Microbial Synergy in Experimental Intra-Abdominal Abscess

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Intra-abdominal sepsis was studied in Wistar rats by using four microbial species: Escherichia coli, enterococci, Bacteroides fragilis, and Fusobacterium varium. These organisms were implanted into the peritoneal cavity singly and in all possible dual combinations. Results were evaluated by mortality rates and the incidence of intra-abdominal abscesses on autopsy following sacrifice after 7 days. Mortality was restricted to recipients of E. coli, thus implicating coliforms in the acute lethality associated with this experimental model. Intra-abdominal abscesses were produced in 61 of 65 (94%) animals that received the combination of an anaerobe and a facultative organism. Abscesses failed to form with any single strain or with E. coli plus enterococci, and they were detected in one of 19 animals receiving B. fragilis plus F. varium. These results suggest that intra-abdominal abscess formation is related to synergy between anaerobes and facultative bacteria.

There are many unanswered questions concerning the pathogenesis of infections involving both facultative and anaerobic bacteria. Intra-abdominal sepsis can be considered a prototype of such mixed infections. Often related to bowel perforation, the subsequent infection includes multiple species of bacteria derived from the intestinal flora. The diversity of this flora has suggested cooperative interactions or synergy among the various microbial partners in the septic process. The present study was designed to determine the role of selected intestinal microbes in an experimental animal model of intra-abdominal sepsis. The test strains were inoculated intraperitoneally in pure cultures and in various combinations. Our results indicate that intra-abdominal abscess is produced by synergy between anaerobes and facultative bacteria.

MATERIALS AND METHODS

Bacteria were implanted intraperitoneally in Wistar rats to determine septic complications; the parameters were mortality and abscess formation. Four strains of bacteria were used: Escherichia coli, enterococci, Bacteroides fragilis, and Fusobacterium varium. There were 12 experimental groups with 20 animals in each group (Table 1). Groups 1 through 4 consisted of pure cultures of the four strains. Groups 5 through 10 included all possible combinations of two strains. The inoculum in group 11 was a mixture of all four test organisms. A final group, which served as controls, received an inoculum of autoclaved intestinal contents and barium sulfate.

Animals. Male Wistar rats (Simonsen Laboratories, Palo Alto, Calif.), weighing between 160 and 180 g, were used for all experiments. Animals were housed in groups of 10 before surgery; after implantation of the bacterial inoculum they were caged separately. All animals were maintained on chow (Ralston Purina, St. Louis, Mo.) and water ad lib.

Microorganisms. The four strains of bacteria used in the various inocula were originally isolated from a culture of an intra-abdominal abscess in a rat produced by intraperitoneal implantation of fecal contents obtained from meat-fed rats (15). Each test organism was isolated in pure culture and then transferred to prerduced peptone-yeast-glucose broth. After anaerobic incubation for 24 to 48 h, the cultures were divided into 5-ml aliquots, placed in gas-tight vials, frozen in liquid nitrogen, and stored at ~40 C until used.

Preparation of inocula. Vials of test bacteria were thawed in the anaerobic chamber and diluted with sterile prerduced peptone-yeast-glucose broth. This dilution was adjusted to give the numbers of microorganisms for the bacterial challenge which are enumerated in Table 1. It should be noted that the number of E. coli is half that for the other three test organisms. This modification was necessary because our preliminary observations showed that the larger challenge with E. coli was universally fatal within 2 days. With this exception, the total number of organisms implanted was constant in all 11 groups. For experiments using combinations of microorganisms, the inocula were made by adding equal volumes of the individually prepared bacterial strains. Diluted broth cultures for all groups were combined with autoclaved rat colonic contents (50% vol/vol) and barium sulfate (10% wt/vol). Aliquots of 0.5 ml of the final product were placed into gelatin
capsules for intraperitoneal implantation. Barium sulfate was added to facilitate localization of the infection. Autoclaved rat colonic contents were added because our preliminary experiments showed that this was necessary to produce intra-abdominal abscesses.

Implantation of inoculum. Rats were prepared for surgical implants by injection of 0.15 ml of Nembutal (50 mg/ml). The abdomen of each animal was shaved and prepped with iodine; a 1-cm anterior midline incision was made through the abdominal wall and peritoneum. A gelatin capsule containing the inoculum was inserted into the pelvic region, and the incision was closed with interrupted silk sutures.

Evaluation of results. After implantation of the inoculum, the animals were returned to separate cages and observed every 12 h for 7 days. Fatalities occurring within 4 h of surgery were ascribed to anesthesia, and these animals were eliminated from the study. All surviving animals were autopsied at 7 days. Results were evaluated by two parameters: mortality during the 7 days of observation and the incidence of abscess formation on autopsy at the termination of the experiment.

Bacteriology. Quantitative bacteriological culture of each inoculum was performed just before implantation. In addition, abscess contents were quantitatively cultured immediately after sacrifice in six animals randomly selected from each group. Samples of 0.1 ml of the inoculum or abscess contents were immediately made into the anaerobic chamber and were assayed on V1 dilution plates (11). Aliquots of 0.1 ml were plated, giving final concentrations of 10*5, 10*4, 10*3, and 10*2 per ml. Media used for detection of anaerobes were brocchia agar base with 8% sheep blood and 10 μg of menadione per ml (BMB); BMB containing 100 μg of neomycin sulfate per ml; and laked-blood agar containing 75 μg of kanamycin and 7.5 μg of vancomycin per ml. These three plates were incubated in the anaerobic chamber at 37 °C for 3 to 5 days. For facultative bacteria the following media were used: MacConkey agar, Pfizer selective enterococcus agar, and blood agar. These plates were incubated in air for 24 to 48 h. After incubation, colony types were enumerated, isolated, and identified.

RESULTS

Implantation of single strains. Each of the four bacterial strains was tested individually to determine its effect on mortality and abscess formation (Table 2). Mortality was restricted to recipients of E. coli, and this effect correlated with the number of bacteria in the inoculum. Using the standard challenge of 5 × 10⁷ organisms, all animals receiving E. coli died within 2 days. A 50% reduction in the inoculum size of this organism decreased mortality to 65%. All experiments using the other three bacterial species with the larger inoculum size failed to produce a single fatality. No abscesses were detected at autopsy in any of the 80 animals receiving a challenge with a single microbial species.

Combinations of two bacterial strains. All possible combinations of two bacterial species were implanted in an effort to define the critical combinations. As in the experiments with single strains of bacteria, mortality was restricted to the animals receiving E. coli as one of the two implanted species (Table 3). Mortality rates ranged from 25 to 37% in the three groups receiving this organism.

Abscess production correlated with the presence of one facultative strain and one anaerobic strain. The incidence of abscess formation was 89 to 100% in each of the four groups that used this type of combination. On the other hand, combining the two facultative bacteria resulted in no abscesses, and a combination of the two anaerobic strains produced only a single abscess in 19 animals (5%).

Cultures of 25 abscesses from sample populations of each group yielded the two implanted species in 20 abscesses. Concentrations of the organisms recovered in each instance ranged

<table>
<thead>
<tr>
<th>Strain</th>
<th>No. of rats</th>
<th>Mortality (no. died/no. tested)</th>
<th>Abscess (no. with abscess/no. tested)</th>
</tr>
</thead>
<tbody>
<tr>
<td>E. coli</td>
<td>20</td>
<td>13/20 (65%)</td>
<td>0/7</td>
</tr>
<tr>
<td>Enterococcus</td>
<td>20</td>
<td>0/20</td>
<td>0/20</td>
</tr>
<tr>
<td>B. fragilis</td>
<td>20</td>
<td>0/20</td>
<td>0/20</td>
</tr>
<tr>
<td>F. varium</td>
<td>20</td>
<td>0/20</td>
<td>0/20</td>
</tr>
</tbody>
</table>

TABLE 1. Experimental groups

<table>
<thead>
<tr>
<th>Group</th>
<th>Inoculum</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>E. coli (2.5 × 10⁷)*</td>
</tr>
<tr>
<td>2</td>
<td>Enterococcus (5 × 10⁷)</td>
</tr>
<tr>
<td>3</td>
<td>B. fragilis (5 × 10⁷)</td>
</tr>
<tr>
<td>4</td>
<td>F. varium (5 × 10⁷)</td>
</tr>
<tr>
<td>5</td>
<td>E. coli (1.3 × 10⁷) + enterococcus (2.5 × 10⁷)</td>
</tr>
<tr>
<td>6</td>
<td>E. coli (1.3 × 10⁷) + B. fragilis (2.5 × 10⁷)</td>
</tr>
<tr>
<td>7</td>
<td>E. coli (1.3 × 10⁷) + F. varium (2.5 × 10⁷)</td>
</tr>
<tr>
<td>8</td>
<td>Enterococcus (2.5 × 10⁷) + B. fragilis (2.5 × 10⁷)</td>
</tr>
<tr>
<td>9</td>
<td>Enterococcus (2.5 × 10⁷) + F. varium (2.5 × 10⁷)</td>
</tr>
<tr>
<td>10</td>
<td>B. fragilis (2.5 × 10⁷) + F. varium (2.5 × 10⁷)</td>
</tr>
<tr>
<td>11</td>
<td>E. coli (6 × 10⁷) + enterococci (1.3 × 10⁷) + B. fragilis (1.3 × 10⁷) + F. varium (1.3 × 10⁷)</td>
</tr>
<tr>
<td>12</td>
<td>Sterile colonic contents and barium sulfate (control)</td>
</tr>
</tbody>
</table>

* Number of organisms.
from $10^7$ to $10^9$/ml. Exceptions included two of 12 animals implanted with *F. varium* in which this organism was not recovered. More difficult to explain are three instances where the abscess yielded an additional bacterial species that was not included in the inoculum. These three isolates were *E. coli*, enterococci, and *Proteus mirabilis*. Cultures of the inoculum before implantation failed to reveal contamination, and the source of these organisms is unknown.

**Implantation of four bacterial strains.** When an inoculum of all four bacteria was tested, 8 of 20 animals (40%) died during the 7 days of observation, and all 12 survivors had discrete intra-abdominal abscesses at the time of sacrifice. Cultures were performed on abscesses from six animals, and these uniformly yielded various combinations of the originally implanted strains. Enterococci were recovered in all six specimens; *B. fragilis*, *E. coli*, and *F. varium* were present in five, four, and three, respectively. Concentrations of these organisms were $10^5$ to $10^6$/ml.

Controls. All animals implanted with autoclaved colonic contents and barium sulfate survived the 7-day period of observation. At autopsy no abscesses were detected.

**DISCUSSION**

There have been several studies of intra-abdominal sepsis using experimental animal models. Inocula have varied from a complex flora of intestinal contents to broth cultures of single strains alone and in various combinations. In most reports, toxicity was measured by acute peritonitis and mortality after intra-peritoneal implantation or by soft tissue lesions after subcutaneous inoculation. Conspicuously absent in these studies was the induction of intra-abdominal abscesses. Although a commonly recognized complication of bowel perforation in humans, localized intraperitoneal purulent collections have proven difficult to reproduce in experimental animals, using colonic flora.

Our initial studies with this animal model used a standardized inoculum of fecal contents obtained from rats fed a meat diet (11, 15). Microbiological analysis of the original inoculum revealed a complex aerobic-anaerobic flora containing 26 bacterial species. This flora simulated the colonic flora in humans eating a Western diet in terms of the organisms present and their relative concentrations (11). Intraperitoneal implantation of gelatin capsules containing the fecal inoculum with barium sulfate produced a two-stage disease. During the initial five postoperative days, there was acute peritonitis associated with *E. coli* bacteremia and a 43% mortality. All animals surviving the acute peritonitis stage developed localized intra-abdominal abscesses by the 7th postoperative day. Microbiological studies showed that four organisms were consistently present and were numerically dominant at infected sites: *E. coli*, enterococci, *B. fragilis*, and *F. varium*.

The present study further clarifies the role of selected bacteria in the pathological events. Instead of the complex intestinal flora, the four organisms that played a preeminent role were implanted singly and in various combinations. Acute mortality was restricted to animals receiving *E. coli* in the inoculum. These data implicate commensals as the major pathogens in the acute, often lethal peritonitis stage in this model and tend to corroborate our earlier findings based on blood culture isolates.

Abscess formation correlated with the combination of an anaerobe and a facultative strain. No abscess resulted when *E. coli* and enterococci were implanted alone or together. Pure cultures of *B. fragilis* and *F. varium* also failed to produce this effect, and only one of 19 animals that received both anaerobes developed abscesses. On the other hand, abscesses occurred in 89 to 100% of animals that received the combination of one facultative strain and one anaerobe.

The addition of autoclaved intestinal contents also proved necessary to produce both mortality and abscesses. Previous studies of experimental intra-abdominal sepsis have also shown potentiation of virulence by incorporating various substances with the bacterial challenge. Additives found effective in this regard included hog gastric mucin (12), a piece of potato (5), gum tragacanth (3), autoclaved tissue (2), and sterile feces (14). Autoclaved fecal contents were used in the present study to simulate in vivo condi-
tions encountered with colonic perforation. However, the mechanism of enhanced virulence with these heterogenous, particulate substances remains to be elucidated.

The observation that abscess formation correlated with the combination of a facultative strain and an anaerobe represents an example of bacterial synergy, i.e., combinations of bacteria causing a pathological lesion that cannot be reproduced by the component organisms alone. This effect cannot be ascribed to the additive numbers of bacteria since the size of the bacterial challenge was similar in all experiments.

Previous studies have documented the synergistic effect of intestinal bacteria in experimental infections. In 1938 Altemeier reported his observations in bacteriological studies of peritonitis after appendiceal rupture (1). Among 100 cases, 96 yielded a mixed aerobic-anaerobic flora. Pure cultures of the individual isolates were relatively innocuous when implanted subcutaneously in animals, but combinations of facultative and anaerobic strains greatly increase virulence (2). Similar observations have been reported by Meleney et al. (9) and Hite et al. (6).

There are several possible mechanisms to explain the increased virulence of facultative and anaerobic bacteria at infected sites. With Meleney's synergistic gangrene, it has been shown that both Staphylococcus and micro-aerophilic streptococci are required to reproduce typical lesions in experimental animals (8). Subsequent studies have suggested that staphylococci produce hyaluronidase, which permits the micro-aerophilic streptococcus to invade tissue (10).

Another synergistic mechanism is the production of critical growth factors by one organism that permit survival of the pathogen at the infected site. This effect has been demonstrated in studies of gingivitis by MacDonald et al. (7). These investigators recovered 17 bacterial species from typical lesions and inoculated various components into animals. Bacteroides melaninogenicus proved to be an indispensable agent for transferable pathogenicity, but a second microbe was required to supply its need for vitamin K. Similarly, it has been shown that diphtheroids provide a necessary nutrient for Fusobacterium nucleatum, the principal pathogen in infective bulbar neerosis of sheep (13).

An alternative explanation for the synergetic effect of aerobic-anaerobic combinations depends on lowering local oxygen concentrations and the oxidation-reduction potential by the aerobe. The resultant physical conditions are appropriate for replication and invasion by the anaerobic component of the infection. Such environmental factors are known to be critical for anaerobic growth in vitro and may apply with equal relevance to in vivo experimental animal studies. Mergenhagen et al. noted that the infecting dose of anaerobic streptococci was significantly lowered when the inoculum was supplemented with chemical reducing agents (10). A similar effect may be produced by facultative bacteria. According to Filides, tetanus spores failed to germinate if inoculated directly into the skin of guinea pigs due to the high tissue oxygen tension (4). When facultative bacteria were introduced concurrently there was a reduction in oxidation-reduction potential, and the tetanus spores germinated and then produced toxin (16). In this manner, the facultative bacteria provided the proper conditions for establishment of an anaerobic infection at previously well-oxygenated sites.

LITERATURE CITED


