of anaerobes per ml was used, a value which corresponds to the *B. fragilis* concentration inoculated in our study. Thus, increased concentrations of intracellular and cell-related *E. coli* within the peritoneal cavity of the animals that had been given enterococci, as reported herein, might, to some extent, be attributable to inhibition of bacterial killing. However, these conclusions need to be confirmed by in vitro studies.

The major pathological changes associated with enterococcal infection are the increased dissemination and prolongation of the peritoneal inflammation. This increased inflammatory response might be the result of a specific effect of these organisms or might be related to an inoculum effect. To clarify this point, we studied the effects of a heavy bacterial inoculum

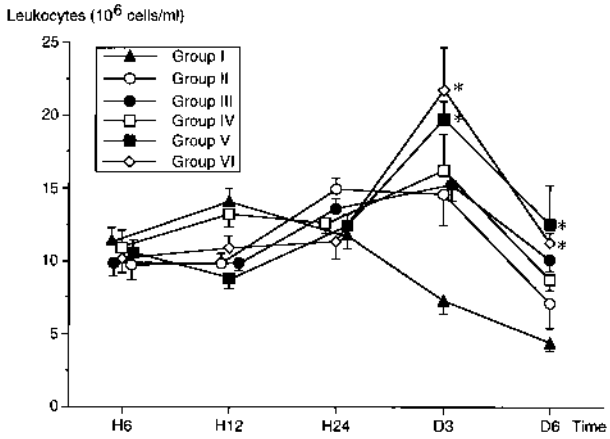

FIG. 2. Leukocyte counts (mean ± SEM) collected at the time of sacrifice within the peritoneal cavity in animals receiving sterile inoculum (group I), a combination of *B. fragilis* and *E. coli* at low dose (group II) or high dose (group III) alone or associated with *E. faecalis*, 10^7 (group IV) or 10^8 (group V) CFU/ml or heat-inactivated enterococci (group VI). *, $P < 0.01$ compared to group II.

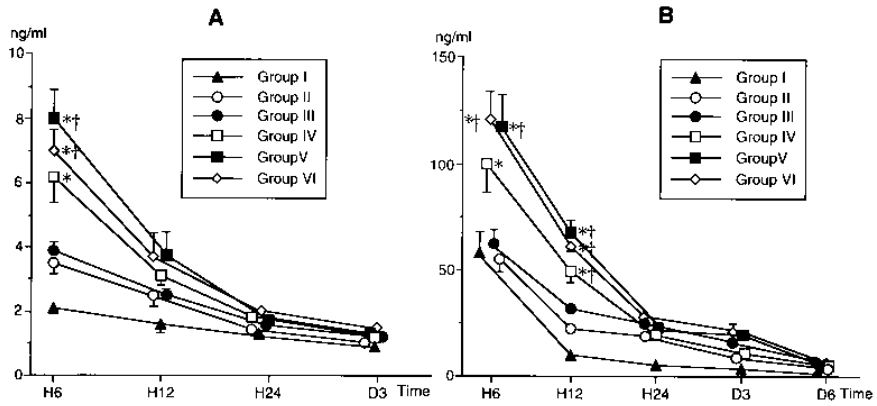

FIG. 3. Peritoneal concentrations of TNF (A) and IL-6 (B) (mean ± SEM) at the time of sacrifice in animals receiving sterile inoculum (group I), combination of *B. fragilis* and *E. coli* at low dose (group II) or high dose (group III) alone or associated with *E. faecalis*, 10^7 (group IV) or 10^8 (group V) CFU/ml or heat-inactivated enterococci (group VI). *, $P < 0.01$ compared to group II; †, $P < 0.01$ compared to group III.

without enterococci (group III) and mixed inoculum with heat-inactivated enterococci (group VI). The microbiological features in these two groups of animals were quite similar. On the other hand, the results of leukocyte and cytokine measurements in these two groups suggest that enterococci exert an effect on the inflammatory response.

In vitro studies demonstrated that enterococcal pheromones and their corresponding inhibitory peptides possess the potential to serve as chemoattractants for polymorphonuclear neutrophils and to trigger a respiratory burst (9). An elevated peritoneal leukocyte count was reported for a prolonged period in our study in the animals that had received high enterococcus inocula or heat-inactivated enterococci. These data suggest that enterococci play a role in the increased count of phagocytes during intra-abdominal infection.

Interestingly, Bhakdi et al. (4) demonstrated the ability of lipoteichoic acid from gram-positive pathogens to stimulate the production of IL-1β, IL-6, and TNF-α by cultured monocytes. They reported that lipoteichoic acids from *Staphylococcus aureus* and *Streptococcus pneumoniae* failed to induce cytokine production whereas lipoteichoic acids at concentrations ranging from 0.5 to 5 μg/ml from several enterococcal species led to the release of all three cytokines. In addition, the levels of these mediators were similar to those synthesized by monocytes after exposure to gram-negative lipopolysaccharides.

Most studies involving experimental models to evaluate the cytokine levels produced locally in response to focal infections have been performed with monomicrobial infection (31) or polymicrobial infection without calibrated inocula (3). In the present study, we detected elevated concentrations of proinflammatory cytokines (TNF and IL-6) in peritoneal fluid when high concentrations of *E. faecalis* had been inoculated. However, low concentrations of *E. faecalis* also generated a trend toward higher peritoneal concentrations of IL-6 compared to the enterococcus-free inoculum, suggesting a specific and dose-dependent inflammatory effect of enterococci. This effect was confirmed in the group receiving heat-inactivated enterococci.

In the present study, the selective increases of the TNF and IL-6 concentrations in peritoneal fluid after bacterial challenge were observed for a prolonged period. Similar compartmentalization of the inflammatory response has previously been reported in experimental models of intra-abdominal infections (3, 31) and in clinical infections (32). In our study, the concentrations of TNF measured in plasma were almost always below the limit of detection despite positive blood cultures. Since 6 h was the first period studied after bacterial challenge, a transient release of TNF in the blood before 6 h cannot be excluded. The prolonged inflammatory response observed herein differs from what is reported in experimental models involving bolus injection of pathogens. Although clinical data available in peritoneal infections are scarce, our results might, to some extent, provide relevant information on the inflammatory response in the course of intra-abdominal infection.

Our data suggest that enterococci might play a direct role in the pathophysiological mechanisms observed during intra-abdominal infection by limiting phagocytosis and intracellular killing of other pathogens and by spreading and prolonging the peritoneal inflammatory response. The use of highly diffusible antibiotics with strong intracellular uptake might be the ideal treatment to counteract the intracellular effects of enterococci on phagocytosis.

However, the increasing frequency of enterococci in clinical isolates and the emergence of highly resistant strains of these pathogens prompt the investigation of other therapeutic approaches. Among the new strategies available, treatments aimed at enhancing local defenses or modulating the inflammatory peritoneal response could represent possible ways of improving the peritoneal response during intra-abdominal infections involving enterococci, but they remain to be evaluated.

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REFERENCES

1. **Aarden, L. A., E. R. De Groot, O. L. Schaap, and P. M. Lansdorp.** 1987. Production of hybridoma growth factor by human monocytes. Eur. J. Immunol. **17:**1411–1416.
2. **Arduino, R. C., B. E. Murray, and R. M. Rakita.** 1994. Roles of antibodies and complement in phagocytic killing of enterococci. Infect. Immun. **62:**987–993.
3. **Ayala, A., M. M. Perrin, J. M. Kisala, W. Ertel, and I. H. Chaudry.** 1992. Polymicrobial sepsis selectively activates peritoneal but not alveolar macrophages to release inflammatory mediators (interleukins-1 and -6 and tumor necrosis factor). Circ. Shock **36:**191–199.
4. **Bhakdi, S., T. Klonisch, P. Nuber, and W. Fischer.** 1991. Stimulation of monokine production by lipoteichoic acids. Infect. Immun. **59:**4614–4620.
5. **Brakenhoff, J. P., E. R. de Groot, R. F. Evers, H. Pannekoek, and L. A. Aarden.** 1987. Molecular cloning and expression of hybridoma growth factor in *Escherichia coli*. J. Immunol. **139:**4116–4121.
6. **Contrepois, A., J. M. Vallois, J. J. Garaud, B. Pangon, J. Mohler, A. Meulemans, and C. Carbon.** 1986. Kinetics and bactericidal effect of gentamicin and latamoxef (moxalactam) in experimental *Escherichia coli* endocarditis. J.

Antimicrob. Chemother. **17:**227–237.
7. **Dunn, D. L., R. A. Barke, N. B. Knight, E. W. Humphrey, and R. L. Simmons.** 1985. Role of resident macrophages, peripheral neutrophils, and translymphatic absorption in bacterial clearance from the peritoneal cavity. Infect. Immun. **49:**257–264.
8. **Elsbach, P.** 1980. Degradation of microorganisms by phagocytic cells. Rev. Infect. Dis. **2:**106–128.
9. **Ember, J. A., and T. E. Hugli.** 1989. Characterization of the human neutrophil response to sex pheromones from *Streptococcus faecalis*. Am. J. Pathol. **134:**797–805.
10. **Harvey, B. S., C. J. Baker, and M. S. Edwards.** 1992. Contributions of complement and immunoglobulin to neutrophil-mediated killing of enterococci. Infect. Immun. **60:**3635–3640.
11. **Hay, H., and J. Cohen.** 1989. Studies on the specificity of the L129 cell bio assay for the measurement of tumor necrosis factor. J. Clin. Lab. Immunol. **29:**151–155.
12. **Ingham, H. R., P. R. Sisson, D. Tharagonnet, J. B. Selkon, and A. A. Codd.** 1977. Inhibition of phagocytosis in vitro by obligate anaerobes. Lancet **ii:** 1252–1254.
13. **Jacob, A. E., and S. J. Hobbs.** 1974. Conjugal transfer of plasmid-borne multiple antibiotic resistance in *Streptococcus faecalis* var. *zymogenes*. J. Bacteriol. **117:**360–372.
14. **Kelton, J. G., R. Ulan, C. Stiller, and E. Holmes.** 1978. Comparison of chemical composition of peritoneal fluid and serum: a method for monitoring dialysis patients and a tool for assessing binding to serum proteins in vivo. Ann. Intern. Med. **89:**67–70.
15. **Kramer, S., M. Cerver, and S. Apperson.** 1986. Comparison of TNF and TNF cytolytic biological activities in a serum-free bioassay. Lymphokine Res. **5**(Suppl.)**:**S139–S143.
16. **Lorber, B., and R. M. Swenson.** 1975. The bacteriology of intra-abdominal infections. Surg. Clin. North Am. **55:**1349–1354.
17. **Matlow, A. G., J. M. A. Bohnen, C. Nohr, N. Christou, and J. Meakins.** 1989. Pathogenicity of enterococci in a rat model of fecal peritonitis. J. Infect. Dis. **160:**142–145.
18. **Meager, A., H. Leung, and J. Wooley.** 1989. Assays for tumor necrosis factor and related cytokines. J. Immunol. Methods **116:**1–17.
19. **Moellering, R. C. Jr.** 1992. Emergence of enterococcus as a significant pathogen. Clin. Infect. Dis. **14:**1173–1178.
20. **Montravers, P., A. Andremont, L. Massias, and C. Carbon.** 1994. Investigation of the potential role of *Enterococcus faecalis* in the pathophysiology of experimental peritonitis. J. Infect. Dis. **169:**821–830.
21. **Montravers, P., R. Gauzit, C. Muller, J. P. Marmuse, A. Fichelle, and J. M. Desmonts.** 1996. Emergence of antibiotic-resistant bacteria in cases of peritonitis after intraabdominal surgery affects the efficacy of empirical antimicrobial therapy. Clin. Infect. Dis. **23:**486–494.
22. **Murray, B. E.** 1990. The life and times of the enterococcus. Clin. Microbiol. Rev. **3:**46–65.
23. **Nichols, R. L., and A. C. Muzik.** 1992. Enterococcal infections in surgical patients: the mystery continues. Clin. Infect. Dis. **15:**72–76.
24. **Onderdonk, A. B., J. G. Bartlett, T. Louie, N. Sullivan-Seigler, and S. L. Gorbach.** 1976. Microbial synergy in experimental intra-abdominal abscess. Infect. Immun. **13:**22–26.
25. **Rotstein, O. D., T. L. Pruett, and R. L. Simmons.** 1985. Mechanisms of microbial synergy in polymicrobial surgical infections. Rev. Infect. Dis. **7:**151–170.
26. **Snedecor, G. W., and W. G. Cochran.** 1967. Statistical methods, 6th ed., p. 258–298. The Iowa State University Press, Ames, Iowa.
27. **Steel, R. G. D., and J. H. Tovrie.** 1987. Principles and procedures of statistics: a biometrical approach, p. 172–194. McGraw-Hill Book Co., New York, N.Y.
28. **Weinstein, W. M., A. B. Onderdonk, J. G. Bartlett, and S. L. Gorbach.** 1974. Experimental intra-abdominal abscesses in rats: development of an experimental model. Infect. Immun. **10:**1250–1255.
29. **Weinstein, W. M., A. B. Onderdonk, J. G. Bartlett, T. J. Louie, and S. L. Gorbach.** 1975. Antimicrobial therapy of experimental intraabdominal sepsis. J. Infect. Dis. **132:**282–286.
30. **Wicher, J., and E. Inghan.** 1990. Cytokine measurements in body fluids. Eur. Cytokine Network **4:**239–243.
31. **Zanetti, G., D. Heumann, J. Gerain, J. Kohler, P. Abbet, C. Barras, R. Lucas, M. P. Glauser, and J. D. Baumgartner.** 1992. Cytokine production after intravenous or peritoneal Gram-negative bacterial challenge in mice. J. Immunol. **148:**1890–1897.
32. **Zeni, F., B. Tardy, M. Vindimian, C. Comtet, Y. Page, I. Cusey, and J. C. Bertrand.** 1993. High levels of tumor necrosis factor-α and interleukin-6 in the ascitic fluid of cirrhotic patients with spontaneous bacterial peritonitis. J. Infect. Dis. **17:**218–223.

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