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Significance of Encapsulated *Bacteroides melaninogenicus* and *Bacteroides fragilis* Groups in Mixed Infections

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Organisms of the *Bacteroides melaninogenicus* and *Bacteroides fragilis* groups are often found mixed with facultatively anaerobic organisms in infections. The relative importance of these *Bacteroides* groups and facultative anaerobic pathogens in mixed infections was investigated in a subcutaneous abscess model in mice. This was determined by observing the effect of antimicrobial therapy directed against one or both organisms present in the abscess. Clindamycin or metronidazole was used for treatment of infections caused by *Bacteroides* species, and either gentamicin, penicillin, ampicillin, or oxacillin was used for treatment of infections caused by facultative flora. In almost all instances the aerobic counterparts in the infection were more important than the unencapsulated *Bacteroides* species. On the other hand, encapsulated *B. melaninogenicus* group organisms were found to be more important in abscess formation than were group A streptococci, *Streptococcus pneumoniae*, *Klebsiella pneumoniae*, *Haemophilus influenzae*, and *Staphylococcus aureus*. Encapsulated *B. fragilis* group organisms were found to be more important than or as important as *Escherichia coli* and group D streptococci and less important than *S. aureus*, group A streptococci, and *K. pneumoniae* in induction of subcutaneous abscesses. This study demonstrates that encapsulated *Bacteroides* species are a factor that should be considered in the treatment of mixed infections with antibiotics.

Intraabdominal, pelvic, and cutaneous infections often contain *Bacteroides fragilis* mixed with aerobes such as *Escherichia coli*, *Streptococcus faecalis*, *Klebsiella pneumoniae*, *Staphylococcus aureus*, and group A streptococci (1, 2, 4, 5). *Bacteroides melaninogenicus* also occurs in mixed infections but is more often isolated at respiratory and maxillofacial sites than in pelvic or cutaneous infections.

Although anaerobes are components of mixed infections, their relative importance in the disease process is often not considered. It has been demonstrated, however, that optimal therapy of intraabdominal infections is best achieved with regimens which show good in vitro activity against both coliforms and *B. fragilis* (1, 5).

For reasons given above, we have conducted experiments to demonstrate that *Bacteroides* species may be significant pathogens in mixed infections and that clinical approaches of their control must take into account the presence of encapsulated anaerobes. A subcutaneous abscess model in mice was used to demonstrate that antibiotic treatments not directed against these organisms may be of limited success.

MATERIALS AND METHODS

Organisms. All aerobic and anaerobic bacterial strains were recent clinical isolates. These included six strains of the *B. melaninogenicus* group (two of *B. intermedius*, *B. asaccharolyticus*, and *B. melaninogenicus*) and six strains of the *B. fragilis* group (two strains each of *B. fragilis*, *B. ovatus*, and *B. vulgatus*). The aerobic strains included two strains of *Haemophilus influenzae* type B and 1 strain each of *Pseudomonas aeruginosa*, *K. pneumoniae*, *E. coli*, *S. aureus*, group A beta-hemolytic streptococci, group D streptococci, and *Streptococcus pneumoniae*. The bacteria were kept frozen in skim milk at −70°C. The bacterial strains were identified by standard criteria (7, 12) and processed as previously described (3). The presence of a capsule was established by Hiss staining (7) and confirmed by electron microscopy after staining with ruthenium red (6). Ruthenium red staining demonstrated that a homogeneous polysaccharide capsule was external to the cell wall. Capsular stains revealed the presence of a capsule in 6 of the 12 *Bacteroides* strains and all the aerobic strains except *E. coli*, *P. aeruginosa*, and 1 strain of *H. influenzae*.

Animals. Male swiss albino mice weighing 20 to 25 g were obtained from the Naval Medical Research Institute mouse colony. The mice were raised under conventional conditions.

Inoculum preparation. 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 (3) for the *Bacteroides* sp. or in 5% CO_2 for the aerobic bacteria. Twenty-four hours before injection, the bacterial strains were inoculated onto chocolate agar or Trypticase soy (BBL Microbiology Systems, Cockeysville, Md.)-5% sheep blood agar. From these media cotton swabs were used to pick colonies from the plates and transfer them to normal saline. The suspensions of organisms prepared were equivalent to a 0.5 MacFarland standard. CFUs were determined by plate count in brain heart infusion agar enriched with vitamin K_1 and hemin.

Abscess induction. Mice were inoculated subcutaneously in the right groin with 0.1 ml of each of the appropriate bacterial suspensions in saline containing ca. 10^8 of each organism. With the exception of *P. aeruginosa*, *E. coli*, and *S. pneumoniae*, most encapsulated organisms tested produced abscesses, and unencapsulated organisms did not. *S. pneumoniae* was encapsulated but produced no abscesses, whereas *E. coli* and *P. aeruginosa* produced abscesses even though they were unencapsulated. Abscesses were usually formed within 24 to 72 h of inoculation, 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 sizes as a consequence of mixed infections were larger (22 to 24 mm) than those caused by single organisms.

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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 (<10%) for all challenged mice. Only surviving mice were included in the statistical analyses.

Determination of abscess size. The size of the individual abscesses was determined during necropsy on day 7 after inoculation. Although the volumes of these abscesses could not be determined with accuracy, their sizes could be compared by measurement of two perpendicular diameters representing maximum length and width. The abscess was assumed to be an irregular prolate spheroid, so the product of the length and width was approximately proportional to the outer surface of the abscess. This product, expressed as square millimeters, was arbitrarily selected as the means of comparison. With this criterion, at day 7 without antibiotic therapy, the abscesses caused by a single organism achieved an outer surface size of ca. 250 mm^2 (range, 180 to 300 mm^2), and abscesses caused by two organisms achieved an outer surface size of ca. 375 mm^2 (range, 225 to 475 mm^2).

Cultivation of organisms isolated from abscesses. Animals were sacrificed by cervical dislocation, and the abscess material was removed aseptically. The site and the histology of the abscesses were confirmed by hematoxylin and eosin staining. The pus specimen was diluted in 1 ml of enriched brain heart infusion broth and swabbed onto an enriched brain heart infusion agar plate. Bacterial growth on the plates was estimated semiquantitatively after 48 h at 37°C in aerobic and anaerobic environments. Characteristic colonies of all organisms were identified by Gram stain and biochemical tests (7, 12).

Antimicrobial agents and therapy. Dosages of antimicrobial agents were selected based on pharmacokinetic studies on untreated animals (Table 1). Serum levels of antibiotics were measured by agar diffusion assay (13) at 30, 60, 120, and 180 min after administration of antibiotics. Abscess levels were measured 1 h after administration of the last dose of antimicrobial agent on day 7. In most instances, the dose of antimicrobial agent approximated in milligrams per kilogram the usual recommended dose for patients.

TABLE 1. Pharmacokinetic properties of six antimicrobial agents in serum and abscesses of mice infected subcutaneously with *B. fragilis*

Antimicrobial agent	Dose per day (mg/kg)	Levels of drug (mean ± SD; n = 10)	
		Mean peak serum level[a] (μg/ml)	Abscess fluid level[b] (μg/g)
Penicillin G	100.0	27.5 ± 8.2[c]	7.4 ± 2.1
Ampicillin	100.0	4.8 ± 1.6	2.1 ± 0.8
Oxacillin	100.0	8.2 ± 3.4	2.8 ± 0.8
Clindamycin	40.0	8.9 ± 3.2	8.4 ± 4.0
Metronidazole	50.0	28.0 ± 6.4	12.2 ± 3.4
Gentamicin	7.5	5.4 ± 2.2	3.8 ± 2.4

[a] Determined on the basis of pharmacokinetic studies on 10 animals with serum obtained at 0.5, 1.4, and 8 h after intramuscular administration of antimicrobial agents.

[b] The expected peak levels.

[c] Results are based on measurement of levels of antimicrobial agents after administration of agents on day 7 of therapy. The antibiotic levels were determined on day 7 of antibiotic administration in mice infected with *B. fragilis*. The serum and abscess fluid levels were measured at time of peak.

Levels of clindamycin, oxacillin, ampicillin, and penicillin were measured by agar diffusion assay (13) with *Micrococcus luteus* ATCC 9341 (American Type Culture Collection, Rockville, Md.), and the levels of gentamicin were measured with *Bacillus subtilis* ATCC 0633. Metronidazole was assayed with high-pressure chromatography (14).

The daily dosage of the antibiotics (administered in divided dosages every 8 h) was as follows: penicillin G, 100 mg/kg (E. R. Squibb and Sons, Princeton, N.J.); gentamicin, 7.5 mg/kg (Schering Laboratories, Kenilworth, N.J.); clindamycin, 40 mg/kg (The Upjohn Co., Kalamazoo, Mich.); metronidazole, 50 mg/kg (G. D. Searle & Co., Chicago, Ill.); and ampicillin or oxacillin, 100 mg/kg (Bristol Laboratories, Syracuse, N.Y.). Treatment was initiated 2 h after inoculation with bacteria and continued for 7 days. All agents were given intramuscularly.

Susceptibility tests. The MIC for all isolates was determined by the agar dilution technique (12). Beta-lactamase activity was determined for all organisms by the chromogenic cephalosporin analog 87/312 methodology (8). All *Bacteroides* sp. were resistant to penicillin (MIC, ≥64 μg/ml) and produced beta-lactamase. They were susceptible to metronidazole (MIC, <0.5 μg/ml) and clindamycin (MIC, <1 μg/ml) and resistant to gentamicin (MIC, ≥256 μg/ml). *S. aureus* was susceptible to gentamicin (MIC, 1 μg/ml), oxacillin (MIC, 0.5 μg/ml), and clindamycin (MIC, 2 μg/ml) and resistant to penicillin (MIC, >32 μg/ml). Group A streptococci and *S. pneumoniae* were susceptible to penicillin (MIC, <0.25 μg/ml). Group D streptococci were mildly susceptible to penicillin (MIC, 2 μg/ml). *H. influenzae* was susceptible to ampicillin (MIC, 1 μg/ml), and *K. pneumoniae*, *E. coli*, and *P. aeruginosa* were susceptible to gentamicin (MIC, <0.5 μg/ml). All aerobic bacteria were resistant to metronidazole (MIC, >128 μg/ml). There was no difference in antibiotic susceptibility between encapsulated and unencapsulated strains.

Experimental design. All challenge experiments were run in duplicate with groups of 10 mice each. Groups were challenged with single aerobic organisms, single encapsulated or unencapsulated *Bacteroides* sp. or a mixture of aerobe and anaerobe. Each infected group was given either no treatment or antibiotics directed against aerobe only, anaerobe only, both aerobe and anaerobe. Antimicrobial agents against anaerobes were clindamycin and metronidazole, whereas those effective against aerobes were gentamicin, penicillin, ampicillin, and oxacillin. Different combinations were used in each experiment, depending on the susceptibility of the organisms. Clindamycin and gentamicin were used for the treatment of *Bacteroides* sp. and *K. pneumoniae*, *P. aeruginosa*, or *E. coli*; metronidazole and ampicillin were used against the *B. melaninogenicus* group and *H. influenzae*; metronidazole and penicillin were used against *Bacteroides* sp. and streptococcal species; and metronidazole and oxacillin were used against *Bacteroides* sp. and *S. aureus*. The relative importance of the organisms present in the abscess was determined by comparing the abscess sizes in the animals treated with antibiotics directed against one or both organisms with those in untreated mice. For example, an abscess caused by a single organism (e.g., *Bacteroides* sp. or an aerobe) which is treated with an antimicrobial agent effective against that organism is predicted to respond to therapy and be smaller than that in an untreated animal. For an abscess caused by organisms when therapy is directed against only one organism at a time, the comparative reductions caused by treatment with separate antibiotics were utilized to decide the relative importance of the two organ-

isms in mixed infections. The abscess should be significantly smaller when treated appropriately, when the susceptible organism is the greater contributor to the abscess. The organisms of greater significance were those for which the abscess size was the smaller of the two ($P < 0.05$). When no statistical difference was noted, the two organisms were considered to be equally significant in abscess induction. Statistical analysis was done with the Student t test.

RESULTS

Significance of encapsulated or unencapsulated *Bacteroides* sp. in mixed infections. Antibiotic therapy was applied to the various combinations of *Bacteroides* sp. and aerobic organisms, and the relative significance of the anaerobe in the abscesses produced was determined (Tables 2 and 3). Abscesses caused by single organisms always responded to specific therapy, and therefore these data are not shown.

Of 15 combinations of mixed infections between the *B. melaninogenicus* group and their aerobic counterparts, the aerobes were more important than the unencapsulated *Bacteroides* species in 13 instances (Table 2). The anaerobe was of equal importance to the aerobe only in mixtures containing *S. aureus* and *B. melaninogenicus* or *H. influenzae* and *B. asaccharolyticus*.

In contrast to data outlined with unencapsulated *B. melaninogenicus*, encapsulated members of the group were more important than the aerobic counterparts in 15 of 18 combinations, equal in 1 combination, and less important in 2 combinations. *P. aeruginosa* was of greater importance in combination with *B. melaninogenicus* and of equal importance in combination with *B. intermedius*.

Not shown in Table 2 are mixtures of the three encapsulated *B. melaninogenicus* species used and an unencapsulated *H. influenzae*. The anaerobe was more significant than the aerobe in all three of these combinations.

In mixed infections of each of the three species belonging to the *B. fragilis* group (Table 3), the aerobic counterpart was more important than the unencapsulated anaerobe in 14 of the 15 combinations tested. An exception was the equal importance seen between *S. aureus* and unencapsulated *B. fragilis*.

Six of the 15 encapsulated *Bacteroides* sp. were more important in abscess induction than were the aerobes: *B. ovatus* was more important than *K. pneumoniae* and *E. coli*; *B. vulgatus* was more important than *S. faecalis*; and *B. fragilis* was more important than *E. coli*, *S. faecalis*, and *S. aureus*. In five combinations the aerobes were more important than the encapsulated *Bacteroides* sp. In the other four combinations the aerobes were of equal importance to the encapsulated *Bacteroides* sp.

TABLE 2. Significance of *B. melaninogenicus* group and other flora in mixed infections

Bacteroides sp. inoculated	Importance of aerobic flora inoculated[a]					
	K. pneumoniae	*P. aeruginosa*	*H. influenzae*	Group A streptococci	*S. pneumoniae*	*S. aureus*
Capsule positive						
B. asaccharolyticus	L	M	M	M	M	M
B. intermedius	M	E	M	M	M	M
B. melaninogenicus	M	L	M	M	M	M
Capsule negative						
B. asaccharolyticus	L	L	E	L	NA	L
B. intermedius	L	L	L	L	NA	L
B. melaninogenicus	L	L	L	L	NA	E

[a] M, *Bacteroides* sp. more important than counterpart; L, *Bacteroides* sp. less important than counterpart; E, both organisms of equal importance; NA, no abscess formed.

TABLE 3. Significance of *B. fragilis* group and other flora in mixed infections

Bacteroides sp. inoculated	Importance of aerobic flora inoculated[a]				
	E. coli	*K. pneumoniae*	Group A streptococci	Group D streptococci	*S. aureus*
Capsule positive					
B. fragilis	M	E	L	M	M
B. vulgatus	E	L	E	M	L
B. ovatus	M	M	L	E	L
Capsule negative					
B. fragilis	L	L	L	L	E
B. vulgatus	L	L	L	L	L
B. ovatus	L	L	L	L	L

[a] M, *Bacteroides* sp. more important than counterpart; L, *Bacteroides* sp. less important than counterpart; E, both organisms of equal importance.

Culture of abscess contents. Bacterial strains inoculated into mice were recovered from the abscesses by necropsy of untreated animals. Single antimicrobial therapy of mixed infections did not completely eliminate the organisms against which it was directed, although a marked reduction in recovery of these organisms was seen. Combined antimicrobial therapy was more effective in reducing the number of bacteria recovered from single or mixed infections, and in several instances this therapy eradicated one or both of the infecting organisms.

Complete sterilization of abscesses caused by mixed flora was achieved in 13 instances. In four instances one of the organisms in the abscesses belonged to the *B. fragilis* group, and in nine instances one of the organisms belonged to the *B. melaninogenicus* group. In 11 of the combinations, the *Bacteroides* sp. were unencapsulated. These combinations were between *B. vulgatus* or *B. fragilis* and *K. pneumoniae*, between *B. ovatus* or *B. fragilis* and group D streptococci, between *B. intermedius* and group A streptococci or *S. aureus*, between *B. intermedius* and *P. aeruginosa*, between all three members of the *B. melaninogenicus* group and *K. pneumoniae*, and between *B. asaccharolyticus* and *H. influenzae*. In only three instances were the *Bacteroides* sp. encapsulated, and that was in the combination of all three members of the *B. melaninogenicus* group and *H. influenzae*.

DISCUSSION

These data demonstrate that in our model encapsulated *Bacteroides* sp. can be significant pathogens in mixed infections and that optimal antimicrobial therapy must be directed against both anaerobic and aerobic constituents of these infections. With few exceptions, encapsulated *Bacteroides* sp. were more than or at least equally as important as their aerobic counterparts in induction of subcutaneous abscesses. However, one must consider the limitation of our data, since the only parameters observed in our study were the relative sizes of the abscesses and semiquantitative microbiology. Another limitation of our model was the use of only two organisms in inducing an abscess. Generally, more than

that number are recovered from intraabdominal or pelvic abscesses (5).

In the mixed infection between aerobes and the encapsulated *B. fragilis* or *B. melaninogenicus* group, when antimicrobial therapy was directed only at the aerobic component, it failed to reduce the abscess size in 6 of 15 instances and in 15 of 18 instances, respectively. Of interest was the observed superiority of the encapsulated *B. fragilis* group when mixed with *E. coli* or group D streptococci, with which they are frequently isolated in intraabdominal infection (5). This is in contrast to findings with mixtures containing *S. aureus*, group A streptococci, and *K. pneumoniae* in which the *B. fragilis* group was of secondary importance. Encapsulated members of the *B. melaninogenicus* group, however, were almost always more important in mixed infections than their aerobic counterparts.

Although other virulence factors may also be of importance, detection of the presence of a capsule in a clinical isolate may add significance to the possible role of the organism as a pathogen in the infection. The observed inability of antimicrobial therapy to completely sterilize abscesses in which the *Bacteroides* sp. were encapsulated further supports the role of encapsulated *Bacteroides* sp. in mixed infections. Our observation that the presence of capsular material is an important factor influencing the pathogenicity of the *B. fragilis* and *B. melaninogenicus* groups agrees with studies by Onderdonk et al. (10) that correlated virulence of *Bacteroides* strains with the presence of capsules. Thlis may be due in part to decreased clearance of encapsulated *Bacteroides* sp. (9, 11). Implication of capsular polysaccharide as a factor associated with virulence is not unique to *Bacteroides* sp. and has been observed in other bacterial strains (15).

The observed importance of encapsulated *Bacteroides* sp. in our mixed infection model may be of clinical relevance. Detection of the presence of a capsule in a clinical isolate may add significance to the possible role of the organism as a pathogen in the infection and would warrant the administration of antimicrobial therapy effective against the organism.

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