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Comparison of the In Vitro Bactericidal Activity of Human Serum and Leukocytes Against *Bacteroides fragilis* and *Fusobacterium mortiferum* in Aerobic and Anaerobic Environments

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In aerobic and anaerobic in vitro environments, *Fusobacterium mortiferum* was killed by human leukocytes or serum alone or in combination; clinical isolates of *Bacteroides fragilis* were killed only by leukocytes in the presence of serum.

Recently there has been a growing awareness of the increased incidence of infections caused by gram-negative, nonsporulating anaerobic microorganisms (1, 2, 7-14, 17, 19, 20). Many of these infections occur in patients post-operatively, particularly after trauma to the bowel or female pelvic organs. Gram-negative anaerobes have also been isolated in pure or mixed culture from cases of thrombophlebitis, skin and soft tissue infections, pulmonary infections, brain and liver abscesses, endocarditis, and many other infections.

Minimal information is available regarding specific humoral and cellular host defense mechanisms against gram-negative anaerobic microorganisms. Phagocytosis and killing by polymorphonuclear leukocytes have been shown to be of primary importance in host defense against extracellular and facultative aerobic microorganisms. Serum bactericidal activity against gram-negative aerobic enteric bacilli is also well recognized. The purpose of the present study was to determine if these bactericidal mechanisms were operative against strains of *Bacteroides fragilis* and *Fusobacterium mortiferum* in vitro under aerobic or anaerobic conditions.

A minor modification of the method of Hirsch and Strauss (16) was used for measuring the effects of human serum and peripheral leukocytes on the killing of *F. mortiferum* 9817, *B. fragilis* subspecies fragilis 1249, and *B. fragilis* subspecies thetaiotaomicron 1309. *F. mortiferum* 9817 was obtained from the American Type Culture Collection, Washington, D.C., and *B. fragilis* strains 1249 and 1309 were isolated from clinical specimens in the Surgical Bacteriology Laboratory of the University of Cincinnati Medical Center. Diluent used in the experiments was Hanks balanced salt solution (Microbiological Associates Inc., Bethesda, Md.) containing 0.1% gelatin that was deoxygenated by boiling immediately before the experiments. Preliminary experiments indicated that survival of each of the bacterial strains in diluent in plastic-capped tubes was excellent over a 3-h incubation period at 37°C in room air. Bacteria were maintained in thioglycolate medium at −70°C; before each experiment, a tube of thioglycolate medium was inoculated from a frozen culture. The tube was incubated for 4 days at 37°C, and then transferred to a tube of deoxygenated medium containing equal parts of Trypticase soy broth and brain heart infusion broth that was incubated at 37°C overnight. The bacteria were washed once and diluted in deoxygenated diluent immediately before the experiments. Leukocytes were prepared from the plasma of dextran-sedimented heparinized blood obtained from healthy adult volunteers. The leukocyte-rich plasma was divided and centrifuged at 200 × *g* for 10 min, and the supernatants were discarded. The leukocytes were washed twice and suspended in deoxygenated or untreated diluent depending on whether the experiments were to be conducted in an aerobic or anaerobic environment.

Various combinations of pooled normal human serum (10% concentration), leukocytes (5×10^6), bacteria (1.0×10^6), and diluent in a final volume of 1 ml were added together in plastic-capped tubes. For the experiments conducted in the anaerobic environment, the diluent added to the reaction mixtures was deoxygenated; for the experiments conducted in room air, untreated diluent was used. For the experiments performed in the anaerobic environment, the reagents were added to the tubes in an anaerobic glove box (Coy Manufacturing Co., Ann Arbor, Mich.). In addition, the tubes

to be used were equilibrated overnight in the glove box, and the serum was equilibrated in the glove box on ice for 3 to 4 h before its addition to the respective reaction mixtures. The internal atmosphere of the glove box contained 85% nitrogen, 10% hydrogen, 5% carbon dioxide, and not over 25 ppm of oxygen. The pH and pO_2 in each of the different reaction mixtures was measured before incubation at 37°C. The pH of the anaerobic mixtures was 6.95 ± 0.05, and the pO_2 ranged from 19 to 25 mm of Hg. The pH of the aerobic mixtures was 6.95 ± 0.05, and the pO_2 ranged from 152 to 169 mm of Hg. A blood gas analyzer (Radiometer BMS3 MK2) was used for the pO_2 determinations.

The next steps in the procedure were carried out entirely in or out of the glove box. The tubes were incubated for 3 h at 37°C on a rotating platform, and samples were removed at 0 time, 30, 90, and 180 min. The samples were diluted in deoxygenated distilled water, and the dilutions were plated in thioglycollate agar by the pour plate method. The plates from all experiments were incubated anaerobically in GasPak jars for 4 days, and the colonies were enumerated to determine the total number of bacteria surviving in each reaction mixture. In some experiments, the number of surviving extracellular bacteria was determined by plating the supernatants of the reaction mixtures after centrifugation at 100 × *g*.

F. mortiferum 9817 was killed directly by serum alone or in the presence of serum and leukocytes; similar results were obtained when the experiments were carried out in or out of the anaerobic glove box (Fig. 1). A 3-log reduction in bacterial counts by leukocytes in the absence of serum was demonstrated in the aerobic environment in comparison to a 1-log reduction in bacterial counts when this reaction was carried out in the glove box. In both environments, *B. fragilis* 1249 was killed by leukocytes

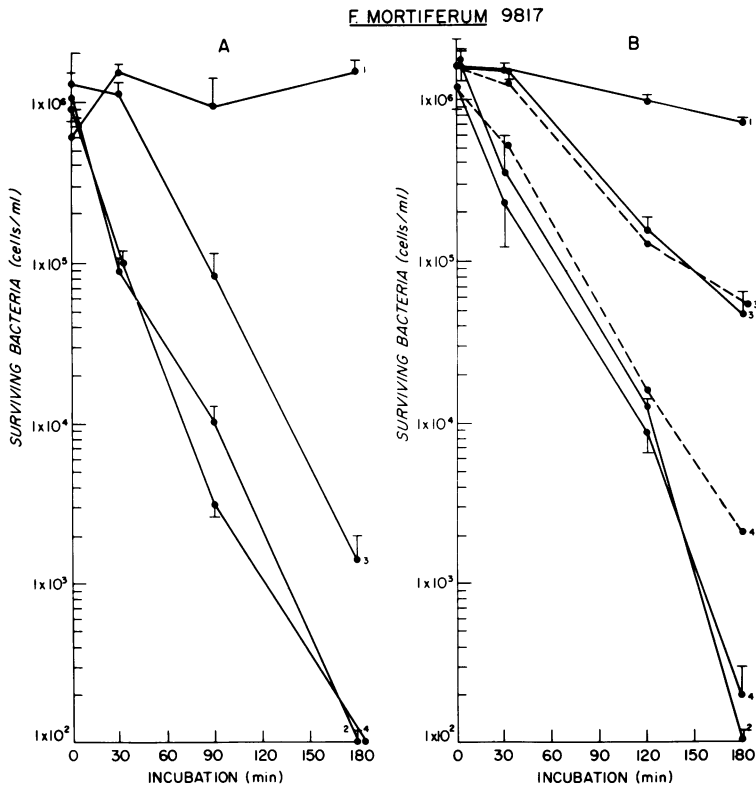

FIG. 1. *Effect of human serum and leukocytes on the killing of F. mortiferum 9817. In (A), experiments were performed in room air; in (B), experiments were performed in the anaerobic glove box. The following reaction mixtures were tested: (i) diluent and bacteria; (ii) serum bacteria; (iii) leukocytes and bacteria; (iv) serum, leukocytes, and bacteria. The solid lines represent total surviving bacteria, and the dotted lines represent surviving extracellular bacteria. The vertical bars represent the standard error of the mean of duplicate determinations.*

in the presence of serum but not by serum alone (Fig. 2). Killing of this microorganism by leukocytes in the absence of serum occurred in the aerobic environment, but not under anaerobic conditions. In both environments, *B. fragilis* 1309 was only killed by leukocytes in the presence of serum and not by either leukocytes or serum alone (Fig. 3). In the anaerobic environment, total counts in the reaction mixtures containing leukocytes, bacteria, and serum or leukocytes and bacteria alone were compared to bacterial counts in the supernatants of these reaction mixtures. For all three bacterial strains, similar counts were obtained, suggesting that phagocytosis of the microorganisms was rapidly followed by intracellular killing.

Preliminary studies were also performed to determine if killing of *B. fragilis* 1249 by aerobic leukocytes in the absence of serum was dependent upon phagocytosis. In the aerobic environment, extracellular and total counts in reaction mixtures containing leukocytes and the bacteria were compared. Similar counts were obtained indicating that the microorganisms were phagocytosed before killing. Because killing of the bacteria by leukocytes did occur in the anaerobic environment in the presence of serum, the difference in the results in the anaerobic and aerobic environments in the absence of serum appears to be the result of a difference in phagocytosis.

The strains of *B. fragilis* subspecies fragilis and thetaiotaomicron used in our study are subspecies that are common clinical isolates. Our observation that both of these strains were phagocytosed and promptly killed intracellularly by leukocytes in the presence of normal serum but not by serum alone confirms the findings of Casciato et al. (6). They demonstrated that under aerobic conditions clinical isolates of *B. fragilis* were not susceptible to the bactericidal activity of human serum but were killed by human leukocytes in the presence of normal serum. Our study shows that this bactericidal mechanism is also operative under anaerobic conditions, a finding of considerable clinical significance.

B. fragilis has the same serum requirements for opsonization as the opportunist pathogens such as *Pseudomonas aeruginosa* (3–5) and *Escherichia coli* (16) and may be handled by the host in a similar manner. In this regard, infec-

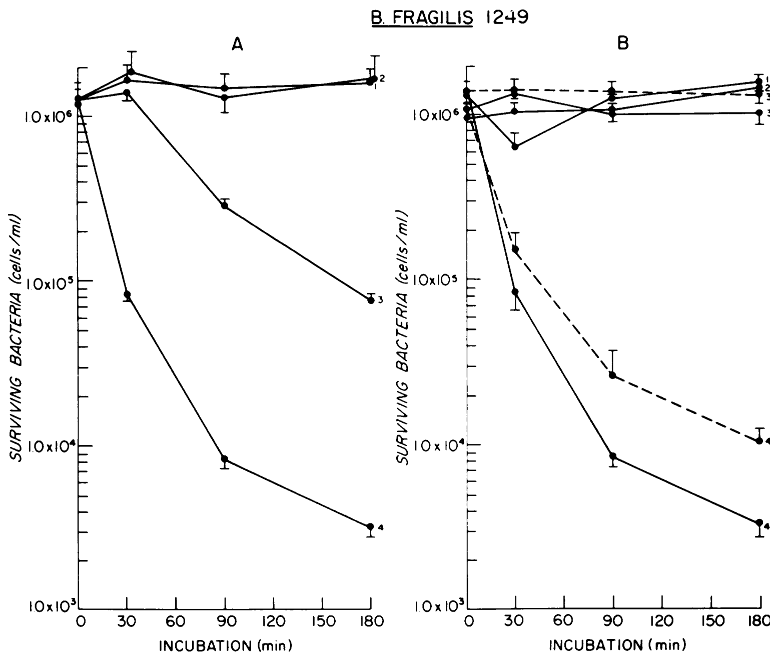

FIG. 2. *Effect of human serum and leukocytes on the killing of B. fragilis 1249. In (A), experiments were performed in room air; in (B), experiments were performed in the anaerobic glove box. The following reaction mixtures were tested: (i) diluent and bacteria; (ii) serum and bacteria; (iii) leukocytes and bacteria; (iv) serum, leukocytes, and bacteria. The solid lines represent total surviving bacteria, and the dotted lines represent surviving extracellular bacteria. The vertical bars represent the standard error of the mean of duplicate determinations.*

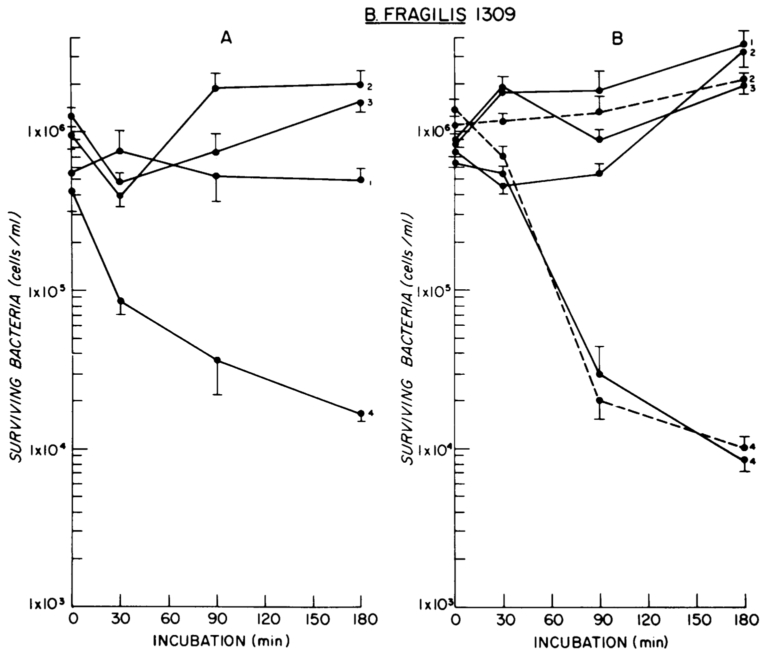

FIG. 3. *Effect of human serum and leukocytes on the killing of B. fragilis 1309. In (A), experiments were performed in room air; in (B), experiments were performed in the anaerobic glove box. The following reaction mixtures were tested: (i) diluent and bacteria; (ii) serum and bacteria; (iii) leukocytes and bacteria; (iv) serum, leukocytes, and bacteria. The solid lines represent total surviving bacteria, and the dotted lines represent surviving extracellular bacteria. The vertical bars represent the standard error of the mean of duplicate determinations.*

tions caused by *B. fragilis* have been seen with increased frequency in the compromised host (8, 10), and the invasiveness of *B. fragilis* probably depends to a large extent upon the functional integrity of the host's phagocytic cells and serum opsonins, as well as on the virulence factors elaborated by this microorganism. The finding that *F. mortiferum* was killed directly by normal human serum in the absence of leukocytes, or by leukocytes alone, or by leukocytes and serum suggests that this strain of *F. mortiferum* is probably not invasive unless the host's humoral and cellular functions are markedly abnormal.

Our observation that gram-negative anaerobic microorganisms were killed by leukocytes in an anaerobic environment supports the findings of Mandell (18). His investigation showed that a variety of aerobic and anaerobic microorganisms were killed efficiently by human polymorphonuclear leukocytes made anaerobic by nitrogen washout, suggesting that mechanisms other than those dependent on hydrogen peroxide may be important in intracellular killing by leukocytes. The finding in our study that *B. fragilis* subspecies fragilis was killed to some extent by leukocytes in the absence of serum in the aerobic but not anaerobic environment remains to be explained, but appears to be dependent upon an ingestion mechanism that occurs independently of serum opsonins.

Because of the recent appreciation of the limitations and complications of antibiotic therapy, it is becoming increasingly important to thoroughly understand host defense mechanisms against opportunist microorganisms. Attention needs to be focused now upon defining the human serum opsonic and leukocyte bactericidal mechanisms that protect against gram-negative nonsporulating anaerobes. Studies in this area will provide fundamental knowledge and may lead to new therapeutic approaches for the treatment and prevention of infections caused by these microorganisms.

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