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Enhancement of Growth of Aerobic and Facultative Bacteria in Mixed Infections with *Bacteroides* Species

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The potential for mutual enhancement of growth of the *Bacteroides fragilis* and *B. melaninogenicus* groups and the aerobic and facultative organisms commonly isolated with them in mixed infections was evaluated. Enhancement was studied by measuring the relative increase in CFU of the two bacterial components inducing subcutaneous abscesses in mice. Of the 42 combinations between three isolates each of the *B. fragilis* and *B. melaninogenicus* groups and seven aerobic or facultative organisms, *Bacteroides* spp. were enhanced in only 8 and inhibited in 4. The aerobic and facultative bacteria were enhanced in 31 of the 42 combinations and depressed in 2. The organisms uniformly enhanced by all of the *Bacteroides* spp. were group A streptococci and *Escherichia coli* (all six instances), followed by *Staphylococcus aureus* and *Klebsiella pneumoniae* (five of six instances), *Pseudomonas aeruginosa* (four instances), group D streptococci (in three instances only by the *B. fragilis* group), and *Haemophilus influenzae* (one instance). It is apparent that the growth rate of facultative and aerobic bacteria is enhanced much more in mixed infections with *Bacteroides* spp. than that of their anaerobic counterparts.

Several organisms are generally recovered from infectious sites where *Bacteroides* spp. are also recovered (3). Organisms of the *Bacteroides fragilis* group are usually recovered from intraabdominal (8) and pelvic (3, 8) infections and from abscesses near the anal area (4), while organisms of the *B. melaninogenicus* group are generally isolated from infections in and around the oral cavity and from the upper and lower portions of the respiratory tract (2).

Previous studies have reported that the relationships between these *Bacteroides* spp. and their aerobic and facultative organisms are synergistic (1, 5–7, 9, 12, 15, 17). However, the criteria for synergy were judged by the induction of sepsis (1, 9, 12, 15), the enumeration of mortality (9, 15, 17), or the ability to induce abscesses (5–7). None of these previous studies defined the role of each of the bacterial strains involved in the mixed infection. In this study we evaluated the potential for mutual enhancement and synergy between *Bacteroides* spp. and other bacteria commonly present with them in clinical infections. This was done by measuring the relative increase in CFU of each bacterial component of mixed infections in subcutaneous abscesses.

All aerobic and anaerobic bacterial strains used in the experiments were recent clinical isolates. These isolates included three strains of the *B. melaninogenicus* group (*B. intermedius*, *B. asaccharolyticus*, and *B. melaninogenicus*) and three strains of the *B. fragilis* group (*B. fragilis*, *B. ovatus*, and *B. vulgatus*). The aerobic or facultative strains included one strain each of *Haemophilus influenzae* type b, *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, *Escherichia coli*, *Staphylococcus aureus*, and Lancefield group A and group D streptococci. The bacteria were kept frozen in skim milk at −70°C. The bacteria were identified by standard criteria (13, 18) and processed as previously described (5). The presence of a capsule was established by Hiss staining (13) and confirmed by electron microscopy after staining with ruthenium red (11). Ruthenium red staining demonstrated a homogenous polysaccharide capsule that was external to the cell wall. Capsular stains revealed the presence of a capsule in all of the *Bacteroides* strains and all of the aerobic strains except *E. coli* and *P. aeruginosa*.

The mice used were male Swiss albino mice weighing 20 to 25 g obtained from the Naval Medical Research Institute mouse colony. The mice were raised under conventional conditions.

Frozen bacterial suspensions were thawed to room temperature, subcultured onto chocolate or Schaedler anaerobic blood agar, and incubated for 48 h at 37°C in an anaerobic glove box (18) for the *Bacteroides* spp. or in 5% CO_2 for the aerobic bacteria. Twenty-four hours before injection into mice the bacterial strains were inoculated onto chocolate agar or Trypticase soy 5% sheep blood agar (BBL Microbiology Systems, Cockeysville, Md.). Cotton swabs were used to pick colonies from these media and transfer them to normal saline. The suspensions of organisms prepared were equivalent to a 0.5 McFarland standard. CFUs were determined by plate count in brain heart infusion agar enriched with vitamin K_1 and hemin to support the growth of *Bacteroides* organisms.

The ability of the organisms to cause an abscess was determined by subcutaneous inoculation of pure cultures of 10^8 CFU of each organism into groups of six mice for each isolate.

To determine relationships between the various organisms in mixed infections, each *Bacteroides* sp. was injected in combination with one species each of the other facultative or aerobic organisms (Table 1). Six mice were included in each experimental and control group. This experimental design was run three times with each organism or combination of organisms.

Mice were inoculated subcutaneously in the right groin with 0.1 ml of each of the appropriate bacterial suspensions in saline that contained 10^8 of each organism. To induce combined infection, two inoculates were mixed and each animal received 0.2 ml containing 0.1 ml of each bacterial suspension.

The effect of inoculation of *Bacteroides* spp. together with other bacteria was tested by counting the total CFU of each

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TABLE 1. Changes in numbers of *Bacteroides* organisms and other bacteria in an abscess[a]

Aerobic or facultative organisms	*B. asaccharolyticus* (8.5 ± 0.7)	*B. intermedius* (8.1 ± 0.9)	*B. melaninogenicus* (7.7 ± 1.1)	*B. fragilis* (8.6 ± 0.8)	*B. vulgatus* (7.6 ± 0.8)	*B. ovatus* (7.8 ± 0.6)	Increase/decrease of aerobes and facultatives
S. aureus (8.8 ± 0.9)	2.8[b]/2.1[b]	3.5[b]/−0.2	2.0[b]/0.3	3.1[b]/0.8	3.3[b]/1.0	−0.8/−0.5	5/0
Group A streptococci (9.2 ± 0.5)	3.2[b]/0.4	2.6[b]/0.5	3.0[b]/−0.4	2.9[b]/0.3	3.5[b]/1.6[b]	3.1[b]/0.2	6/0
Group D streptococci (7.1 ± 1.8)	−1.2/−0.8	0.2/−1.8[b]	−2.6[b]/−2.4[b]	1.8[b]/0.4	2.5[b]/0.8	2.3[b]/0.6	3/1
E. coli (9.0 ± 0.4)	3.3[b]/1.0	3.3[b]/−0.6	2.8[b]/0.4	3.1[b]/2.0[b]	2.9[b]/1.4[b]	2.8[b]/1.0	6/0
K. pneumoniae (7.2 ± 1.2)	2.4[b]/0.8	2.8[b]/1.0	0.5/0.6	3.2[b]/1.4[b]	3.4[b]/2.0[b]	2.9[b]/0.6	5/0
P. aeruginosa (8.1 ± 1.3)	2.2[b]/1.2	0.8/0.9	0.4/1.4[b]	3.6[b]/0.6	2.8[b]/0.4	2.9[b]/0.8	4/0
H. influenzae (6.3 ± 0.8)	2.1[b]/1.8[b]	1.8[b]/0.6	1.2/0.3	0.6/−0.9	−1.6[b]/−2.6[b]	0.4/−3.4[b]	2/1
Increase/decrease of bacteroides	2/0	0/1	1/1	2/0	3/1	0/1	

[a] Change (compared to control) in number of aerobic or facultative organisms (expressed in $\log_{10}$ CFU)/change (versus control) in number of *Bacteroides* organisms (expressed in $\log_{10}$ CFU). The numbers in parentheses indicate the $\log_{10}$ CFU (± standard deviation) of CFU of organisms in the subcutaneous abscess induced by the single organism when injected alone. Abscesses were induced by subcutaneous injection into the right groin of a 0.1-ml volume of suspension containing 10^9 of each bacterium per ml. The abscesses were cultured and bacterial counts were performed on day 5 after inoculation. Six mice were included in each experimental group.

[b] Significant differences between single and mixed infections $P < 0.05$.

organism in abscesses induced by a single organism or mixed flora. Animals were sacrificed by cervical dislocation at day 5 after the inoculation of bacteria, and the abscess material was aseptically removed. The site and histology of the abscesses were confirmed in two mice of each experimental group by staining a tissue sample with hematoxylin and eosin. The CFU of bacteria in each abscess were determined individually. The abscesses were homogenized inside a glove box in 1.0 ml of sterile saline in a ground-glass tissue homogenizer. Tenfold serial dilutions of the homogenates were made with sterile saline, and 0.1 ml of each dilution was spread in triplicate on enriched brain heart infusion and blood agar plates. Colonies were counted after 48 h of aerobic or anaerobic incubation at 37°C to determine the number of each species in the abscess. Numbers of bacteria were expressed as $\log_{10}$ CFU. Characteristic colonies of all organisms were picked and identified by Gram stain and biochemical tests (13, 18). The Student *t* test was used for statistical analysis.

All organisms that were tested caused abscesses. Abscesses were usually formed within 24 to 72 h of inoculation. They reached a maximum diameter of 12 to 18 mm within 5 to 7 days and began to drain between 10 and 20 days. Maximum abscess diameters resulted from mixed infections were larger (22 to 24 mm) than those of abscesses caused by single organisms (12 to 15 mm).

Histological examination of abscesses showed a central area of necrotic cells, fibrin, and bacteria surrounded by a band of leukocytes and a distinct collagen capsule. Mortality associated with abscess formation was variable but low (<5%) for all challenged mice. Only surviving mice were included in the statistical analyses. The differences in the numbers of aerobic, facultative, and anaerobic bacteria found between individual and mixed infections were calculated. The differences in numbers in $\log_{10}$ CFU aerobic and facultative bacteria and the differences in numbers of *Bacteroides* spp. are presented as ratios in Table 1.

For the purpose of comparing mixed infections of *Bacteroides* spp. and other bacteria with individual infections, enhancement of growth was defined as a statistically significant difference ($P < 0.05$) when the average CFU of an organism in a mixed infection was significantly higher than the average CFU of the same organism in an individual infection.

Of the 42 combinations, *Bacteroides* spp. were enhanced in 8 and inhibited in 4 (Table 1). The aerobic and facultative bacteria were enhanced in 31 of the 42 combinations and depressed in 2. Mutual enhancement of the *Bacteroides* spp. and the aerobic or facultative bacteria occurred in all but one of the seven instances where *Bacteroides* spp. were enhanced. The combination between *Bacteroides* spp. and other bacteria significantly increased the growth of almost all aerobic and facultative species except *H. influenzae*, which was enhanced only by *B. asaccharolyticus* and *B. intermedius*, and a group D streptococcus which was enhanced only by the *B. fragilis* group. The organisms uniformly enhanced by all the *Bacteroides* spp. were group A streptococci and *E. coli*. *S. aureus* and *K. pneumoniae* were enhanced in five of six combinations. *P. aeruginosa* was enhanced in four, the group D streptococcus in three, and *H. influenzae* in one.

A mutual symbiotic enhancement of growth of either aerobic or facultative bacteria together with *Bacteroides* spp. was noted in our study. This finding supports our previous observation of the synergistic potential between *Bacteroides* spp. and anaerobic and facultative bacteria that was measured by an increase in mortality and abscess formation (6); however, the present study identifies more clearly and specifically the mutual effect of each of the bacterial agents of mixed infections by enumerating the total number of organisms at the infected site.

Although the relationships between various organisms were different, certain trends were observed. It is apparent that the growth of the aerobic and facultative partners in mixed infections with *Bacteroides* spp. was enhanced much more than the growth of their anaerobic partners. *Bacteroides* strains grew in mixed cultures at the same rate as they grew in single infections. However, it appears that aerobic or facultative bacteria grew at a faster rate in a mixed infection with a *Bacteroides* sp. than when injected alone. There were exceptions to this apparent symbiotic effect. One exception was noted when *H. influenzae* was enhanced by *B. melaninogenicus* but depressed by *B. vulgatus*. The other exception was noted when group D streptococcus was suppressed by organisms belonging to the *B. melaninogenicus* group. The mutual suppressive effect of these last two combinations of organisms is supported by the rarity in which these organisms are present in clinical mixed infections.

The enhancing potential between *Bacteroides* spp. and other bacteria that are commonly recovered with them from clinical infectious sites demonstrates the pathogenic poten-

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tials of organisms that belong to the *B. fragilis* and *B. melaninogenicus* groups. The enhancing effect noted between these different bacterial species may be due to protection from phagocytosis and intracellular killing (10), production of essential growth factors (14), or lowering of oxidation-reduction potentials in host tissues (16).

The data presented in this study suggest that aerobic and facultative bacteria benefit even more in mixed infections with anaerobic bacteria than do the anaerobes. The theory explaining synergy between aerobes and anaerobes by the ability of aerobic or facultative organisms to lower the oxidation-reduction potentials in host tissue does not fit our observation. If such a theory was correct, we would have observed a more pronounced increase in the rate of multiplication of anaerobes. However, although slight enhanced growth of the *Bacteroides* spp. was rarely observed, the promotion of the growth of aerobic or facultative pathogens was clearly demonstrated. This finding supports the pathogenic role of *Bacteroides* spp. in mixed infections.

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