Encapsulated Anaerobic Bacteria in Synergistic Infections

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INTRODUCTION

Polymicrobial infections can be more virulent than those involving single organisms (4, 29). Studies of the successful transmission of infections in animals that were given defined mixtures of aerobic and anaerobic organisms have been reported by many investigators (28, 38, 41). The key role of Bacteroides melaninogenicus was established by Rosebury et al. (38). However, none of the earlier studies established the role of encapsulated organisms in the infectious process.

Encapsulated anaerobic bacteria has been recognized as an important virulence factor. Several studies demonstrated the pathogenicity of encapsulated anaerobes and their ability to induce abscesses in experimental animals even when inoculated alone. Onderdonk et al. (34) correlated the virulence of B. fragilis strains with the presence of capsule. Encapsulated B. fragilis strains or purified capsular polysaccharide alone induced abscesses, whereas non-encapsulated strains seldom caused abscesses unless they were combined with an aerobic organism. Simon et al. (40) showed that encapsulated Bacteroides strains resisted netrophil-mediated killing, compared with nonencapsulated strains.

The susceptibility of pathogenic bacteria to phagocytosis and killing by polymorphonuclear leukocytes and macrophages is important in determining the outcome of the host-pathogen interaction. Ingham et al. (19), Toft et al. (42), and Jones and Gemmell (21) reported that both phagocytic uptake and killing of facultative bacterial species were impaired at high concentrations of B. fragilis and B. melaninogenicus.

The presence of a capsule on B. fragilis was shown to provide the organism with a growth advantage in vivo over nonencapsulated isolates (37). Furthermore, encapsulated strains survived better in vitro than nonencapsulated variants when they were grown in an aerobic environment. Another recently described mechanism of protection independent of encapsulation is the inhibition of polymorphonuclear migration due to the production of succinic acid by Bacteroides sp. (39).

The importance of the capsular polysaccharide of B. fragilis as an immunogen was demonstrated when antibodies against it protected animals from early bacteremia (23). However, prevention of the formation of intra-abdominal abscess by this organism was found to be T-cell mediated (35).

This paper summarizes our recent research on the role of encapsulation on anaerobic bacteria in mixed infections. The review includes (i) clinical evidence that supports the role of encapsulated anaerobic bacteria in mixed infections, (ii) experiments that investigated the process of encapsulation of anaerobes, and (iii) studies of the influence of encapsulation on the relationship of anaerobes with their aerobic counterparts in infectious sites.

In addition to capsule, anaerobic bacteria possess other important virulence factors. These include the production of superoxide dismutase and catalase, immunoglobulin proteases, coagulation-promoting and -spreading factors (such as hyaluronidase, collagenase, and fibrinolysin), and adherence factors (4). Other factors that enhance the virulence of anaerobes include mucosal damage, oxidation-reduction potential drop, and the presence of hemoglobin or blood in an infected site. However, this review is devoted only to the role of capsule as a virulence factor.

ENCAPSULATED ANAEROBIC BACTERIA IN CLINICAL INFECTIONS

Aspirates obtained from infections next to mucous membrane surfaces generally contain a complex bacterial population consisting of several species (4, 17). Although anaerobes are components of mixed infections, their role and relative importance in the disease process often are not considered.

Capsule and Infectivity of Organisms from Clinical Specimens

In an attempt to define the important pathogens among isolates recovered from clinical specimens, we studied the virulence and importance of encapsulated bacterial isolates recovered from 13 clinical abscesses (11). This was done by injecting each of the 35 isolates (30 anaerobes and 5 aerobes) subcutaneously (s.c.) into mice alone or in all possible combinations with the other isolates recovered from the same abscess. We then observed their ability to induce or survive in an s.c. abscess. Sixteen of the isolates were encapsulated; 15 of them were able to cause abscesses by themselves and were recovered from the abscesses even

452
when inoculated alone. The other organisms, which were not encapsulated, were not able to induce abscesses when inoculated alone. However, some were able to survive when injected with encapsulated strains. Therefore, the possession of a capsule by an organism was associated with increased virulence, compared with the same organism’s nonencapsulated counterparts, and might have allowed some of the other accompanying organisms to survive. We found this phenomenon to occur in Bacteroides sp., anaerobic gram-positive cocci, Clostridium sp., and Escherichia coli. Detection of a capsule in a clinical isolate may therefore suggest a pathogenic role of the organism in the infection.

Recovery of B. melaninogenicus from Tonsils

Two recent studies support the importance of encapsulated anaerobic organisms in respiratory infections (5b, 9). The presence of encapsulated and abscess-forming organisms that belong to the B. melaninogenicus group was investigated in 25 children with acute tonsillitis and in 23 children without tonsillar inflammation (control) (9). Encapsulated organisms of the B. melaninogenicus group were found in 23 of 25 children with acute tonsillitis versus 5 of 23 controls \( (P < 0.001) \). Inoculation s.c. into mice of the Bacteroides strains that had been isolated from patients with tonsillitis produced abscesses in 17 of 25 instances compared with 9 of 23 controls \( (P < 0.05) \). These findings suggest a possible pathogenic role for the B. melaninogenicus group in acute tonsillar infection and also the importance of encapsulation in the pathogenesis of the infection.

Recovery of Encapsulated Anaerobic Bacteria from Orofacial Abscesses

Orofacial abscesses contain many species of aerobic and anaerobic bacteria, which are also found in high numbers in the normal oral flora (4, 17). It is believed, therefore, that most of these organisms originate from that source and become pathogenic during the inflammatory process, perhaps by selection. An evaluation was recently done of the role of encapsulation among the most frequently recovered anaerobes, the anaerobic cocci and Bacteroides sp. A comparison was made between the recovery rates of encapsulated organisms in chronic inflammatory conditions in and around the oral cavity and in the pharynx of normal individuals (5b).

The presence of encapsulated Bacteroides sp. and anaerobic gram-positive cocci was investigated in 182 patients who had head and neck or chronic orofacial infections and in the pharynx of 26 individuals without inflammation. Forty-nine of the patients had chronic otitis media, 45 had cervical lymphadenitis, 37 had chronic sinusitis, 24 had chronic mastoiditis, 10 had peritonsillar abscesses, and 12 had periodontal abscesses. Of the 216 isolates of B. melaninogenicus and B. fragilis groups, B. oralis, and anaerobic cocci, 170 (79%) were found to be encapsulated in patients with chronic infections compared with only 34 (35%) of 96 controls \( (P < 0.001) \).

The recovery of a greater number of encapsulated anaerobic organisms in patients with acute and chronic orofacial infections provides further support for the potential pathogenic role of encapsulated organisms and the possible conversion of nonencapsulated strains into encapsulated ones during the inflammatory process. Early and vigorous antimicrobial therapy, directed at both aerobic and anaerobic bacteria present in these mixed infections, may abort the infection before the emergence of encapsulated strains that contribute to the chronicity of the infection.

Capsule Formation in Experimental Mixed Infections

Studies were performed to (i) ascertain the effect of the aerobic component in mixed infection on the external cell membrane of the anaerobic bacteria, mostly through appearance of many encapsulated cells in mixed infections (this section); (ii) explore the role of encapsulated Bacteroides spp. and anaerobic cocci in bacteremia and translocation (next section); (iii) evaluate the relative importance of each of the aerobic and anaerobic components of mixed infection through use of selective antimicrobial therapy and the effect of the presence of an encapsulated anaerobe on that relationship (see “Significance of Anaerobic Bacteria in Mixed Infection with Other Flora”); and (iv) investigate the synergistic or antagonistic capabilities of each of the components of mixed infection (last section). The most frequently recovered bacteria were used: Bacteroides, Clostridium, and Fusobacterium spp. and anaerobic and facultative gram-positive cocci (AFGPC).

These studies were conducted in white male albino mice, weighing 20 to 25 g, obtained from the Naval Medical Research Institute mouse colony. The mice were raised under conventional conditions. The animal model used in all experiments was an s.c. abscess model (20) that was suitable for this evaluation because of its simplicity, the ability to monitor the infection while the experiment was in progress, and the relatively easy access for obtaining cultures and measuring abscess size. Unless differently specified, 0.1 ml of each of the appropriate bacterial suspensions \( (10^8 \text{ cells per dose}) \) in saline was inoculated s.c. into the right groin in all animals.

Many of the previous studies of mixed aerobic-anaerobic infections used an intraperitoneal abscess model. Differences exist between the s.c. and intraperitoneal abscess models. While s.c. abscesses appear within 2 to 3 days, intraperitoneal abscesses take at least 7 to 10 days to develop.

The presence of capsules in all organisms was determined by the Hiss stain (26) and confirmed by electron microscopy after staining with ruthenium red (22). Ruthenium red staining demonstrated a homogeneous polysaccharide capsule that was external to the cell wall.

The ability of the aerobic component in mixed infections to enhance the appearance of encapsulated anaerobic bacteria in these infections was studied in an s.c. abscess model in mice. The anaerobic bacteria with which they were inoculated were those commonly recovered in mixed infections (4, 17). B. melaninogenicus group, B. buccalis, B. oris-buccae (8), B. bivius (5a), B. fragilis group (6), and AFGPC (14) did not induce abscess when isolates that contained only a small number of encapsulated organisms (<1%) were inoculated. However, when these relatively nonencapsulated isolates were inoculated, mixed with abscess-forming viable or nonviable bacteria (“helpless”), Bacteroides spp. and AFGPC survived in the abscess and became heavily encapsulated (>50% of organisms had a capsule). Therefore, these heavily encapsulated Bacteroides isolates were able to induce abscesses when injected alone (Fig. 1). Of interest is the observed appearance of pili along with encapsulation in the B. fragilis group after coinoculation with Klebsiella pneumoniae (6).
Most of the "helper" strains were encapsulated; however, several of the strains were not encapsulated, but were able to induce abscesses when inoculated alone. The helper organisms used in conjunction with "oral" Bacteroides spp. (B. melaninogenicus group, B. buccalis, and B. oris-buccae) and AFGPC were Staphylococcus aureus, Streptococcus pyogenes, Haemophilus influenzae, Pseudomonas aerugi-

cosa, E. coli, K. pneumoniae, and Bacteroides sp. (8, 14). For the B. fragilis group, these organisms were E. coli, K. pneumoniae, Staphylococcus aureus, Streptococcus pyo-
gen, and enteroccci (6). Neisseria gonorrhoeae was chosen as a helper for B. fragilis and B. melaninogenicus groups, and also B. bivius (5a). Of interest is the observed inability of N. gonorrhoeae strains to survive in intra-
abdominal abscesses and also their disappearance from s.c. abscesses within 5 days of inoculation with Bacteroides sp. (5a).

The virulence of Fusobacterium sp. was also associated with the presence of a capsule. Only encapsulated strains of Fusobacterium nucleatum, F. necrophorum, and F. vani
were able to induce abscesses when inoculated alone (15). However, following passage in animals of nonencapsulated strains, none of these organisms acquired a capsule.

The presence of a thick granular cell wall (30 to 36 nm) before animal passage was associated with virulence of Clostridium sp. (5c). Such structure was observed before inoculation into animals only in Clostridium perfringens and C. butyricum, the only organisms capable of inducing an s.c. abscess when inoculated alone. This structure was observed in other clostri
dial species only after their coinoculation with encapsulated Bacteroides sp. or K. pneumoniae.

However, other undetermined factors may also contribute to the induction of an abscess, since most isolates of C. difficile were not able to produce an abscess even though they possessed a thick wall.

The selection of encapsulated Bacteroides sp. and AFGPC with the assistance of other encapsulated or nonencapsulated but abscess-forming aerobic or anaerobic organisms may explain the conversion into pathogens of nonpathogenic organisms that are part of the normal host flora or are concomitant pathogens. Although such a phenomenon was not observed in Fusobacterium sp., the presence of a capsule in these organisms was a prerequisite for induction of s.c. abscesses. Some Clostridium spp. also manifested cell wall changes after animal passage that could be associ-
ated with increased virulence. Although the exact nature and chemical composition of the capsule or external cell wall may be different in each of the anaerobic species studied, the changes observed tended to follow similar patterns.

The mechanism responsible for the observed phenomenon is as yet unknown and may be due to either genetic trans-
formation or a process of selection. It is possible that capsular material of aerobes such as K. pneumoniae or an anaerobe such as a Bacteroides sp. enables the selection of a few encapsulated organisms from a predominantly unencapsulated population of the tested anaerobic organism. The presence of capsular material in the inoculum was probably sufficient to prevent phagocytosis of the organisms (33, 40) and allowed the selection of encapsulated forms. An interference can occur in the interaction between polymor-
phonuclear cells and the anaerobic bacteria (21). This process, which required opsonization by both complement and immunoglobulins, can be inhibited by either Bacteroides sp. or aerobic bacteria by either competing for limited opsonins or decreasing intracellular killing (25). We postulated that a selection process was the mechanism responsible for our finding because we were able to recover heavily encapsu-
lated organisms after passage in vivo with a helper organism in all of the slightly encapsulated organisms.

The possibility of some genetic transfer of virulence factors through a bacteriophage from the helper organisms to the Bacteroides sp. can further facilitate the pathogenesis of these organisms. The phage could also have a role in selecting out the encapsulated cells among the population of B. fragilis which are more resistant to it than the unencapsulated cells (3). This phenomenon as well as other virulence factors could account for the ability of B. fragilis (which constitutes only about 0.5% of the normal fecal flora) to become a pathogen present in 70 to 80% of intra-abdominal infections (4, 17). A similar mechanism could account for the virulence of Bacteroides sp. in pelvic inflammation following interaction with encapsulated N. gonorrhoeae. Acquisition of such virulence-associated factors as pili and capsules strengthens the need to apply direct therapy against potential pathogens such as B. fragilis.

The observed change in the cell walls of clostridia may be a result of exposure to another bacterium in an animal environment with possible genetic transfer that occurs only in these circumstances. It may also be due to the selection from the introduced population of a small number of cells that have such a wall structure and possibly have, as a result, some advantage because of an antiphagocytic effect (33), or associated effects such as shortened division time, which allow its progeny to survive and outgrow the others. Another possibility is that the ability to produce such a structure is inherent in these strains and is only expressed in vivo.

FIG. 1. Summary of the cycle of events leading to capsule formation by B. fragilis. "Helper" = viable bacteria or formalinized bacteria or capsular material.

ROLE OF A CAPSULE OF BACTEROIDES SP. AND ANAEROBIC COCCI IN BACTEREMIA

Anaerobic bacteremia account for 5 to 15% of cases of bacteremia (17) and are especially prevalent in polymicrobial bacteremia, associated with abscesses (7).

The role of possession of capsular material in the systemic spread of Bacteroides sp. and AFGPC was investigated in mice following s.c. inoculation of encapsulated strains alone or in combination with aerobic or anaerobic facultative bacteria (I. Brook, J. Med. Microbiol., in press). Encapsu-
lated anaerobes were isolated more frequently from infected animal blood, spleen, liver, and kidney than were non-
encapsulated organisms.

After inoculation with a single encapsulated anaerobic strain, encapsulated organisms were recovered in 163 of 420
(39%) animals, whereas nonencapsulated anaerobes were recovered in only 14 of 420 (39%) animals. Following inoculation of B. fragilis mixed with aerobic or facultative flora, encapsulated B. fragilis was isolated more often and for longer periods of time than was the nonencapsulated strain. Furthermore, encapsulated B. fragilis was recovered more often after inoculation with other flora than it was when inoculated alone.

Therefore, encapsulated strains were more virulent than nonencapsulated strains. These data highlight the importance of encapsulated Bacteroides sp. and AFGPC in increasing the mortality associated with bacteremia and the spread to different organs. A similar pathogenic quality was observed in other bacterial species, such as Streptococcus pneumoniae (43) and H. influenzae (16), in which the encapsulated strains showed greater ability for systemic spread.

Onderdonk et al. (36), who studied the model of intra-abdominal sepsis and abscesses in rats, were able to detect bacteremia due to B. fragilis only during the first few hours after inoculation of B. fragilis and E. coli. However, their model represented an intra-abdominal infection, whereas ours was an s.c. infection. Bennion et al. (2), who induced colonic ischemia in dogs, were able to induce prolonged and persistent bacteremia due to B. fragilis and other anaerobic bacteria.

These data also demonstrate the synergy between Bacteroides sp. and AFGPC and the other flora present with them in mixed infections. Further studies are warranted to explain the pathophysiology of increased virulence of encapsulated Bacteroides sp. and AFGPC expressed by persistent bacteremia and spread to different organs.

**SIGNIFICANCE OF ANAEROBIC BACTERIA IN MIXED INFECTION WITH OTHER FLORA**

Although anaerobic bacteria often are recovered mixed with other aerobic and facultative flora, their exact role in these infections and their relative contribution to the pathogenic process are unknown. The relative importance of the organisms present in an abscess caused by two bacteria (an aerobic and an anaerobe) and the effect of encapsulation on that relationship were determined by comparing the abscess sizes in (i) mice treated with antibiotics directed against one or both organisms and (ii) nontreated animals (12, 13, 15; Brook and Walker, J. Infect., in press).

As judged by selective antimicrobial therapy, the possession of a capsule in most mixed infections involving Bacteroides sp. generally made these organisms more important than their aerobic counterparts. In almost all instances, the aerobic counterparts in the infection were more important than nonencapsulated Bacteroides species (13). Encapsulated members of the B. melaninogenicus group were almost always more important in mixed infections than their aerobic counterparts (Streptococcus pyogenes, Streptococcus pneumoniae, K. pneumoniae, H. influenzae, and Staphylococcus aureus). Encapsulated B. fragilis group organisms were found to be more important than or as important as E. coli and enterococci and less important than Staphylococcus aureus, Streptococcus pyogenes, and K. pneumoniae.

In contrast to Bacteroides sp., encapsulated AFGPC were found more often to be less important than their aerobic counterparts (12). Clostridium sp. and Fusobacterium sp. were found to be less or equal important to enteric gram-negative rods (15; Brook and Walker, in press). Although Fusobacterium sp., AFGPC, and Clostridium sp. were generally equal to or less important than their aerobic counterparts, variations in the relationships existed. However, as determined by the abscess size, most of the anaerobic organisms enhanced mixed infection.

**SYNERGY BETWEEN ANAEROBIC AND AEROBIC OR FACULTATIVE ANAEROBIC BACTERIA**

Several studies documented the synergistic effect of mixtures of aerobic and anaerobic bacteria in experimental infections. Altemeier (1) demonstrated the pathogenicity of bacterial isolates recovered from peritoneal culture after appendiceal rupture. Pure cultures of individual isolates were relatively innocuous when implanted s.c. in animals, but combinations of facultative and anaerobic strains showed increased virulence. Similar observations were reported by Melaney et al. (29) and Hite et al. (18).

We have evaluated (10) the synergistic potentials between aerobic and anaerobic bacteria commonly recovered in mixed infections. Each bacterium was inoculated s.c. into mice alone or mixed with another organism, and synergistic effects were determined by observing abscess formation and animal mortality. The tested bacteria included encapsulated Bacteroides sp., Fusobacterium sp., Clostridium sp., and anaerobic cocci. Facultative and anaerobic bacteria included Staphylococcus aureus, P. aeruginosa, E. coli, K. pneumoniae, and Proteus mirabilis. In many combinations, the anaerobes significantly enhanced the virulence of each of the five aerobes. The most virulent combinations were between P. aeruginosa or Staphylococcus aureus and anaerobic cocci or Bacteroides sp.

The bacterial combinations used in this study often occur clinically in infectious sites (4). The anaerobic species used in this study, B. fragilis, B. asaccharolyticus, AFGPC, C. butyricum, C. perfringens, F. varium, and F. nucleatum, are the most frequently recovered anaerobes from clinical specimens (4, 17). Our data support the finding of other investigators who observed synergy between Bacteroides sp. and enteric bacteria (24). We have been able to demonstrate synergy between the Bacteroides sp. and all of the facultative or aerobic organisms that we tested and between most AFGPC and P. aeruginosa or Staphylococcus aureus. Although there was variability of synergy between combinations of AFGPC strains, we have also demonstrated synergy in the ability to induce s.c. abscesses between AFGPC and Bacteroides sp. or Fusobacterium sp. Such synergy was also demonstrated between Fusobacterium sp. and Bacteroides sp. or Clostridium sp. Little to no synergy was observed between AFGPC and Clostridium sp.

Enhancement of the growth of each bacterial component in mixed infection was evaluated by studying the relative growth of each bacterial component. This was done by comparing the (i) growth of each organism in an abscess when present with other organism to (ii) growth of that bacteria when inoculated alone (5, 15; Brook and Walker, in press).

Streptococcus pyogenes, E. coli, Staphylococcus aureus, K. pneumoniae, and P. aeruginosa were enhanced by B. fragilis, B. melaninogenicus (5), peptostreptococci (14), Fusobacterium sp. (15), and Clostridium sp. (Brook and Walker, in press) except C. difficile. Although mutual enhancement of growth of both aerobic and anaerobic bacteria was noticed, the number of aerobic and facultative bacteria was increased many times more than their anaerobic counterparts. Encapsulated Bacteroides sp. was able to enhance the growth of aerobic and facultative anaerobic bacteria more than nonencapsulated organisms. Exceptions to the
mutual enhancement were noticed in combinations of organisms that generally are not recovered together in mixed infections, such as enterococci and B. melaninogenicus. The above observations suggest that the aerobic and facultative bacteria benefit even more than do the anaerobes from their symbiosis.

Several hypotheses have been proposed to explain microbial synergy. When this phenomenon occurs in mixtures of aerobic and anaerobic flora, it may be due to protection from phagocytosis and intracellular killing (19), production of essential growth factors (27), and lowering of oxidation-reduction potentials in host tissues (30). Obligate anaerobes can interfere with the phagocytosis and killing of aerobic bacteria (19). The ability of human polymorphonuclear leukocytes to phagocytose and kill Proteus mirabilis was impaired in vitro when the human serum used to opsonize the target bacteria was pretreated with live or dead organisms of various Bacteroides sp. (21). B. gingivalis cells or supernatant culture fluid was shown to possess the greatest inhibitory effect among the Bacteroides sp. (32). Supernatants of cultures of B. fragilis group, B. melaninogenicus group, and B. gingivalis were capable of inhibiting the chemotaxis of leukocytes to the chemotactic factors of Proteus mirabilis (31). Another possible mechanism that explains the synergetic effect of aerobic-anaerobic combinations is the lowering of local oxygen concentrations and the oxidation-reduction potential of the aerobic bacteria. 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 cocci was significantly lowered when the inoculum was supplemented with chemical reducing agents (30). A similar effect may be produced by facultative bacteria, which may provide the proper conditions for establishing an anaerobic infection at a previously well-oxygenated site.

The demonstration of the synergetic potentials of anaerobic bacteria such as B. fragilis, B. assacharolyticus, Fusobacterium sp., Clostridium sp., and anaerobic cocci, when mixed with various aerobic and anaerobic bacteria, further mitigate their pathogenic role. Further studies are needed to investigate the exact mechanisms by which such synergy occurs and the mode by which capsular material enhances it.

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LITERATURE CITED

ENCAPSULATED ANAEROBIC BACTERIA