

EXPERIMENTAL ANAEROBIC BACTERIAL INFECTIONS IN MICE

by

Clay B. Walker

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APPROVED:

~~_____~~
T. D. Wilkins, Chairman

W. E. C. Moore

C. S. Cummins

E. M. Gregory

N. R. Krieg

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Blacksburg, Virginia

FOREWARD

This dissertation was written in the form of five papers for publication with the concurrence of my committee.

Two of these papers have been published as follows:

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Archives of Oral Biology

Walker, C. B., S. E. H. West, D. Nitzan, and T. D. Wilkins. Pathogenic components of the microbiota of the gingival crevice area of man.

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ABSTRACT

INTRODUCTION

Although the existence of anaerobic bacteria has been known to microbiologists for over a century, the importance of these organisms in human disease, with the exception of certain clostridia, has only been recognized in the past few years. Even today, many clinicians still feel that there is little evidence that anaerobes per se are pathogenic, except in rare circumstances. Several factors have contributed to this misconception. In many instances, anaerobic bacteria occur in mixed infections involving aerobic or facultative organisms and, without the use of strict anaerobic culture procedures, the anaerobes involved are often not recovered. Another contributing factor has been the insistence by many clinicians that Koch's postulates must be fulfilled. The concept of one organism-one disease is difficult to demonstrate with most anaerobes. Many of these organisms, particularly gram-negative rods, are not readily pathogenic for experimental animals. The inability to establish individual pure culture infections has made it difficult to determine if one particular organism is responsible for the disease or if several organisms are involved in a synergistic relationship.

Since a microorganism can not be implicated in an infection unless the ability to grow the organism exists, anaerobic bacteria are often overlooked by clinical bacteriology laboratories that do not use strict anaerobic techniques. Reports still find their way into the literature describing sterile fluid and sterile abscesses. A more careful examination of these specimens would have revealed all too often that the "sterile" abscesses were colonized by fastidious anaerobes. Unfor-

tunately, antimicrobial therapy is difficult to prescribe for an infection when the causative organism is not known. In cases where only facultative or aerobic microorganisms are recovered, without taking into consideration whether or not anaerobes may be implicated, antimicrobial therapy may do more harm than good. The elimination of the facultatives may result in a resurgence in the growth of the anaerobic bacteria and lead to further complications for the patient.

Clinical Findings

Fortunately many clinicians have been made aware of the importance of anaerobic bacteria due to the efforts of investigators such as the groups headed by Finegold and by Gorbach as well as by technical advances made by the group at Virginia Polytechnic Institute and the Wadsworth V.A. Hospital. These investigators, as well as others, have recovered bacteria from infections involving various sites of the human body. Finegold (9) has reported that anaerobes are found in 70-95% of infections involving the thoracic, intraabdominal, and pelvic regions. Moore et al. (27) and Gorbach (11) have reported anaerobes in 90 to 93% of a diverse group of intraabdominal septic processes.

There are many reports in the literature concerning "sterile" liver and pelvic abscesses. Sabbaj (32) noted that of 41 liver abscess specimens examined in a routine microbiology laboratory, only 15 were found to harbor anaerobes; however, when 6 of those that were presumed negative for anaerobes were cultured in the anaerobic research laboratory, 5 were positive for anaerobes. Similar findings have been reported by Altemeier (2). In the early phase of his study, only 50% or so of the specimens yielded

anaerobes. In the last part of the study, special attention was directed toward anaerobic culture and all 12 specimens examined were found to contain anaerobic bacteria. Similar results have also been obtained in the cases of pelvic abscesses. Sophisticated anaerobic techniques have increased the isolation of anaerobes from approximately 40% to 90% or higher.

Anaerobes are found from other sites of infection at an equally high recovery rate. Four separate groups of investigators (22, 30, 37, 39) have recovered obligate anaerobes from adnexal infections in 63 to 100% of the cases. Swenson et al. (37) and Parker and Jones (29) found that the majority of non-venereal abscesses of Bartholin's glands were caused by anaerobes. Both Gorbach (11) and Finegold (9) have found that post-partum and post-abortion infections are frequently associated with anaerobic bacteria.

Anaerobes have also been found to be involved in a number of infections involving the thoracic region including pulmonary infections, lung abscesses, necrotizing pneumonia, aspiration pneumonia, and empyema. Extensive bacteriological studies have revealed that anaerobes are associated with these infections in 75 to 95% of the cases (3, 4, 12, 35, 36).

Anaerobic bacteria may also be frequently encountered in various other infections of the human body. These may include infections of the central nervous system, skin and soft tissue, bone and joints, bacteremia, endocarditis, and various dental and oral infections including periodontal disease (11, 12, 13).

Clinically Important Non-sporeforming Anaerobic Bacteria

Bacteroides fragilis, Bacteroides melaninogenicus, Fusobacterium nucleatum, and the anaerobic gram positive cocci are four of the most frequently encountered anaerobic bacteria isolated from human clinical specimens. These organisms are recovered in both pure and mixed culture from infections involving various anatomical sites.

Bacteroides fragilis is the most common single anaerobic organism recovered from clinical specimens and is the most resistant to antimicrobial agents. In addition, this organism is more often isolated in pure culture than any other anaerobe. The relative ease with which this organism can be isolated and successfully cultivated is, no doubt, a contributing factor to the frequency with which it is reported from clinical specimens. Nonetheless, B. fragilis is an important pathogen of man and is associated with infections involving various sites of the human body.

Bacteroides melaninogenicus is currently recovered from clinical specimens at almost the same frequency as B. fragilis providing strict anaerobic culture techniques are used. Although this organism is rarely found in pure culture, this may not mean a lack of virulence. Instead it probably represents the dependence of this organism on other organisms for required growth factors. Most strains studied have been found to require vitamin K or some analogue of this vitamin (10). Certain bacteria synthesize naphthoquinones in sufficient quantity to support the growth of B. melaninogenicus when grown together with these organisms. In addition, some strains of B. melaninogenicus produce a collagenase that appears to be inhibited by amino acids and peptides (20). Since these substances

are the end-products of collagen digestion, their removal by other bacteria presumably would encourage the continued production of collagenase by removing such feed-back repressors. This organism, like B. fragilis does not appear to be limited to any particular organ or infection site. The organism has been recovered from various sites including chanroid bubos (7), pleuropulmonary infections (3), liver abscesses (2), various orofacial infections (8), and various dental infections including periodontal disease (6, 33).

Fusobacterium nucleatum is the most frequently encountered of the fusobacteria from human clinical specimens. This organism, like B. melaninogenicus, is a common inhabitant of the human oral cavity and is frequently isolated from gingival infections along with B. melaninogenicus. The organism is also encountered in pleuropulmonary infections (3), liver abscesses (2), and in blood cultures (43). This organism has been implicated as part of the infective microbiota of periodontal disease and is commonly recovered from cases of advanced periodontitis (33). Although F. nucleatum may be found in pure culture, most often the organism is found in association with B. fragilis, B. melaninogenicus or anaerobic streptococci.

The gram positive anaerobic cocci are commonly isolated from human infections, often in association with F. nucleatum, B. fragilis, and B. melaninogenicus, as well as in pure culture. Clinically important organisms of this group are found in the genera Peptostreptococcus, Peptococcus, and Streptococcus. Most reports of the occurrence of gram-positive anaerobic cocci in clinical specimens do not identify the organisms to species and often not even to genus. However, in a report by Bartlett

and Finegold (3) on anaerobic pleuropneumony infections, peptostreptococci were found in 16% of the cases, peptococci in 17%, and microaerophilic streptococci in 34%. Pien (31) found these organisms to account for approximately 25% of the anaerobes recovered from over 10,000 clinical specimens. It should be noted that although the microaerophilic streptococci are by definition not obligate anaerobes, these organisms are generally considered along with the gram-positive anaerobic cocci and are not recovered except with anaerobic techniques. This group includes S. intermedius (formerly Peptostreptococcus intermedius) which may, on initial isolation, only grow anaerobically. This organism is part of the normal mouth flora and is often found in dental abscesses, periodontitis, and pleuropneumony infections.

The gram-negative anaerobic cocci are generally considered to be of little importance as human pathogens. The one exception to this is the genus Veillonella. These organisms are common inhabitants of the human oral cavity, intestine, and vagina; and are found clinically in soft tissue infections, upper respiratory infections, from wound drainage and from blood cultures. However, since these organisms are rarely found in pure culture in infection, many clinicians consider them to be of minor importance in human infections.

Experimental Animal Infections

Due to its high incidence of recovery from clinical specimens and its resistance to many commonly used antibiotics, B. fragilis has been the subject of perhaps the most extensive recent research of any of the clinically important anaerobes. Little is still known, however, regarding

mechanisms of pathogenicity or of resistance to antimicrobial agents. Although the organism has been subjected to extensive antibiotic susceptibility testing in vitro, clinical treatment of B. fragilis infections have yielded conflicting data as to what are the most effective drugs against this organism. The desirability of an experimental animal infection with a pure culture of B. fragilis has been recognized for some time. Such a model infection would provide a method for studying mechanisms of pathogenicity, mechanisms of resistance to many antibiotics, and the relative efficacies of various antimicrobial agents including new or experimental antibiotics. Unfortunately, B. fragilis, like most gram-negative anaerobic bacilli, has very little virulence for laboratory animals. Although experimental infections with B. fragilis have been developed by a few investigators, these infections either require the presence of other organisms or, if pure, produce a low incidence of infection that yields variable results. Generally, the production of experimental infections with anaerobes require different techniques than do facultatives or aerobes. Whether this is due to a lower degree of virulence of anaerobes for experimental animals or to a requirement for a lower oxidation-reduction potential than is normally found in healthy tissue is not known. Infections with many facultative organisms can be initiated with either intraperitoneal or subcutaneous injection of 10^6 viable cells or less. Some organisms however, may require more extensive methods; e.g., the use of an adjuvant such as mucin or the use of a particular animal species such as the chimpanzee for Neisseria gonorrhoeae or the armadillo for Mycobacterium leprae. Experimental infections with facultative or aerobic bacteria is facili-

tated by a much more thorough knowledge and understanding of the pathogenic mechanisms of these organisms. For example, the presence of capsules in such organisms as some of the streptococci have been shown to be definitely correlated with virulence and non-capsulated strains are avirulent. Similar examples exist with these organisms where virulence can be correlated with the production of extracellular products or with particular morphological structures such as attachment pili or capsules. For the most part, such information is not yet available for the anaerobic bacteria.

Despite the limited knowledge concerning the pathogenic mechanisms of anaerobic bacteria, a number of investigators, aware of the importance of these organisms in human disease, have been successful in establishing experimental infections in animals. Hite, Locke, and Hesseltine (19) were able to establish experimental infections in mice with pure cultures of certain anaerobic bacteria and with mixtures containing anaerobic bacteria in conjunction with aerobic organisms. In their method, bacterial cultures were grown for 48 h in a dextrose brain broth or in casein hydrolysate yeast extract broth media in anaerobic jars. Bacterial injections always consisted of 1.0 ml total volume. Pure cultures were tested by injecting equal volumes of culture and sterile medium. These investigators tested pure cultures of anaerobic streptococci, Bacteroides melaninogenicus, Fusobacterium necrophorum (formerly Bacteroides necrophorum), and F. nucleatum (formerly Bacteroides fusiformis). Although a number of different combinations of these organisms were pathogenic for mice as mixtures, only F. necrophorum and F. nucleatum produced infection as pure cultures. In each case the incidence of infection was extremely low as was the severity of the infection.

In the mid-1950's, MacDonald, et al. were able to establish and determine the pathogenic components of an experimental fusospirochetal infection in guinea pigs. They found that typical fusospirochetal infections could be produced regularly with a combination of 17 different organisms (24). Eventually through a procedure of progressive deletion of organisms, it was found that the infection could be produced by a particular combination of four organisms (25). The four essential organisms included two strains of Bacteroides, one of which was B. melaninogenicus, a gram-negative rod, and a diphtheroid. Socransky and Gibbons (34) found that B. melaninogenicus was the primary pathogen involved. These workers were able to produce a subcutaneous infection in guinea pigs with a mixed culture containing only B. melaninogenicus and a gram-positive coccobacillus. However, MacDonald et al. (23) also obtained a pure culture infection with one strain of B. melaninogenicus. This strain was later shown not to have a requirement for vitamin K as did the previous strains tested and therefore did not require a helper organism such as the coccobacillus to supply the vitamin. This strain also produced infection as a pure culture when injected subcutaneously into 5 germ-free and 4 conventional mice.

Until the early 1970's, this strain of B. melaninogenicus, as described by MacDonald, Socransky, and Gibbons was the only strain of Bacteroides that had been shown to be capable of initiating a pure culture infection in experimental animals. In 1971, Takazoe et al. (38) discovered another strain of B. melaninogenicus that produced pure culture infections when injected intradermally into rabbits or guinea pigs. Inoculum was prepared by suspending, in saline, cells grown for three

days on anaerobic blood agar plates. Two-tenths ml of this cell suspension, containing approximately 10^8 viable cells, was injected intradermally into the abdomens of young rabbits or guinea pigs. A localized abscess developed at the point of injection and persisted for 7-10 days before spontaneously rupturing. These authors correlated the pathogenicity of this strain with the presence of a distinctive slime layer or capsule.

During investigations to determine the pathogenic components of an experimental fusospirochetal infection, MacDonald et al. (25) concluded that oral anaerobic streptococci were not a necessary component of this infection. Reports in the literature varied widely concerning the pathogenic potential of anaerobic streptococci in pure culture. MacDonald et al. (25) were not able to obtain infection in guinea pigs with pure cultures of these organisms. Coleman and Hare (5) tested seven strains by injecting them subcutaneously into mice. Of the seven only two strains gave rise to a small lesion at the site of injection. Weiss and Mercado (41) reported similar results with strains derived from pulmonary abscesses. Mergenhagen et al. (26) found that injection of anaerobic streptococci subcutaneously into rabbits or the groin of guinea pigs did not produce demonstrable lesions; this was in agreement with most previous investigations. These investigators found, however, that the injection of 1.0 ml of a 48 h old thioglycolate broth culture, containing approximately 10^9 viable cells, intracutaneously into mice reproducibly gave lesions. At least 0.5 ml was necessary to elicit infection with all 6 strains tested. Further experiments revealed that a minimum of 3×10^9 viable cells suspended in saline were required.

Since all the strains were obligate anaerobes, these investigators felt that some of the cells were killed by oxygen and that this might account for the high number of cells required to produce infection. This theory was tested by diluting the cells in saline that had been supplemented with 0.1% agar and 0.1% sodium thioglycolate. Both substances had previously been shown to aid in the initiation of growth of these organisms in a synthetic medium, probably by maintaining a sufficiently low oxidation-reduction potential. Only 1/100 to 1/10,000 as many cells were required to produce infection with the agar-thioglycolate inoculum as with the saline inoculum. Thioglycolate was found to be replaceable with other reducing agents such as cysteine or glutathione. However, elimination of either the reducing agent or the agar resulted in a 100 to 10,000 fold increase in the number of cells required to produce infection. These authors concluded that a reducing agent was required to maintain viable cells and that agar was necessary as a physical localizing factor. It was felt that the agar might hold the cells at the site of injection and prevent dilution of the inoculum by tissue fluid; or, the agar might serve to maintain a locus of oxidation-reduction potential suitable for the proliferation of the organisms.

Both the work of MacDonald, Socransky, and Gibbons with B. mel-aninogenicus and the anaerobic streptococci model infection of Mergenhagen, Thonard, and Scherp were begun in the early to mid-1950s and were concerned with the pathogenic mechanisms of the organisms involved and the role that these organisms might have in mixed infections. No attempt was made by either group to determine the efficacies of antibiotics. There were several reasons for this. At the time of the work,

most clinicians had little interest in anaerobes due to the prevailing dogma that anaerobic bacteria were not particularly pathogenic for man. Also, at this time, even most of those clinicians who were aware of the possible significance of anaerobes in human disease, were not aware that antibiotics effective against facultative and aerobic bacteria were often not effective against anaerobes. Widespread interest in antimicrobial therapy of anaerobic infections did not begin to emerge until the 1960s. This interest resulted primarily from technological advances that permitted the cultivation of fastidious anaerobes from clinical material and the clarification of the taxonomy of these organisms. Once clinicians gradually became aware of the significance of the recovery of anaerobes from clinical specimens, interest in the treatment of such infections with antimicrobial agents also increased. The incidence of recovery of B. fragilis from human infections and the discovery of the resistance of this organism to many commonly used antibiotics also increased interest in this area.

In the early to mid 1970s, several different experimental animal infections were developed with anaerobic bacteria. A number of these experimental infections were developed with other organisms when efforts to develop a pure B. fragilis infection failed. Some of these model infections mimic human infections and many have been used to determine the effectiveness of antimicrobial agents against the infection.

In 1974, Hill et al. (18) described the production of progressive intrahepatic abscesses in mice following injection of single and combined species of nonsporulating anaerobic bacteria. One ml volumes

of inocula consisting of equal volumes of 20 h old bacterial cultures and sterile mucin were injected intraperitoneally into mice. Depending on the species used, the inoculum contained from 2×10^8 to 5×10^8 viable cells. All the strains were originally isolated from human clinical specimens and included 5 strains of B. fragilis, and single strains of B. melaninogenicus, F. necrophorum, F. nucleatum and Peptostreptococcus CDC group 1. Although pure cultures of all of these strains produced liver abscesses, the incidence of infection with the single exception of F. necrophorum, was 50% or less, and in most instances was 25% or less. Only F. nucleatum and F. necrophorum, when injected singly, reproducibly gave an incidence of infection of 50%. Combinations of two species generally gave a more severe, reproducible, and progressive disease than did any one strain given singly. These infections were not readily suitable for in vivo testing of antimicrobial agents due to the low incidence of infection and to the necessity of performing necropsy to determine if infection had occurred. The model infection, however, did provide an example of a progressive experimental infection of an internal organ. Since the same site was infected regardless of whether pure or mixed cultures were used, this model provided a method for studying and evaluating both pathogenicity and synergism of different anaerobic species in the formation of liver abscesses.

Later, in 1974, Wilkins and Smith (42) described the production of an experimental infection in mice with F. necrophorum in which the disease resulted in the death of the mice if untreated. This infection was initiated with 0.1 ml of washed cells from a 15-18 h broth culture. Infection was produced regardless of whether the bacteria were given intraperitoneally

or subcutaneously, although a higher number of cells was required intraperitoneally to produce infection. These authors tested the effectiveness of four antibiotics in the treatment of these infections. Although therapy of the intraperitoneal infection was possible, there were considerable variation in the results. The authors postulated that the variation may have been due to variation in the loci of the infection. Abscesses were found in the liver, mesentery, pancreas, peritoneal membrane, and gonads of different mice. In contrast, subcutaneous injection reproducibly resulted in a single localized abscess at the point of injection. Antibiotics were tested on a weight to weight basis and the amount of each required to protect 50% of the mice from infection (the ED₅₀ value) was calculated for several different treatment schedules. The major advantage of this model infection was that the exceptional virulence of the strain of F. necrophorum used made it possible to have death or survival as the sole criterion of the effectiveness of antimicrobial therapy. This meant that a larger number of animals could be included in an experiment since time consuming autopsies were not required to determine the presence of infection.

Kuch (21), in 1975, reported on the efficacies of several antimicrobial agents including several experimental antibiotics against a F. necrophorum infection in mice. The infection model used was essentially a combination of the models described by Hill et al. (18) and Wilkins and Smith (42). Antibiotics were tested on a weight to weight basis as well as at dosages that yielded serum levels in the mice that were clinically achievable in man. This last method of evaluating antimicrobial agents gives a more meaningful comparison of different antibiotics since clinically achievable blood levels are different from one antibiotic to another.

Abe et al (1) has described a model infection in mice initiated with F. necrophorum that is very similar to that described by Hill et al. (18) except that mucin was not required. The fusobacteria were given by intraperitoneal injection and resulted in chronic liver abscesses in about two weeks post-injection.

In late 1974, Weinstein et al. (40) reported an elegant model infection that simulated large bowel perforation with subsequent abscess formation. The infection was produced by surgical implantation of a double gelatin capsule containing a polymicrobial inoculum consisting of the pooled cecal and intestinal contents of rats. The infection produced was biphasic; peritonitis occurred during the first week following implantation and abscesses formed after this. An extensive bacteriological study revealed that Escherichia coli was the principal isolate during the initial peritonitis stage and B. fragilis and Fusobacterium were the major isolates from the abscesses (28). These investigators found that essentially the same course of infection could be produced with an inoculum of B. fragilis, F. varium, E. coli, and an enterococcus mixed in sterile cecal contents along with barium sulfate. The barium sulfate was used to increase the local inflammatory response in order to restrict dissemination of the cecal contents. This model infection is significant for several reasons. It provides a model for intra-abdominal sepsis which often may occur in man following large bowel perforation. It demonstrates the importance of both facultative and anaerobic bacteria in such an infection and the role of each in the stages of the infection. It also provides a model for determining the efficacies of various antimicrobial agents against a defined mixed in-

fection involving both facultatives and anaerobes. Subsequent work has been done by these investigators using this model to determine the effectiveness of antimicrobial agents and the relationship of the organisms involved to the pathogenicity of the mixed inoculum (A. Onderdonk, personal communication). Unfortunately this model does have some apparent disadvantages. Surgical implantation of the gelatin capsule is time consuming and requires some surgical skill; both of which limit the number of animals that can be used in a single experiment. It is not known if surgical implantation is absolutely necessary or if the inoculum could be injected directly into the peritoneal cavity. Although the incidence of infection is relatively high, autopsy is required for evaluation of abscess formation and determining the effectiveness of antimicrobial therapy. Since the inoculum consists of a mixture of bacteria and sterile cecal contents, it is difficult to be sure that each individual inoculum contain the components in the same proportions.

At the annual meeting of the American Society for Microbiology in 1975, Renz et al. (K. J. Renz, G. J. Miraglia, and D. W. Lambe, 1975, Absts. Annu. Meet. Am. Soc. Microbiol., Abst. A16, p. 3) reported on establishing experimental infections in mice with several species of anaerobic bacteria including B. fragilis. The method involved injection of 0.5 ml of 5% hog gastric mucin intraperitoneally into mice. Fifteen minutes later approximately 10^8 viable cells of B. fragilis were injected by the same route. Abscesses were produced in approximately 50% of the mice and were located primarily within the liver and, to a lesser extent, the spleen. This model infection was developed for the testing of new antimicrobial agents that might be effective against anaerobic

bacteria and particularly against B. fragilis. The model is not significantly different from that described by Hill et al. (18) except that the incidence of infection was slightly higher. Both models require mucin for the initiation of the infection, have a low incidence of infection, and require necropsy to determine the presence of infection.

Both Wilkins and Hill have used their individual models to determine the effects of therapy against anaerobic infections. In 1975, Hackman and Wilkins (14, 15) described the efficacies of several β -lactam antibiotics against a mixed infection in mice initiated with F. necrophorum and B. fragilis. This work arose out of an attempt to produce a pure culture infection with B. fragilis by injecting F. necrophorum and B. fragilis together and attempting to kill the fusobacteria with penicillin. These investigators had previously tried unsuccessfully to infect mice with a number of strains of B. fragilis. They thought that since B. fragilis alone did not seem to be pathogenic for mice and F. necrophorum had been shown to be infective, an inoculum consisting of both might allow the B. fragilis to become established in the infection along with F. necrophorum. Since B. fragilis is generally extremely resistant and F. necrophorum is susceptible to penicillin, penicillin therapy was initiated to eliminate the fusobacteria. It was found, however, that B. fragilis protected the fusobacteria from the effects of the penicillin. Since B. fragilis is often found in mixed culture with other anaerobes in clinical specimens, the ability of B. fragilis to protect other organisms from β -lactam antibiotics may be of importance in clinical treatment of these infections.

Hill (16) has reported on the effect of hyperbaric oxygen exposure in the treatment of progressive liver abscesses produced by injection of

combined cultures of F. necrophorum plus either B. fragilis or F. nucleatum. The method used to produce infection was essentially the same as described previously by Hill et al. (18). Twenty-hour old broth cultures of the anaerobic species were mixed in equal volumes and then an equal volume of sterile mucin was added. The mice were injected intraperitoneally with 0.5 ml containing 10^7 to 10^8 viable cells.

Hill (17) later used this model for the therapeutic evaluation of minocycline and tetracycline against a mixed infection in mice initiated with F. necrophorum and F. nucleatum. Different treatment regimens were tested including a regimen begun three weeks after bacterial injection to determine the effect of the antibiotics against well established abscesses. This may give a more realistic evaluation of the antibiotics since in clinical situations the abscesses encountered are more likely to have been present for some time.

In the past two years, several additional experimental infections have been developed and previously described models have undergone further development and refinement. Onderdonk, et al. (A. B. Onderdonk, D. L. Kasper, J. G. Bartlett, and R. L. Cisneros. 1976. Abst. Annu. Meet. Am. Soc. Microbiol., Abst. B77, p. 23) were successful in obtaining a pure B. fragilis infection following peritoneal implantation of a gelatin capsule in rats. Infection was produced in approximately 95% of the animals when encapsulated strains of B. fragilis were used but only 25% of the rats developed infection when unencapsulated strains were used. This model is essentially the same as that described by Weinstein et al. (40) with the exception that a monomicrobial rather than a polymicrobial inoculum is used.

A systemic infection was produced in atherosclerotic rabbits with a pure culture of B. fragilis (D. M. Klurfel, M. J. Allison, E. Gerszten, and H. P. Dalton. 1976. Absts. Annu. Meet. Am. Soc. Microbiol., Abst. B20, p. 14). This model involved the intravenous injection of washed B. fragilis cells into atherosclerotic rabbits. The atherosclerotic condition appeared to be required in order to establish infection since normal rabbits injected with B. fragilis failed to develop infection.

An unusual experimental model has been utilized for the production of infection with several anaerobic species by Stalons and Swenson (D. R. Stalons and R. M. Swenson. 1976. Absts. Annu. Meet. Am. Soc. Microbiol., Abst. B19, p. 14). The technique used consisted of the implantation of a polyethylene chamber (practice golf ball) in the dorso-lumbar region of a rabbit. After 30 to 45 days the chamber was encapsulated with tissue and was filled with fluid. At this time, approximately 5×10^8 viable cells of the anaerobic species used were injected into the chamber. According to these investigators, the chamber remained culturally positive for the bacteria for as long as three weeks. This technique has been used previously for the in vivo cultivation of Treponema pallidum with some success. The method appears to result only in the multiplication of the injected organisms without resulting in a disease process. Stalons and Swenson, however, did find in a few cases, that bacteremia resulted. The bacteremia was not associated with any particular organism and appeared to be only a random occurrence.

Most of the experimental animal models currently in use for studying anaerobic bacterial infections have been developed in the past five years. The models have recently been utilized in the study of pathogenic mechanisms

including adherence, clearance from the blood, enhancement of infection by protein compounds, endotoxin activity, and immunologic responses as well as antimicrobial therapy (Abst. Annu. Meet. Am. Soc. Microbiol., 1977. Absts. A17, A18, B12, B16, B17, B18, B19, and B29, pp. 3-4, 17-20).

Statement of Purpose

When the work described in this dissertation was begun in 1975, experimental animal infections with anaerobic bacteria required either a polymicrobial inoculum or else resulted in a low incidence of infection with the exception of a few isolated cases. At this time, there was no single method available that was suitable for producing experimental infections with a number of different anaerobic species. Most importantly, a pure culture infection could not be produced with B. fragilis that was suitable for evaluation of antimicrobial agents against this organism. Although the B. fragilis infection described by Renz et al. was used to some extent for this purpose, the low incidence of infection obtained and the necessity of necropsy in order to determine infection prohibited the use of this model for large scale studies.

The problem, therefore, was to develop an economical method for producing a pure culture infection in an experimental animal with B. fragilis which would be suitable for the evaluation of antimicrobial agents. To fulfill these requirements, it was necessary that the presence of the infection be readily apparent, that the majority of the animals develop the infection, that the infection be amenable to antimicrobial therapy, and that the process be reproducible by other investigators. In addition, it was desirable that the method developed should

also be useful to produce pure culture infections with other clinically significant anaerobic bacteria.

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PART I
Use of Semisolid Agar for Initiation of Pure
Bacteroides fragilis Infection in Mice

SUMMARY

The development of a pure Bacteroides fragilis infection in mice is described. The infection produces large subcutaneous abscesses at the site of injection which can be observed grossly within 7 days after injection. The infection was initiated by injection of pure cultures grown in semisolid agar medium. Similar infections were also produced with pure cultures of B. distasonis, B. ovatus, B. thetaiotaomicron, and B. vulgatus. However, a distinct deoxyribonucleic acid homology group, formerly classified as B. thetaiotaomicron, did not produce abscesses in any of the mice tested.

INTRODUCTION

Bacteroides fragilis is an anaerobic, gram-negative, nonsporing rod that is often encountered in human clinical specimens. This organism is frequently isolated from blood cultures, lung abscesses, soft-tissue infections, and infections of the urogenital tract. Although B. fragilis is often found in pure culture in human infections, attempts to establish pure culture infections in experimental animals have met with only limited success. Both Hill et al. (4) and Renz et al. (K. J. Renz, G. J. Miraglia, and D. W. Lambe, Jr., Abstr. Annu. Meet. Am. Soc. Microbiol. 1975, A16, p. 3) have obtained intraperitoneal abscesses in mice by injection of pure cultures of B. fragilis along with 0.5 ml of hog gastric mucin. Both of these models produce a low frequency of infection and require mucin, and different batches of mucin vary in their ability to promote infection.

In this paper, we present a method for the production of subcutaneous abscesses in mice by injection of pure cultures of B. fragilis contained in soft agar. This method produces localized abscesses in 90% or more of the mice. The culture age optimal for the establishment of the infection and the variability in virulence among different B. fragilis strains were determined. The virulence of the reference strains of the deoxyribonucleic acid (DNA) homology groups of other species of saccharolytic bacteroides (2) was also investigated.

MATERIALS AND METHODS

Organisms. All strains of B. fragilis were from the culture collection of the Virginia Polytechnic Institute and State University (VPI) Anaerobe Laboratory. These strains were isolated from human clinical specimens and were sent to the VPI Anaerobe Laboratory for confirmation of identification. Identifications were done by L. V. Holdeman or W. E. C. Moore according to previously published criteria (5). Reference strains of the DNA homology groups of other saccharolytic bacteroides (2) were provided by J. L. Johnson.

Media. Unless stated otherwise, media and dilution fluids were prepared as described in the VPI Anaerobe Laboratory Manual (5). Chopped-meat carbohydrate broth was prepared without meat particles, and chopped-meat carbohydrate semisolid agar medium was prepared by adding 0.25% (wt/vol) agar (Difco) to chopped-meat carbohydrate broth before sterilization.

Inocula. Stock cultures were maintained in chopped-meat broth, stored at room temperature (25 C), and transferred each week. Cultures

for injection of mice were prepared by inoculating tubes containing 10 ml of broth or semisolid agar medium with 0.1 to 0.2 ml of an 18-20 h chopped-meat broth culture. The culture was incubated for 18 to 20 h before injection except for those experiments where incubation times were being compared.

Concentration of cultures for injection. In experiments where the concentration of cells desired was greater than that obtained in culture, the cultures were concentrated by anaerobic centrifugation. Chopped-meat carbohydrate broth cultures were concentrated by transferring 10 ml of culture anaerobically to a 12-ml-capacity glass centrifuge tube (17.5 by 120 mm) (no. 104, Ivan Sorval Inc., Norwalk, Conn.), sealing the tube with a size 0 rubber stopper, centrifuging at 2,000 x g (Sorval GLC-1) for 10 min, and resuspending the cell pellet in anaerobic dilution fluid (5) to yield the desired concentration. When chopped-meat carbohydrate semisolid agar cultures were concentrated by the same procedure, a gelatinous pellet resulted which comprised one-half the original volume. This pellet was used for injection without dilution.

Injection of bacteria. All cultures were transferred to sterile serum bottles and capped while the bottles were under a stream of O₂-free CO₂. Immediately before injection of the bacterial culture, plastic syringes were flushed several times with CO₂ and then filled from the serum bottles. Subcutaneous injections were made beneath the loose skin of the left groin. Injections consisted of 0.5 ml and for most of the experiments contained approximately 2×10^9 viable cells.

Source of mice. Random-bred Swiss White male mice, Dublin ICR, 18 to 20 g (Flow Laboratories, Dublin, Va.), were used for all experiments.

Viable cell counts. Viable cell counts of the bacterial suspensions were performed by the roll tube procedure in supplemented brain heart infusion agar medium (5). Viable cell counts of the number of bacteria per gram of abscess material were determined by standard plate count procedures on brain heart infusion agar medium. Manipulations were done in an anaerobic chamber similar to that described by Aranki and Freter (1). Approximately 1 g of abscess was homogenized with 99 ml of anaerobic dilution fluid (5) in a blender within the anaerobic chamber. Additional dilutions were done in the anaerobic dilution fluid (5), and then triplicate plates were spread with 0.1 ml per plate.

Heat-killed cells. An 18 to 24 h culture of B. fragilis strain VPI 9032 was killed by heating at 60 C for 1 h, allowing to cool for 1 h, and again heating for 1 h at 60 C.

Anaerobic procedures. Except where stated otherwise, all cultural manipulations were performed while being gassed with O₂-free CO₂ using the techniques described in the VPI Anaerobe Laboratory Manual (5).

Cultural examination of abscesses. Pus from abscesses was streaked directly onto brain heart infusion agar roll tubes. The criteria used for designation of pure culture infections were colony morphology, observation of Gram stains, analysis of fermentation products by gas chromatography, and biochemical reactions as determined by the micro-method described by Wilkins et al. (6, 7).

RESULTS

Chopped-meat carbohydrate concentrated broth culture. Previous work in this laboratory in which 0.1 ml (3) or 0.5 ml of broth culture had been injected into mice was unsuccessful in producing B. fragilis infections. To increase the number of viable cells injected without increasing the volume above 0.5 ml, a maximally turbid broth culture, 18 to 20 h old, of B. fragilis strain VPI 9032 was concentrated four-fold by anaerobic centrifugation. One-half milliliter of this suspension, containing approximately 4×10^9 viable cells, was injected subcutaneously. Visible abscesses were present within 3 to 5 days. The infection was characterized by a spreading abscess that began at the site of the injection and rapidly spread across the abdomen, resulting in the loss of hair and skin in this area and often producing an open sore. Generally, the infection spontaneously healed between the 2nd and 4th weeks. However, in 5 to 10% of the infected mice, death occurred due to invasion of B. fragilis into the peritoneal cavity. The number of mice that developed an infection was variable, ranging from 55 to 80% in different experiments.

Chopped-meat carbohydrate semisolid agar culture. We did not consider the above method satisfactory as a model for studying B. fragilis infections because of the difficulty in concentrating the culture and because of the variability of the infection. Next, we tested an inoculum in a semisolid or soft agar medium. Our rationale was that a semisolid agar medium might help protect the bacteria from the immune mechanisms of the mouse. Strain VPI 9032 was grown overnight (18 to 20 h) in semisolid agar medium, and 0.5 ml of culture containing approximately

2×10^9 viable cells was injected subcutaneously. Abscesses occurred in 90 to 100% of the mice. The abscesses were visible by the 4th to 7th day as an enlarged area approximately 5 mm in diameter at the point of injection. In approximately 30% of the mice, the abscesses spontaneously ruptured and drained through the skin within 10 to 14 days after injection. The abscesses present in the remaining infected mice remained localized and progressively increased in size. These abscesses were 15 to 20 mm in diameter by the end of the 8th week, which was the maximum time the mice were kept. The abscesses were filled with a viscous pus. When 0.1 ml of this pus was injected subcutaneously into other mice, similar abscesses developed. The abscesses (4th week) contained 3×10^{10} to 6×10^{10} viable bacteria per g. B. fragilis was the only organism isolated from the abscesses. Injection of 0.5 ml of a heat-killed culture in semisolid agar medium did not result in abscess production.

Strain variability. Twenty-five strains of B. fragilis were tested for pathogenicity by injecting 0.5 ml of 18 to 20 h semisolid agar culture subcutaneously in mice. Of these 25 strains, 21 produced abscesses in some of the mice (Table 1), and 13 strains produced abscesses in 50% or more of the mice. Three strains (VPI 8429, VPI 8708, and VPI 9032) produced abscesses in 90 to 100% of the mice. These abscesses persisted for up to 8 weeks, which was the longest time the mice were kept. The incidence of spontaneous healing was 30% or less for all three strains.

Requirement for soft agar. The necessity of using semisolid medium to initiate infections was evaluated by determining the incidence of infection with broth cultures compared with that with semisolid agar cul-

Table 1. Production of subcutaneous abscesses by strains of Bacteroides fragilis

VPI strain no.	No. of mice developing abscesses ^a
2553 (ATCC 25285)	6
3625	5
4255	4
4489	6
4736	4
4909	4
4912	2
4948	0
5001	0
5002	0
6957	4
7310	5
7428	7
7713	1
8002-1	2
8429	10
8662B	8
8708	10
9032	10
9035	6
9132	0
9309	9
9439	4
9525	6
9836A	6

^aTen mice per group.

tures. Strain VPI 9032 was grown in both media for 18 to 20 h, and the cultures were adjusted to yield viable cell numbers of approximately 1×10^9 , 2×10^9 , and 4×10^9 cells per 0.5 ml of culture injected. Two groups of 10 mice each were injected subcutaneously with each concentration. The incidence of abscess development is given in Table 2. In each case, the semisolid agar cultures produced a significantly higher incidence of infection than did the comparable number of cells in broth cultures.

Effect of culture age. We had observed in our early experiments that a higher incidence of infection was produced with 18 to 20 h cultures than with 12 h cultures. Viable cell counts, however, demonstrated that the older cultures did not contain more viable cells than the 12 h cultures. To investigate this observation, 100 ml of semisolid agar medium was inoculated with 1.0 ml of a 20 h broth culture of strain VPI 9032. Samples (5 ml) were taken from this culture every 2 h for a period of 24 h. From each sample, viable cell numbers were determined and five mice were injected subcutaneously. The number of mice developing abscesses was compared with the culture age and the viable cell numbers (Figure 1). The maximum number of viable cells was reached between 10 and 12 h, which corresponded to the end of logarithmic phase of growth. Samples taken during this period produced abscesses in a maximum of only three of the five mice injected. The first sample to produce abscesses in all five of the injected mice was taken at 18 h of incubation. Samples taken at 20, 22 and 24 h also produced abscesses in all of the mice. The number of viable cells remained constant from 12 to 24 h. A repeat experiment gave similar results.

Table 2. Comparison of chopped-meat carbohydrate broth and chopped-meat carbohydrate semisolid agar cultures of B. fragilis

Inoculum	No. of viable cells	No. of mice with abscesses	Percent abscess formation
Chopped-meat carbohydrate broth	1×10^9 ^a	1 ^b	5
	2×10^9	6	30
	4×10^9	13	65
Chopped-meat carbohydrate semisolid agar	1×10^9	7	35
	2×10^9	18	90
	4×10^9	19	95

^aNumber of viable cells/0.5 ml of inoculum.

^bTwenty mice in each group.

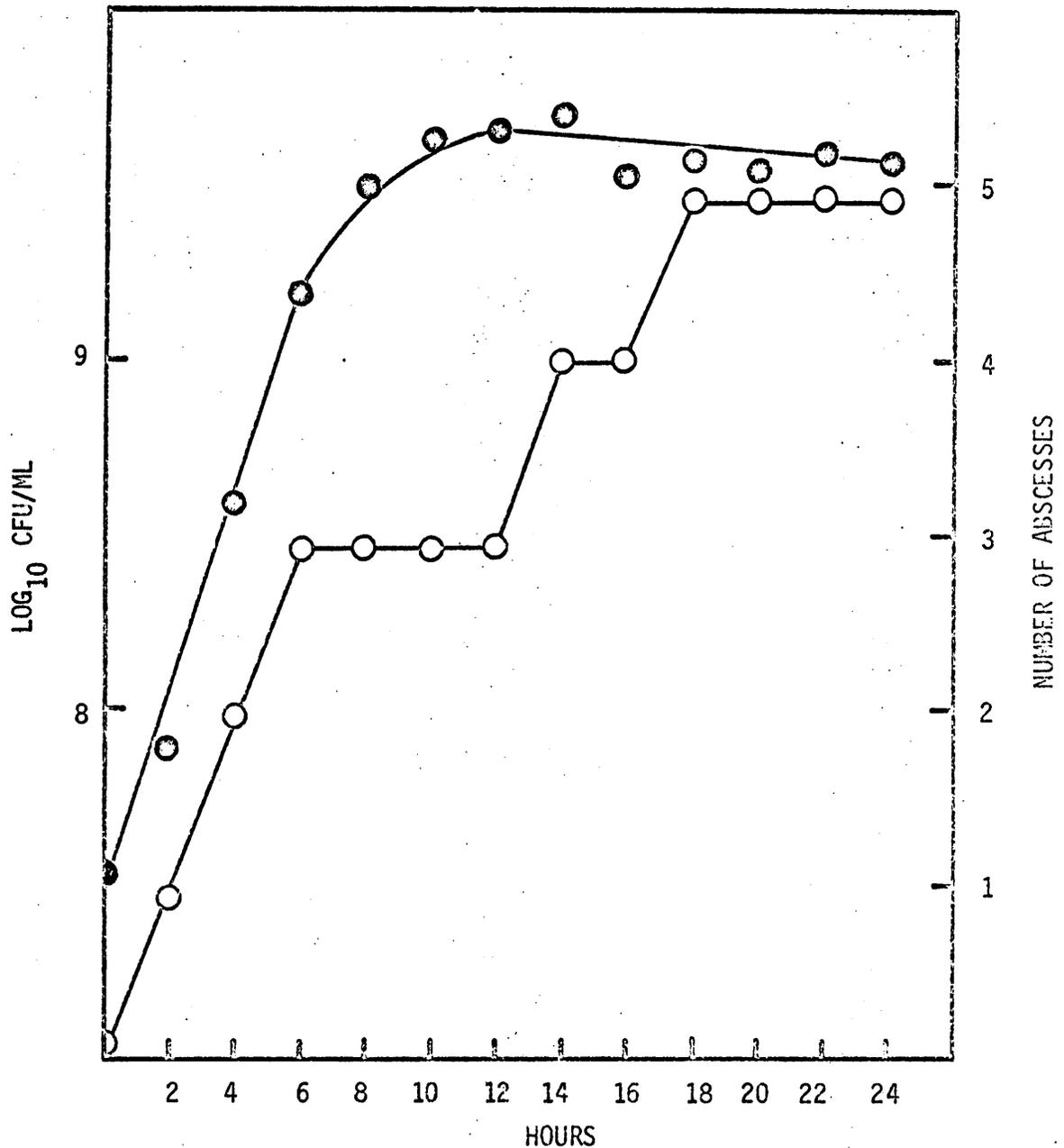


Figure 1. Determination of the culture age optimal for the establishment of *B. fragilis* subcutaneous abscesses in mice. Samples of the culture were taken at 2-h intervals, the number of viable bacteria per milliliter was determined, and five mice were injected from each sample. Symbols: (●) Viable cell number; (○) number of mice developing abscesses at 7 days after injection.

Intraperitoneal injections. Five strains (VPI 6957, VPI 8662B, VPI 8708, VPI 8429 and VPI 9032) of B. fragilis were grown in both broth and semisolid agar media for 18 to 20 h, and 0.5 ml was injected intraperitoneally into five mice. None of these strains produced death or intraperitoneal abscesses with either inoculum.

Strains VPI 8708 and VPI 9032 were grown in broth and concentrated fourfold by anaerobic centrifugation, and 0.5 ml (4×10^9 to 5×10^9 viable cells) was injected intraperitoneally. Both strains produced multiple 1 mm abscesses on the liver and along the intestines. In some of the experiments, strain VPI 8708 killed all five of the mice injected within 5 days, whereas in other experiments only one or two of the mice died. Strain VPI 9032 only killed one or two out of the five mice injected. Of a total of 25 mice that were tested with each strain, strain VPI 8708 killed 11 of the mice and strain VPI 9032 killed 6. Mice that did not die from the infection were killed and autopsied 14 days after injection. These mice had small abscesses on the liver and intestines. In other experiments, mice that appeared to be close to death during the first 5 days were killed, and approximately 0.1 ml of cardiac blood was cultured in chopped-meat broth. Blood cultures were positive for B. fragilis in three of seven mice. Blood cultures were negative from six mice that appeared healthy but were found to have small intraperitoneal abscesses present on autopsy.

Semisolid agar cultures of two strains, VPI 8708 and VPI 9032, were concentrated twofold, and 0.5 ml (4×10^9 viable cells) was injected intraperitoneally. Both strains produced small multiple liver and intestinal abscesses in mice, but rarely were any of the mice killed

by the infection. In 25 mice injected separately with each strain, strain VPI 8708 killed five mice and strain VPI 9032 killed three. Intraperitoneal abscesses were present in 21 of the 25 mice injected with strain VPI 8708 and in 18 of the 25 injected with VPI 9032.

Pathogenicity of different DNA homology groups. The reference strains of the DNA homology groups (2) representing the species B. distasonis, B. ovatus, B. thetaiotaomicron, and B. vulgatus were tested for pathogenicity by subcutaneous injection of 0.5 ml of 18 to 20 h semisolid agar cultures. Approximately 50 mice were injected with each reference strain. The incidence of infectivity for these strains is given in Table 3. B. thetaiotaomicron reference strain VPI 5482 was the only strain that gave a low incidence of infectivity. To determine whether this finding was limited only to this reference strain, two other strains of B. thetaiotaomicron were tested. In addition, three strains (VPI 0061-1, VPI 3699, and VPI 6387) were also tested that were originally classified as B. thetaiotaomicron, but because these organisms have a higher guanine plus cytosine content and comprise a distinct DNA homology group, they have been temporarily designated as the "high theta" group. Ten mice were injected with each strain, including the reference strain VPI 5482 originally tested. Strain VPI 5482 produced abscesses in 3 of the 10 mice, which agreed closely with the earlier data. The other two strains (VPI 2302 and VPI 3051) of B. thetaiotaomicron produced three and four abscesses, respectively. The three "high theta" strains did not produce abscesses in any of the mice injected. The abscesses caused by the three strains of B. thetaiotaomicron were smaller in size (maximum diameter of 5 to 7 mm) compared

Table 3. Pathogenicity of DNA homology reference strains of Bacteroides species

DNA homology group	VPI strain no.	No. of mice tested	No. of mice infected	Percent infected
<u>B. distasonis</u>	4243	49	48	98
<u>B. ovatus</u>	0038-1	49	44	90
<u>B. thetaiota-omicron</u>	5482	47	17	36
<u>B. vulgatus</u>	4245	47	36	77

with the other Bacteroides species tested, which produced abscesses of 15 to 20 mm in diameter.

DISCUSSION

The intraperitoneal route of injection produced small internal abscesses similar to those described in the mouse models developed by Hill et al. (4) and by Renz et al. (Abstr. Annu. Meet. Am. Soc. Microbiol. 1975, A16, p. 3). Although our intraperitoneal model did not require mucin for initiation, it did suffer from most of the disadvantages associated with these other two models: (i) the rates of infection and mortality were variable, and (ii) autopsy was required to determine the presence of abscesses, which were often so small that a careful, time-consuming dissection was required to determine infection. In addition, our model required anaerobic concentration of the cultures. A subcutaneous route of infection with a concentrated broth culture inoculum had similar drawbacks. The preparation of inocula was cumbersome and the incidence of infection was variable. This method was not suitable for long-term studies, since the infection usually resolved itself within 2 to 3 weeks.

The method that we found most satisfactory was subcutaneous injection of cultures grown in semisolid agar. We were able to reproducibly initiate infection in 90% or more of the mice tested. Abscesses were localized at the injection site and were visibly present within 1 week. The use of semisolid medium permitted direct injection of the culture without further manipulations. We found that 70% of the abscesses persisted for at least 2 months. Therefore, this method should be suitable for both long-term and large-scale investigations.

The exact mechanism of action of the semisolid medium is not known. It may protect the bacteria from phagocytosis or other immune responses of the host. Unlike hog gastric mucin, semisolid agar media are easily prepared, and agar should not cause the variability that is often attributed to different preparations of mucin.

We are unable to explain why 18 to 20 h cultures were more virulent than 10 to 12 h maximally turbid cultures. We are currently trying to determine whether an invasion factor is released by lysis of the older cells, whether the younger cells are more oxygen sensitive, or whether the older cells are more invasive due to capsule formation or some other mechanism.

Our data on the pathogenicity for mice of the Bacteroides species that were previously classified as subspecies of B. fragilis are preliminary. Of the five reference strains of the DNA homology groups that we tested, four infected at least 30% or more of the mice injected. The reference strain of the "high theta" group, strain VPI 0061-1, was the only reference strain that was not pathogenic for mice. Two other strains of this group were also nonpathogenic in these tests. This "high theta" group appears to be extremely rare in human infection, but is commonly found in the human colon. This correlation between the pathogenicity for mice and humans did not hold up as well for the other groups. Both B. distasonis and B. ovatus are relatively uncommon in human infections, but the reference strains infected 90% or more of the mice injected. Of the species tested, B. thetaiotaomicron is second to B. fragilis in occurrence of isolates from human clinical specimens. However, B. thetaiotaomicron strain VPI 5483 infected only 30 to 36%

of the mice challenged; the other two strains of this group that we tested yielded similar results. Further experiments with more strains are required to verify these results.

We hope that the soft agar injection technique which we have developed for the initiation of B. fragilis infections will be of use in the evaluation of antimicrobial agents and the study of pathogenic mechanisms of B. fragilis. This method may also prove useful in the initiation of other types of experimental infections.

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PART II
Chemotherapy of an Experimental Bacteroides fragilis
Infection in Mice

SUMMARY

The efficacies of five common antimicrobial agents were determined for a pure Bacteroides fragilis infection in mice. Therapy was begun 4 h after bacterial injection and given every 8 h thereafter for 5 days. Blood levels were determined over an 8 h period for each concentration of antibiotic tested. Clindamycin and tetracycline were the most effective in preventing the formation of abscesses. Chloramphenicol, penicillin G, and cephalothin were not effective in protecting the mice from infection.

INTRODUCTION

Bacteroides fragilis is an anaerobic, gram-negative, nonspore-forming bacillus frequently encountered in human clinical specimens. Although the in vitro susceptibility of this organism has been determined to many antimicrobial agents, the lack of a suitable experimental animal model has prohibited the testing of the efficacies of these agents in vivo.

We have recently described the production of a pure B. fragilis infection in mice that results in an easily visible subcutaneous abscess (12). This model provides the first opportunity for the testing of antimicrobial agents against a pure B. fragilis infection in an experimental animal. Using this model, we have tested the efficacies of five common antimicrobial agents: clindamycin, chloramphenicol, tetracycline, penicillin G, and cephalothin.

MATERIALS AND METHODS

Source of organisms. B. fragilis VPI 9032 was originally isolated from a human clinical specimen.

Bacterial inoculum. Before this series of experiments, a chopped-meat broth culture of B. fragilis VPI 9032 was lyophilized in a number of ampoules. One ampoule was opened for each experiment into chopped-meat broth. The culture was grown overnight (18 to 20 h), and 0.1 ml was inoculated into 10 ml volumes of chopped-meat-carbohydrate semisolid medium (12). The chopped-meat-carbohydrate semisolid culture was incubated for 18 to 20 h and then injected into mice.

Injection of bacteria. One-half milliliter of the chopped-meat-carbohydrate semisolid culture was injected subcutaneously under the loose skin of the groin on the left side of the mouse as previously described (12).

Source of antibiotics. The antibiotics used and the sources were: penicillin G, potassium salt (1,595 U/mg) and tetracycline-hydrochloride, Sigma Chemical Co., St. Louis, Mo.; chloramphenicol-sodium succinate, and chloramphenicol-hydrochloride, Parke-Davis, Detroit, Mich.; cephalothin, Eli Lilly and Co., Indianapolis, Ind.; clindamycin-phosphate and clindamycin-hydrochloride, The Upjohn Co., Kalamazoo, Mich.

Injection of antibiotics. All antibiotics were dissolved in sterile distilled water and given in 0.1 ml volumes. All solutions were made up fresh daily and stored at 4 C with the exception of tetracycline-hydrochloride, which was made up fresh for each injection time. The antibiotics were given intraperitoneally (i.p.) on

the animal's right side. Tetracycline-hydrochloride was also given by oral gavage. Two groups of approximately 25 mice each were tested with each concentration of antibiotic. For each experiment, at least 25 mice were used as a control group. The mice received the bacterial injection before being randomly selected for treatment or as controls; the latter received 0.1 ml of sterile distilled water in place of the antibiotic.

Schedule of injections. Antibiotic therapy was begun 4 h after the bacterial injection and then given every 8 h thereafter for a period of 5 days.

Determination of MICs. Minimal inhibitory concentrations (MICs) were determined by the agar dilution procedure (13) with the medium of Wilkins and Chalgren (14). Chloramphenicol-hydrochloride and clindamycin-hydrochloride were used for determination of MICs to chloramphenicol and clindamycin.

Determination of antibiotic blood levels. The amount of antibiotic present in the blood was determined by the method described by Sabath and Toftegaard (11). For each concentration of antibiotic tested, blood levels were determined in two separate groups of five mice each. Blood was collected from each mouse by tail bleeding at 0.5, 1, 2, 3, 4.5, 6 and 8 h after the last injection of the antibiotic. Ten microliters of blood from each mouse was placed on a 6.35 mm paper disk (Schleicher and Schuell, no. 740-E). The disks were placed on the assay medium (11) and incubated in an anaerobic chamber similar to that described by Aranki and Freter (2). The assay plates were incubated at 37 C for 3 to 5 h until hemolysis of the blood had

occurred. The assay medium was made 1 day before use and stored at 4 C. The levels of antibiotics in the blood were determined by comparison with standard curves constructed by exponential regression with a computer. Clindamycin-hydrochloride and chloramphenicol-hydrochloride were used to construct the standard curves for clindamycin and chloramphenicol. Standard curves were constructed by dissolving known amounts of antibiotic in whole sheep blood. Whole sheep blood was used routinely for the standard curves because preliminary trials indicated that no significant differences were detectable between the quantitation of the antibiotics in sheep blood and in mouse blood.

Assay organisms. The strains of Clostridium perfringens used as assay organisms for the different antibiotics are given in Table 1.

Anaerobic techniques and media. The media for the cultivation of B. fragilis were prepared prereduced (5), and all culture manipulations were performed as described in the VPI Anaerobe Laboratory Manual (5).

Mice. Swiss white mice, ICR strain, 18 to 20 g (Flow Laboratories, Dublin, Va.), were used for all experiments. The mice were caged in groups of 25 with a light-dark cycle of 12 h and were allowed to stabilize for at least 48 h before being used in any experiment.

Determination of effects of therapy. The mice were examined daily beginning on the 4th day after bacterial injection and continuing through the 14th day for the presence of a localized subcutaneous abscess. Maximum abscess formation was found to occur by the 7th to 10th day (11). The mice were not kept past the 14th day.

RESULTS

MICs. The MICs for the five antibiotics tested are given in Table 1. B. fragilis VPI 9032 was susceptible to clindamycin, tetracycline, and chloramphenicol and relatively resistant to both penicillin G and cephalothin in vitro.

Blood levels. The antibiotic concentrations in the blood are given in Table 2 for an 8 h period after the final injection of the antibiotic. For all of the antibiotics tested except tetracycline, the peak blood level occurred within the first 0.5 h after injection.

Clindamycin. Intraperitoneal injections of 37.5, 75, 150, or 300 mg of clindamycin per kg were given every 8 h for 5 days. Table 3 gives the percentage of mice protected from infection by each dosage and the peak blood levels obtained. Peak blood levels for clindamycin in humans are generally considered to fall within a range of 3 to 14 $\mu\text{g/ml}$ (7). To obtain comparable blood levels in mice, we found it necessary to give 75 to 150 mg/kg. These dosages gave peak blood levels of 3 and 11 $\mu\text{g/ml}$ and protected 69 to 74% of the mice, respectively. Increasing the dosage to 300 mg/kg yielded peak blood levels of 32 $\mu\text{g/ml}$ and protected 84% of the mice. Although the lowest dosage tested, 37.5 mg/kg, gave a peak blood level of only 0.44 $\mu\text{g/ml}$, it protected 45% of the mice. This result is not surprising since the MIC for the test organism was only 0.015 $\mu\text{g/ml}$.

Chloramphenicol. Intraperitoneal injections of 75, 150, or 300 mg of chloramphenicol per kg were given as described for clindamycin. The 150 mg/kg dosage gave peak blood levels of 25 $\mu\text{g/ml}$, which were comparable to blood levels of 22 $\mu\text{g/ml}$ usually achieved in humans (6).

Table 1. Susceptibilities of various strains of C. perfringens and B. fragilis (VPI 9032) to diverse antimicrobial drugs

Organism and strain	MIC ($\mu\text{g/ml}$)				
	Clindamycin	Chloramphenicol	Cephalothin	Tetracycline	Penicillin G
<u>C. perfringens</u>					
ATCC 13124	0.015				
SAL 249 ^a		4-8			
VPI 8050A			0.25	0.125-0.25	0.125
SAL 19					
<u>B. fragilis</u> VPI 9032	0.015	4	64	0.5	32

^aSAL strains were obtained from F. Tally, Tufts Medical Center, Boston, Mass.

Table 2. Concentrations of various antimicrobial agents in whole blood of normal mice after administration of doses used in treating infected mice

Drug administered	Route ^a	Concn. (mg/kg)	μg of drug/ml of whole blood \pm SD ^b							
			0.5	1	2	3	4.5	6	8	
Clindamycin	i.p.	37.5	0.44 \pm 0.17	0.40 \pm 0.16	0.16 \pm 0.08	0.13 \pm 0.1	< 0.1	< 0.1	< 0.1	
		75.0	2.9 \pm 1.1	0.8 \pm 0.2	0.2 \pm 0.07	0.1 \pm 0.03	< 0.1	0.15 \pm 0.02	< 0.1	
		150.0	11 \pm 7	3.0 \pm 0.5	0.6 \pm 0.03	0.5 \pm 0.3	< 0.1	0.16 \pm 0.1	< 0.1	
		300.0	32 \pm 6	11 \pm 3	2.9 \pm 0.9	2.7 \pm 1.0	2 \pm 1	0.5 \pm 0.4	0.3 \pm 0.3	
Chloramphenicol	i.p.	75.0	12 \pm 6	9 \pm 6	4 \pm 2	3 \pm 1	ND ^d	ND	ND	
		150.0	25 \pm 8	18 \pm 8	14 \pm 12	5 \pm 3	< 3	ND	ND	
		300.0	48 \pm 34	34 \pm 24	15 \pm 11	7 \pm 4	< 3	< 3	< 3	
		500.0	114 \pm 40	44 \pm 18	1.3 \pm 0.25	0.3 \pm 0.09	0.2 \pm 0.05	0.4 \pm 0.2	ND	
Penicillin G	i.p.	1,000.0	228 \pm 64	543 \pm 104	10 \pm 4	0.5 \pm 0.1	0.3 \pm 0.1	0.3 \pm 0.2	0.6 \pm 0.8	
		1,000.0	170 \pm 73	70 \pm 42	18 \pm 16	6 \pm 4	4 \pm 3	< 3	ND	
Cephalothin	i.p.	62.5	34 \pm 28	35 \pm 26	9 \pm 9	6 \pm 5	1.3 \pm 0.9	0.7 \pm 0.5	0.5 \pm 0.2	
		125.0	90 \pm 41	92 \pm 20	41 \pm 13	55 \pm 20	28 \pm 11	28 \pm 13	43 \pm 26	
Tetracycline	o.p.	125.0	1.6 \pm 1.1	1.8 \pm 0.6	0.9 \pm 0.7	0.5 \pm 0.3	< 0.3	ND	ND	
		250.0	2.1 \pm 0.6	2.3 \pm 1.1	1.0 \pm 0.5	0.7 \pm 0.3	0.3 \pm 0.05	< 0.2	1.2 \pm 0.9	
		500.0	3.3 \pm 1.7	6.6 \pm 0.8	4.1 \pm 2.3	3.3 \pm 2.2	1.0 \pm 0.5	1.2 \pm 0.9	0.3 \pm 0.07	

^a i.p., Intraperitoneal; o.p., oral gavage.

^b SD, Standard deviation

^c Time in hours after drug administration

^d ND, None detected

Table 3. Correlation of protection achieved by administration of various antimicrobial agents with peak concentrations of the agents in whole blood

	Route ^a	Concentration (mg/kg)	% of mice protected		μg of drug/ml of whole blood + SDb (peak blood level)
			Day 7	Day 10	
Clindamycin	i.p.	37.5	45	45	0.4 ± 0.2
		75.0	69	69	3.0 ± 1.0
		150.0	74	74	11.0 ± 7.0
Chloramphenicol	i.p.	300.0	84	84	32.0 ± 6.0
		75.0	33	17	12.0 ± 6.0
		150.0	43	23	25.0 ± 8.0
Penicillin G	i.p.	300.0	51	36	48.0 ± 34.0
		500.0	14	16	114.0 ± 40.0
		1,000.0	37	18	543.0 ± 104.0
Cephalothin	i.p.	1,000.0	26	24	170.0 ± 73.0
Tetracycline	i.p.	62.5	45	45	34.0 ± 28.0
		125.0	55	55	1.8 ± 0.6
		250.0	64	64	2.3 ± 1.1
	o.p.	500.0	77	77	6.6 ± 0.8

^{a,b} See Table 2.

The test organism had an MIC of 4 $\mu\text{g/ml}$. This dosage protected 43% of the mice that were examined on the 7th day after bacterial injection. By the 10th day, however, the number of mice protected had dropped to 23%. Similar results showing an increase in the number of visible abscesses after cessation of therapy were also observed with dosages of 75 and 300 mg/kg (Table 3).

Penicillin. High dosages of penicillin have been reported by some workers to be effective against B. fragilis infections (E. J. Benner, Prog. Abstr. Intersci. Conf. Antimicrob. Agents Chemother., 17th, San Francisco, Calif., Abstr. 396, 1974). We tested the efficacy of very high dosages of penicillin by injecting 500 and 1,000 mg/kg i.p. every 8 h for 5 days. These doses corresponded to 797,500 and 1,595,000 U/kg. The MIC for the test organism was 32 $\mu\text{g/ml}$. At 500 mg/kg, 84 to 86% of the mice developed abscesses (Table 3), which was not significantly different from an 88% incidence of infection in the respective control group. The 1,000 mg/kg dosage conferred limited protection through the 7th day after bacterial injection; however, by the 10th day, 82% of these mice had developed abscesses (Table 3); this finding also was not significantly different from that in the control group.

Cephalothin. Cephalothin had an MIC of 64 $\mu\text{g/ml}$ for the test organism and was tested in vivo at a very high dosage of 1,000 mg/kg i.p. every 8 h for 5 days. This antibiotic conferred only limited protection, protecting approximately one-fourth of the mice from infection (Table 3). This protection, unlike that of penicillin G, remained consistent, without the increase in the incidence of abscesses that occurred from the 7th to the 10th day with penicillin.

Tetracycline. We attempted to inject this antibiotic i.p. as we had with the previous antibiotics. Because of the toxicity of the larger dosages, however, we also gave the drug by oral gavage. The mice injected i.p. received either 62.5 or 125 mg/kg every 8 h for 5 days, and the mice treated by oral gavage received 125, 250, or 500 mg/kg. As expected, the dosages given i.p. produced much higher blood levels due to greater absorption of the drug (Table 3). The 125 mg/kg i.p. dosage killed all of the mice by the 6th day after bacterial injection. The 62.5 mg/kg i.p. dosage caused the mice some discomfort but did not result in death. These mice recovered rapidly after cessation of drug therapy on the 5th day. This dosage protected 45 to 50% of the mice. Of the oral injection, only the 500 mg/kg dosage produced any notable toxicity. These mice, like those that received 62.5 mg/kg i.p., recovered as soon as treatment was terminated. Even though the blood levels achieved with the oral dosages were much lower than those found with the 62.5 mg/kg i.p. dosage, all three of the antibiotic concentrations given by oral injection conferred a higher degree of protection than did the 62.5 mg/kg i.p. dosage. The degree of protection in the oral treatment groups ranged from 55% for the 125 mg/kg dosage to 77% for the 500 mg/kg dosage.

DISCUSSION

In the series of experiments reported here, clindamycin had the highest efficacy of the antimicrobial agents tested. The efficacy observed may be correlated, in part, with the retention of the anti-

biotic in the blood in concentrations exceeding the MIC of the test organism. We found that all four dosages of clindamycin tested were sufficient to maintain blood levels well above the MIC for most strains of B. fragilis for at least 8 h. Our results showed, however, that the larger dosage protected more mice from infection. This increase in protection cannot be explained entirely by increased blood levels since the lowest dosage (37.5 mg/kg) tested gave blood levels seven to eight times higher than the MIC as late as 8 h after administration of the drug. A twofold increase in the dosage to 75 mg/kg gave a dramatic increase in the number of mice protected. The only significant difference in the blood levels produced by these two dosages, however, occurred within 1 to 2 h after injection of the drug. One possible explanation of this observation is that higher blood levels during the 1st or 2nd hour may influence the quantity of the antibiotic that enters the abscess.

For many years tetracycline was the drug of choice for the treatment of anaerobic bacterial infections. Lately its use has decreased because of an increase in the number of anaerobes found to be resistant to it, in particular clinical isolates of B. fragilis (7). The B. fragilis strain used in these experiments was susceptible to tetracycline. The dosages of tetracycline sufficient to give peak blood levels equivalent to those usually obtained in humans protected the mice from infection almost as well as did clindamycin. On a weight-to-weight basis, however, higher dosages were required for tetracycline than for clindamycin. This finding resulted from the need to use the oral route of administration for tetracycline and

from the limited absorption of this drug from the gastrointestinal tract (9). The differences in the amount of protection conferred by oral dosages of tetracycline correlated with the length of time that blood levels of the antibiotic were kept above the MIC. The highest oral dosage tested, 500 mg/kg, was sufficient to maintain blood levels above the MIC for up to 7 h. The lowest oral dosage, 125 mg/kg, only gave blood levels above or equal to the MIC for 4 h. The failure of the i.p. dosage of 67.5 mg/kg to give protection comparable to that of the oral dosages cannot be explained by failure to maintain adequate blood levels. This dosage gave a peak blood level of 34 $\mu\text{g/ml}$ within the first 30 min and the blood level was still equal to or slightly above the MIC of the drug for the test organism 8 h later. Although toxicity was noted in these animals, it was not grossly different from the toxicity observed in the group that was given 500 mg/kg orally. It is possible that differences existed that were not noted.

Most strains of B. fragilis are susceptible to chloramphenicol in vitro. The strain that we used had an MIC of 4 $\mu\text{g/ml}$, which is in the range found for most strains of B. fragilis (15). Treatment with this antibiotic, even at levels that gave peak blood levels approximately double that seen in humans, was not very effective against the infection. This result may have been due to the fact that even the highest dosage that we tested gave blood levels that were just equal to or slightly less than the MIC within 3 to 4 h after administration of the drug. The MIC for most anaerobes is only slightly below achievable blood levels, and the antibiotic level is difficult to

maintain above the MIC. In addition, the concentration achieved in the abscess may be less than the blood level. The bacteriostatic nature of this antibiotic also appeared to be reflected in the results with all three concentrations tested. Approximately 15 to 20% of the abscesses were not grossly evident during the treatment period or for up to 2 days after cessation of therapy. Previously undetected abscesses were observed as early as 3 days after termination of therapy and rapidly increased in size, so that no differences existed between the sizes of the abscesses present in the control group and in the treatment groups by the 10th day after the bacterial injection. It appeared that chloramphenicol, although present, was somewhat bacteriostatic for the test organisms; but, once therapy was terminated, the organisms were able to continue normal growth.

Penicillin G and cephalothin are both β -lactam antibiotics to which strains of B. fragilis are relatively resistant in vitro. Although both these antibiotics were tested at very high dosages, little efficacy was found with either. This agrees with our earlier finding that mixed infections containing B. fragilis could not be treated successfully with penicillin or cephalothin (3, 4). Whether this resistance of B. fragilis is due to production of β -lactamase or to the rapid clearance from the blood is not known. Several investigators have reported the presence of β -lactamase in B. fragilis; however, the levels of the enzyme detected were very low (1, 8, 10). Both these antibiotics initially gave relatively high peak blood levels; however, by the end of 1 to 2 h, blood levels of each had dropped well below the MICs of penicillin G and cephalothin. Although we initially found

a higher incidence of protection with the 1,000 mg/kg dosage of penicillin during the period of therapy, there was no significant difference between the 1,000 mg/kg dosage and the 500 mg/kg dosage of penicillin a few days after termination of therapy. In this instance, it appears that the high dosage of penicillin did have a very limited effect on the infection as long as therapy was maintained.

Although caution must be exercised in the extrapolation of data obtained from experimental animals to humans, the results obtained from these experiments reflected very well the clinical findings with these antibiotics against infection in humans with B. fragilis. This model may be useful, therefore, for the evaluation of new antimicrobial agents by providing a means for the rapid and economical determination of their efficacies. The model infection provides a method for the testing of antibiotics against B. fragilis that may be more relevant to the clinical use of the drugs than the routine testing of in vivo susceptibility.

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PART III

Relative Efficacies of Various Antimicrobial Agents Against An Experimental Bacteroides fragilis Infection in Mice

SUMMARY

The efficacies of antimicrobial agents were determined for a Bacteroides fragilis infection in mice. Dosages were based on the concentration of antibiotic required to give peak blood levels in mice similar to those recommended for man. Chemotherapy was begun 4 h after bacterial injection and was given every 8 h thereafter for 5 days. Blood levels were determined over an 8 h period for each concentration of antibiotic tested. Minocycline, doxycycline, and tetracycline were each tested against a tetracycline resistant strain and a tetracycline susceptible strain of B. fragilis. Other antibiotics tested were metronidazole, cefoxitin, carbenicillin, and rosamicin, a new macrolide antibiotic. Erythromycin was tested along with rosamicin for comparison purposes. Minocycline was more effective than either doxycycline or tetracycline regardless of which strain of B. fragilis was used. Metronidazole was the most effective antibiotic tested, protecting up to 100% of the mice from infection. Rosamicin protected approximately 50% while erythromycin protected less than 30%. Cefoxitin and carbenicillin were equally effective; each protected about 40% of the mice.

INTRODUCTION

Bacteroides fragilis, a gram negative, nonsporing anaerobic bacillus, is the causative agent of a number of serious infections in the

human body. This organism is the anaerobe most commonly isolated from human clinical specimens and accounts for approximately 20-25% of all anaerobic bacteria from such specimens. Unfortunately, besides being the most common anaerobe found in clinical specimens, B. fragilis is also the most resistant to antimicrobial agents. This resistance to many of the common antibiotics currently in use and the undesirable side effects associated with several of these antibiotics, creates a demand for new drugs that are effective against B. fragilis.

There has been a need for a method that provides a means of evaluating new or experimental drugs against this organism that falls between in vitro testing and clinical trials in humans. We have previously described an experimental infection in which a localized abscess forms following injection of a pure culture of B. fragilis into mice (17). In a recent paper, the relative efficacies of five commonly used antibiotics were determined in this model (18). The results of that study agreed closely with clinical usage of the drugs. In this paper, we have examined the effectiveness of several relatively new or experimental antibiotics against this B. fragilis infection. The drugs tested were: metronidazole, rosamicin, cefoxitin, carbenicillin, doxycycline, and minocycline. Tetracycline and erythromycin were also tested for comparison purposes.

MATERIALS AND METHODS

Source of organisms. Both of the Bacteroides fragilis strains, VPI 9032 and VPI 8708, used in this series of experiments were originally isolated from human clinical specimens. To increase virulence,

strain VPI 8708 was animal passaged once. On reisolation, a culture in chopped meat broth (5) was lyophilized in a number of ampoules. A culture of VPI 9032 was also lyophilized prior to the series of experiments reported here.

Bacterial inoculum. One ampoule of either VPI 9032 or VPI 8708 was subcultured for each experiment in chopped meat broth. The culture was grown overnight and 0.1 ml was inoculated under 100% CO₂ into 10 ml volumes of chopped meat carbohydrate semisolid medium (17). The chopped meat carbohydrate semisolid culture was incubated 18 to 20 h and then injected into mice.

Injection of bacteria. One-half ml of the chopped meat carbohydrate semisolid culture was injected subcutaneously under the loose skin of the groin as previously described (17).

Anaerobic techniques and media. The media for the cultivation of *B. fragilis* were prepared pre-reduced (5) and all culture manipulations were performed as described in the VPI Anaerobe Laboratory Manual (5).

Source of antibiotics. The antibiotics used and the sources were: erythromycin and tetracycline HCl, Sigma Chemical Co., St. Louis, Mo.; metronidazole, Searle Laboratories, Chicago, Ill.; rosamicin and rosamicin phosphate, Schering Corp., Bloomfield, N. J.; cefoxitin, Merck Institute, Rahway, N.J.; minocycline, Lederle Laboratories, Pearl River, N.Y.; carbenicillin and doxycycline, Pfizer, Inc., New York, N.Y.

Injection of antibiotics. All antibiotic solutions were made fresh prior to each treatment time and were given in 0.1 ml volumes. Tetracycline, minocycline, doxycycline, carbenicillin, and cefoxitin were dissolved in sterile distilled water. Metronidazole was finely

ground with a mortar and pestle and suspended in propylene glycol (Sigma Chemical Co., St. Louis, Mo.). Rosamicin, rosamicin phosphate, and erythromycin were dissolved in 1 part 95% ethanol and brought to the desired volume with 7-8 parts of propylene glycol. Carbenicillin, cefoxitin, and rosamicin phosphate were injected intraperitoneally. All other antibiotics were given by oral gavage. Two groups containing 25-35 mice each were tested with each concentration of antibiotic. For each experiment, a minimum of 25 mice were used as a control group. The mice received the bacterial injection prior to being randomly selected for treatment or as controls; the latter received 0.1 ml of the solvent in place of the antibiotic solution.

Schedule of injections. Antibiotic therapy was begun 4 h following the bacterial injection and then given every 8 h thereafter for a period of 5 days.

Determination of minimal inhibitory concentration (MIC). MICs were determined by an agar dilution procedure (20) with the medium of Wilkins and Chalgren (21).

Determination of antibiotic blood levels. The amount of antibiotic present in the blood was determined as previously described (18). For each concentration of antibiotic tested, blood levels were determined at 0.5, 1, 2, 3, 4.5, 6, and 8 h following the last injection of the antibiotic. Blood level concentrations were determined in a minimum of two separate groups of five mice each. The strains of Clostridium perfringens used as assay organisms for the different antibiotics are given in Table 1.

Table 1. Susceptibilities of assay strains of *C. perfringens* and test strains of *B. fragilis* to 8 antibiotics

Organism and Strain	Minimal Inhibitory Concentration (mcg/ml)							
	Tetracycline	Minocycline	Doxycycline	Carbenicillin	Cefoxitin	Metronidazole	Rosamicin	Erythromycin
<i>C. perfringens</i>								
SAL* 19	0.06	0.03	0.03	0.125 - 0.25	--	--	0.06	0.5
VPI 8050A	--	--	--	--	0.25	0.5	--	--
<i>B. fragilis</i>								
VPI 9032	0.5	0.03	0.06	16	4	1	0.5	1
VPI 8708	16 - 32	4	4	128	4	1	0.5	1

*SAL strain obtained from Dr. F. Tally, Tufts Medical Center.

Mice. Swiss white male mice, ICR strain, 18-20 g, (Flow Laboratories, Dublin, Va.) were used for all experiments. The mice were caged in groups of 25-30 with a light-dark cycle of 12 h, and allowed to stabilize for at least 48 h prior to being used in any experiments.

Determination of efficacy. The mice were examined daily beginning on the fourth day following bacterial injection and continuing through the 14th day for the presence of a localized subcutaneous abscess. Maximum abscess formation was found to occur by the seventh to the tenth day (17). The mice were not kept past the 14th day.

RESULTS

Minimal inhibitory concentrations. The MICs for the antibiotics tested against the two B. fragilis strains are given in Table 1. B. fragilis VPI 9032 was considered relatively susceptible to all eight antibiotics. The other test strain, VPI 8708, was considered to be susceptible to cefoxitin, metronidazole, rosamicin, and erythromycin; intermediate to minocycline and doxycycline; and relatively resistant to tetracycline and carbenicillin in vitro.

Blood levels. The antibiotic concentrations in the blood are given in Table 2 for an 8 h period following the final injection of the drug.

Controls. The incidence of infection with each of the B. fragilis test strains, VPI 8708 and VPI 9032, was approximately 88%.

Metronidazole. Metronidazole was originally tested against VPI 9032 at 31.25, 62.5, 125, and 250 mg/kg. The drug was given by oral gavage every 8 h for 5 days. Table 3 gives the relative efficacy and the peak blood level obtained with each concentration. Recommended

Table 2. Concentrations of various antimicrobial agents in whole blood in mice following administration of doses used in treating a *Bacteroides fragilis* infection

Drug Administered	Mg/Kg Route ^a	Mcg drug per ml whole blood + S.D. ^b (Hour after drug administration)							
		0.5	1	2	3	4.5	6	8	
Metronidazole	250 (po)	63 ± 13	73 ± 23	55 ± 14	39 ± 10	28 ± 5	22 ± 7	15 ± 7	
	125 (po)	34 ± 11	34 ± 15	22 ± 6	13 ± 3	10 ± 5	10 ± 4	5 ± 3	
	62.5 (po)	26 ± 8	21 ± 9	9 ± 7	10 ± 6	6 ± 4	9 ± 6	8 ± 6	
Cefoxitin	31.25 (po)	17 ± 10	11 ± 5	9 ± 5	6 ± 3	1 ± 3	5 ± 4	1 ± 3	
	500 (ip)	110 ± 56	45 ± 17	19 ± 8	7 ± 2	11 ± 4	6 ± 2	6 ± 3	
Carbenicillin	1000 (ip)	280 ± 27	150 ± 28	32 ± 8	11 ± 6	9 ± 3	6 ± 1	< 6	
Tetracycline	500 (po)	12.5 ± 5.7	8.8 ± 3.1	6.1 ± 2.2	3.7 ± 1.2	2.3 ± 0.8	3.1 ± 1.4	2.3 ± 0.4	
	250 (po)	6.2 ± 2.8	5.5 ± 1.3	5.4 ± 1.7	2.3 ± 0.6	~ 1.2	2.1 ± 0.8	~ 1.2	
Minocycline	300 (po)	6.6 ± 4.4	7.4 ± 4.7	6.6 ± 4.3	4.8 ± 4.4	4.4 ± 4.3	3.0 ± 2.9	2.8 ± 2.0	
	150 (po)	6.4 ± 1.2	6.2 ± 1.4	4.5 ± 1.3	2.2 ± 1.4	1.7 ± 1.3	1.7 ± 1.1	1.0 ± 0.5	
	75 (po)	5.4 ± 1.0	4.5 ± 1.4	2.5 ± 1.0	1.4 ± 0.8	1.0 ± 0.4	0.4 ± 0.1	0.5 ± 0.4	
	37.5 (po)	1.1 ± 0.4	0.7 ± 0.5	0.8 ± 0.4	0.4 ± 0.1	0.1 ± 0.02	< 0.1	< 0.1	
Doxycycline	19 (po)	0.6 ± 0.2	0.5 ± 0.1	0.5 ± 0.3	0.4 ± 0.1	0.1 ± 0.02	< 0.1	< 0.1	
	150 (po)	7.4 ± 2.2	8.6 ± 3.4	6.1 ± 2.6	2.8 ± 1.5	4.8 ± 1.9	4.1 ± 1.3	1.9 ± 0.9	
Rosamicin base	75 (po)	5.6 ± 1.4	4.4 ± 1.2	2.8 ± 0.4	1.4 ± 0.3	1.4 ± 0.3	0.7 ± 0.3	0.9 ± 0.3	
	250 (po)	2.5 ± 0.6	4.2 ± 2.3	4.9 ± 3.2	3.6 ± 0.5	3.9 ± 0.5	2.4 ± 1.0	N.D. ^c	
Rosamicin-P0 ₄	150 (po)	0.8 ± 0.7	0.7 ± 0.6	1.4 ± 0.6	2.0 ± 1.0	1.1 ± 0.2	0.8 ± 0.6	0.7 ± 0.5	
	100 (po)	0.2 ± 0.06	0.2 ± 0.05	0.3 ± 0.2	0.3 ± 0.2	0.2 ± 0.1	0.2 ± 0.1	< 0.1	
Erythromycin	250 (po)	2.0 ± 1.2	1.5 ± 0.6	1.4 ± 0.7	1.4 ± 0.9	1.1 ± 0.6	1.2 ± 0.7	~ 0.4	
	150 (po)	0.5	0.6 ± 0.3	0.7 ± 0.3	0.6 ± 0.2	~ 0.5	~ 0.5	< 0.5	
	75 (ip)	13.3 ± 9.0	9.2 ± 4.4	7.4 ± 4.7	8.9 ± 6.4	5.5 ± 3.0	6.6 ± 5.0	6.5 ± 2.5	
Erythromycin	37.5 (ip)	5.1 ± 4.4	3.0 ± 2.4	2.6 ± 2.0	3.5 ± 2.6	2.8 ± 1.9	2.0 ± 1.2	2.4 ± 0.9	
	250 (po)	7.5 ± 3.7	8.0 ± 4.4	5.1 ± 3.5	~ 2.4	~ 2.4	0.4 ± 0.3	0.4 ± 0.2	

^aip, intraperitoneal; po, per osium

^bS.D., standard deviation

^cN.D., none detected

Table 3. Correlation of protection achieved by administration of various antimicrobial agents with peak concentrations of the agents in whole mouse blood

Compound	Drug administered		% of mice protected		Mcg drug/ml of whole blood + S.D. ^a (peak blood level)
	Route	Mg/Kg	Day 7	Day 10	
Carbenicillin	i.p. ^b	1000	32	39	280 ± 30
Cefoxitin	i.p.	500	38	39	110 ± 55
Metronidazole	p.o.	250	80	82	73 ± 23
		125	93	96	34 ± 15
		62.5	100	100	26 ± 8
		31.25	100	100	17 ± 10
Rosamicin base	p.o.	150	52	48	2.0 ± 1.0
		100	51	52	0.3 ± 0.2
Rosamicin phosphate	p.o.	250	64	70	2.0 ± 1.2
		150	45	46	0.7 ± 0.3
		100	44	52	N.D. ^c
Erythromycin	p.o.	250	28	28	8.0 ± 4.4
		150	21	21	N.D.
		100	26	30	N.D.

^aS.D., Standard deviation

^bi.p., Intraperitoneal; p.o., per osium

^cN.D., None detected, blood levels below limits of the assay.

peak blood levels for this drug in the treatment of anaerobic infections in man have not been established. For our purposes, we considered a peak blood level of 25 to 40 mcg/ml to be desirable (16). To obtain these levels in mice, it was necessary to give from 62.5 to 125 mg/kg. These dosages gave peak blood levels of 26 and 34 mcg/ml respectively and protected 90 to 100% of the mice. We found that a lower dosage of 31.25 mg/kg, which gave a peak blood level of 17 mcg/ml, was equally as effective in conferring protection as were the two previous concentrations. The highest concentration tested, 250 mg/kg, gave a peak blood level of approximately 70 mcg/ml but had less efficacy than the previous concentrations. These concentrations were also tested against B. fragilis strain, VPI 8708, with identical results.

Carbenicillin. We had previously tested penicillin at a high dosage of 1000 mg/kg (1,595,000 U/kg) against B. fragilis VPI 9032 and found that the drug was not effective (18). For comparison purposes, carbenicillin was tested at this same concentration against the same strain. The MIC of carbenicillin against this strain was 16 mcg/ml. The drug, however, protected only 30-40% of the mice from infection even though a peak blood level of 280 mcg/ml was achieved (Table 3).

Cefoxitin. Cefoxitin had an MIC of 4 mcg/ml for each of the test organisms. This antibiotic was tested at 500 mg/kg against VPI 9032. This dosage produced a peak blood level of 110 mcg/ml which is comparable to that recommended in human therapy (6). The drug protected 38-39% of the mice (Table 3).

Tetracycline, minocycline, and doxycycline. Each of these three drugs were tested against a tetracycline susceptible strain of B.

fragilis, VPI 9032, which had an MIC of 0.5 mcg/ml; and against a tetracycline resistant strain, VPI 8708, with an MIC of 16 mcg/ml. The MIC of minocycline and doxycycline for VPI 8708 was 4 mcg/ml for each drug. For the tetracycline susceptible strain, the MICs were \leq .03 and 0.06 mcg/ml for minocycline and doxycycline respectively. In this series of experiments, a dosage of 250 mg/kg of tetracycline resulted in a peak blood level of approximately 6 mcg/ml which correlated with that recommended for humans (7). A 75 mg/kg dosage for both minocycline and doxycycline gave peak blood levels similar to the 250 mg/kg tetracycline dosage. Minocycline was the most effective of the three drugs against both the tetracycline resistant and susceptible strains (Table 4). Minocycline at 75 mg/kg protected 96% of the mice infected with the susceptible strain and 54% of those infected with the resistant B. fragilis. Correspondingly, tetracycline (250 mg/kg) protected 64% and 35% and doxycycline (75 mg/kg) protected 57% and 44% respectively. Doubling of the tetracycline dosage to 500 mg/kg increased the protection conferred against both test strains, but some toxicity resulted at this level. Doubling the dosage of doxycycline to 150 mg/kg did not result in any apparent toxicity but the efficacy was not greatly increased. In these experiments, minocycline dosages of 150 and 300 mg/kg not only failed to increase the efficacy but were less effective than the 75 mg/kg dosage in protecting the mice from infection.

Rosamicin and erythromycin. Rosamicin, a new macrolide antibiotic, was tested in this model both as the base and the phosphate form. For comparison purposes, erythromycin base was also tested.

Table 4. Correlation of protection achieved with tetracycline, minocycline, or doxycycline against tetracycline resistant and susceptible *B. fragilis* strains with peak concentrations of these drugs in whole mouse blood

Drug administered Compound	Mg/Kg	% of mice protected		mcg drug/ml of whole blood + S.D. ^a (peak blood level)
		Tetracycline susceptible strain (9032)	Tetracycline resistant strain (8708)	
Tetracycline	500	77	51	12.4 ± 5.7
	250	64	35	6.2 ± 2.8
Minocycline	300	80	-- ^b	7.4 ± 4.7
	150	86	40	6.4 ± 1.2
	75	96	54	5.4 ± 1.0
	37.5	86	48	1.1 ± 0.4
Doxycycline	19	84	--	0.6 ± 0.2
	150	66	48	10.6 ± 2.4
	75	57	44	5.6 ± 1.4

^aS.D., Standard deviation

^bNot tested

Initially we planned to give each drug orally at 250 mg/kg every 8 h for 5 days; but, rosamicin base and erythromycin were both toxic at these levels. With rosamicin, 18 out of 30 mice died within 5 days following the start of chemotherapy. On autopsy, the intestinal tracts of these mice were grossly distended by gas. Twelve of the 30 mice that received erythromycin also died, as did five of the 30 mice that received the phosphate form of rosamicin. These mice, however, did not have the gaseous distention.

The toxicity noted with the relatively high dosage of rosamicin, was avoided by giving lower dosages of 50, 100, and 150 mg/kg. Blood levels achieved with the 50 mg/kg dosage were below the detection limits of our assay (< 0.1 mcg/ml) but this concentration protected 31% of the mice from infection (Table 3). The 100 mg/kg dosage produced blood levels of approximately 0.2-0.3 mcg/ml and protected approximately 50% of the mice. The blood levels with 150 mg/kg were 3-4 fold higher than with the 100 mg/kg dosage (Table 2) but the efficacy was no greater (Table 3). No gross pathological changes were seen with these 3 concentrations either while the mice were receiving the drug or on autopsy following the therapy period.

Rosamicin phosphate given orally (150 and 250 mg/kg) gave lower blood levels (Table 2) than the base; and, blood levels achieved with the 100 mg/kg dosage were below 0.1 mcg/ml. The relative efficacies of the 100 and 150 mg/kg concentrations were approximately the same as those obtained with the same concentrations of rosamicin base (Table 3). A dosage of 250 mg/kg of the phosphate form increased the efficacy 20-30% over that of the 150 mg/kg dosage. This dosage,

however, appeared to be relatively toxic since approximately 15% of the mice receiving this concentration died.

Since the phosphate derivative is a parenteral form of the drug, this form also was tested at 37.5, 75, and 150 mg/kg both by intraperitoneal and subcutaneous injection. Originally, treatment was scheduled every 8 h for 5 days; however, due to the adverse condition of the mice, therapy was stopped on the 3rd or 4th day. Blood levels were determined for each dosage (Table 3), but due to the number of mice that died in each of the treatment groups, efficacies could not be determined. Autopsy was performed immediately following death but no gross pathological changes were apparent. Death or other toxic side effects did not occur in the controls which received the ethanol-propylene glycol solvent for the entire 5 day period.

DISCUSSION

Metronidazole was found to confer the greatest protection of the antimicrobial agents tested. The high degree of efficacy observed may be related, at least in part, with the retention of this drug in the blood at concentrations that were above the MIC for the test organisms. We found the two dosages, (62.5 and 125 mg/kg) which produced peak blood levels of 26 and 34 mcg/ml respectively, to be sufficient to maintain blood levels well above the MIC for most strains of B. fragilis for a minimum of 8 h. The lowest dosage (31.25 mg/kg) tested also produced blood levels above or equal to the MIC for our two test strains. Although we found higher efficacies with the two lower dosages, the differences were relatively small. The highest dosage, 250 mg/kg, gave

less protection than the other dosages. We did not see any apparent symptoms of toxicity associated with this dosage; but, the growth of the mice was slower in comparison to the control groups and the lower dosage treatment groups. The elevated blood levels produced by the 250 mg/kg dosage in our experiments may indicate that the drug was not being metabolized as readily as were the lower dosages. The high levels of antibiotic may have lowered the natural resistance of the mice sufficiently to allow the infection to become established in some animals.

Cefoxitin is a β -lactam antibiotic which, due to the nature of its structure, is thought to have greater resistance to β -lactamase enzymes than do either penicillin G, cephalothin, or carbenicillin. We tested both cefoxitin and carbenicillin against a B. fragilis strain which would be considered susceptible to either by in vitro susceptibility testing. This strain had MICs of 4 and 16 mcg/ml to cefoxitin and carbenicillin respectively. At the concentrations tested, each drug protected approximately 40% of the mice. This was approximately a 15-20% increase in efficacy over that found for either penicillin G or cephalothin as determined against the same test strain (18). Although the protection achieved with either cefoxitin or carbenicillin was higher than that conferred by penicillin or cephalothin, neither of these two drugs could be considered particularly effective in this model. The low degree of protection conferred by carbenicillin was not surprising. Even though a very high blood level was achieved within 30 min after injection, the quantity of the drug in the blood dropped below the MIC for the test strain within 3 h. Cefoxitin is not

as easily explained. Based on obtainable blood levels and the MIC of the test organism, a higher degree of protection would normally be expected. Weinrich and DeI Bene (19), in a study with 10 clinical isolates of B. fragilis, found that an enzyme was present that hydrolyzed cefoxitin as well as other β -lactam antibiotics in vitro.

Darland and Birnbaum, however, in a survey of 79 strains of B. fragilis, reported that none of these strains hydrolyzed cefoxitin (3). In our study, we found biologically-active concentrations of cefoxitin to be present in the blood for 6 to 8 h above or equal to the in vitro MIC of the drug. However, cefoxitin only protected approximately 40% of the mice from infection. Since this antibiotic was present in our studies at concentrations that were effective against the organism in vitro, this may indicate that other mechanisms of resistance are involved rather than enzymatic hydrolysis of the antibiotic. One possibility may be that the minimal inhibitory concentrations for β -lactam antibiotics are different in vivo than in vitro. It may be that the organism is not as dependent on maintaining the integrity of the cell wall in an infection to the extent that it is in artificial media.

Approximately 60% of the current clinical isolates of B. fragilis are resistant to tetracycline (13). Because of this, increasing interest is being expressed in tetracycline analogues that have a greater activity against these tetracycline resistant organisms. Doxycycline and minocycline have both been shown to be more active than tetracycline in vitro against B. fragilis (2, 15). Minocycline has been reported to be more effective than either doxycycline or tetracycline in vivo against fusobacteria infections. Hill (4) tested minocycline against

a mixed fusobacteria infection in mice and found that this antibiotic was more effective than tetracycline both in the prevention of abscess formation and in the treatment of developed abscesses. In another study conducted by Kuck, minocycline was reported to be more effective than either doxycycline or tetracycline in the prevention of F. necrophorum infection in mice (8). In our study, when all three of these drugs were administered at dosages that produced peak blood levels of approximately 6 mcg/ml, minocycline was the most effective against B. fragilis. The most significant difference was observed with the tetracycline susceptible strains. All three drugs, at dosages which gave peak blood levels of around 6 mcg/ml, were found in the blood at concentrations exceeding the MICs for at least 8 h. Minocycline, however, protected 30% more of the mice than did tetracycline and 40% more than doxycycline. Minocycline was more effective against the tetracycline resistant strain; but, the differences observed were not nearly as great. Unlike the results reported by Kuck (8), we did not find that minocycline gave higher blood levels than equal dosages of doxycycline. Therefore, blood concentrations of these two drugs could not account for the higher efficacy of minocycline, particularly for the large difference seen with the tetracycline susceptible strain.

We noted that high dosages of minocycline resulted in a decrease in efficacy. We saw a similar effect with metronidazole in this regard. With minocycline, we did not observe any obvious symptoms of toxicity with these high dosages as we have previously reported for high dosages of tetracycline (18). It is quite probable, however,

that these higher dosages of minocycline were sufficient to compromise the host either by interfering with mammalian protein synthesis or by causing hepatic dysfunction.

Rosamicin, a new macrolide antibiotic, has been reported by several investigators to have greater activity in vitro than erythromycin against a number of anaerobic bacteria (12, 14). Sutter and Finegold (14) found that all 37 strains of B. fragilis they tested were inhibited by 4 mcg/ml or less of rosamicin. Sixty percent of these were found to be inhibited by 0.5 mcg or less per ml which was the MIC for the two test strains of B. fragilis reported here. In our experiments, 0.5 mcg/ml appeared to be close to the maximum concentration that could be safely achieved in the blood of mice without introducing toxic side-effects. Dosages of either rosamicin base or phosphate that gave blood levels of approximately 0.5 mcg/ml protected about 50% of the mice when the drug was administered orally.

Although we experienced toxicity that appeared to be dose related to the drug, other investigators have shown that rosamicin produced no toxic effects in either monkeys that received 200 mg/kg daily over a 3 month period or in human volunteers that were given 250 mg four times daily for 10 days (unpublished data, Schering Corp.). In vitro data have shown rosamicin to be more effective than erythromycin against a number of clinically important anaerobic bacteria (12, 14). Our in vivo data have also demonstrated a greater efficacy with rosamicin than with erythromycin against B. fragilis.

Of the antimicrobial agents that we tested, several produced results in vivo that could not have been predicted by in vitro determin-

ations. Carbenicillin and cefoxitin were not particularly effective in protecting the mice from infection even though the peak blood levels achieved were 15-20 times the MIC of each of these drugs. The MICs of minocycline and doxycycline are approximately the same for a number of bacteria (2, 13); and recommended blood levels in man are also similar (1, 9, 10). However, minocycline, in our study as well as that of other investigators (8, 11) has been shown to be more effective than doxycycline. Although data obtained with any drug in mice can obviously not be directly extrapolated to humans, the data obtained from such studies do provide needed information concerning the expected effect of a drug that cannot be obtained from in vitro susceptibility testing alone. Studies such as this will hopefully be of use in the evaluation of new or experimental antibiotics prior to clinical trials.

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PART IV

Concentrations of Antimicrobial Agents in Blood and Abscesses of Mice Infected with Bacteroides fragilis

SUMMARY

The concentrations of biologically active antibiotic within localized abscesses were compared with blood levels of the drug over an 8 h period. Clindamycin, metronidazole, penicillin G, rosamicin, tetracycline, doxycycline, and minocycline were tested in mice infected with Bacteroides fragilis. Subcutaneous abscesses, approximately 1 cm in diameter, were sampled at 6-7 days following the bacterial injection. The antibiotic was given as a single dose, either intraperitoneally or orally. Blood and abscess samples were collected at 1, 2, 4, 6, and 8 h after the drug was given. With all antibiotics, except clindamycin, concentrations in the abscesses were generally lower than the blood levels for the first 1-2 h. From 2-8 h after drug administration, abscess levels were equal or slightly higher than blood levels. Clindamycin differed from the other antibiotics in that it consistently gave a higher concentration in the abscesses than in the blood. Concentrations of clindamycin at 1-2 h were at least twice as high in the abscesses as in the blood and were 8-10 times blood level by the 4th h. Even after 8 h, concentrations in the abscesses were 4-8 times higher than in the blood.

INTRODUCTION

Quantitation of the amount of biologically active antibiotic present in vivo has generally been based on the quantity of the drug that is in the blood. There have been some reports in which concentrations

of antibiotics have been determined in tissue, bone, bone marrow, and interstitial fluid as well as blood or serum (1, 3, 4, 9, 16, 18). Unfortunately there is very little information available concerning antibiotic concentrations achieved within localized abscesses. Infections with anaerobic bacteria may result in the formation of localized abscesses that are not always amenable to surgical drainage, either due to location or the size of the abscesses. The relative penetration of antibiotics into such abscesses could be of importance in the therapy of such infections.

In the series of experiments reported here, we have utilized an experimental mouse infection, initiated with a pure culture of Bacteriodes fragilis which produces a relatively large (1 cm) localized subcutaneous abscess (19). We have determined the quantity of antibiotic present within these abscesses and in the blood for 8 h following the administration of each of 7 antibiotics. In most instances, the antibiotic was tested at a dosage that gave blood levels in mice similar to those normally encountered in man.

MATERIALS AND METHODS

Bacterial strains. B. fragilis VPI 9032 and VPI 8708 were originally isolated from human clinical specimens.

Bacterial inocula and injection. An ampoule containing a lyophilized culture of VPI 9032 or VPI 8708 was subcultured into chopped meat broth (5) prior to each experiment. The culture was grown overnight (18-20 h), and 0.1 ml was inoculated into 10 ml volumes of chopped meat carbohydrate semisolid medium (19). The chopped meat

carbohydrate semisolid cultures were incubated 18 to 20 h and then 0.5 ml volumes were injected subcutaneously in the groin of mice as previously described (19).

Anaerobic techniques. The media for the cultivation of B. fragilis were prepared prereduced (5) and all culture manipulations were performed as described in the VPI Anaerobe Laboratory Manual (5).

Antibiotics. The antibiotics used and their sources were: penicillin G, potassium salt (1595 U/mg), and tetracycline HCl, Sigma Chemical Co., St. Louis, Mo.; clindamycin phosphate and clindamycin HCl, The Upjohn Co., Kalamazoo, Mich.; metronidazole, G. D. Searle and Co., Chicago, Ill.; minocycline HCl, Lederle Laboratories, Pearl River, N.Y.; doxycycline hydrate, Pfizer, Inc., New York, N.Y.; and rosamicin, Schering Corp., Bloomfield, N.J.

Administration of antibiotics. All antibiotics, with the exception of metronidazole and rosamicin, were dissolved in sterile distilled water. Metronidazole was finely ground with mortar and pestle and suspended in propylene glycol (Sigma Chemical Co., St. Louis, Mo.). Rosamicin was dissolved in 95% ethanol and brought up to the desired volume with 7-8 parts of propylene glycol. Penicillin G and clindamycin were given intraperitoneally (i.p.). All other antibiotics were given by oral gavage (p.o.). All antibiotics were given in 0.1 ml volumes. Antibiotic solutions or suspensions were made fresh immediately prior to administration.

Determination of MICs. Minimal inhibitory concentrations (MICs) were determined by an agar dilution procedure (21) with Wilkins-Chalgren medium (22). Clindamycin-HCl was used for determining the MICs of clindamycin.

Determination of antibiotic concentrations in blood and pus.

Quantitation of the amount of biologically active antibiotic present in blood or pus was performed by the method described by Sabath and Toftegaard (13). The strains of Clostridium perfringens used as assay organisms for the different antibiotics are given in Table 1. Abscesses were allowed to develop for 6 to 10 days. A single dose of the antibiotic was given; blood and pus specimens were collected at 1, 2, 4, 6, and 8 h. For each concentration of antibiotic tested, concentrations in both blood and pus were determined in two separate groups of 5 mice each for each sampling time. Blood was collected by tail bleeding, the mouse was killed by cervical dislocation, the abscess was opened, and pus was collected. For each antibiotic concentration tested, except rosamicin, 10 μ l of blood or pus was placed on a 6.35 mm paper disk (Schleicher and Schuell, No. 740-E). Due to the sensitivity of the assay for rosamicin, 20 μ l samples were used rather than 10 μ l. The disks were placed on the assay medium (13), pre-incubated aerobically at room temperature for 30-60 min to facilitate diffusion of the antibiotic, and then incubated in an anaerobic chamber similar to the one described by Aranki, et al. (2). The assay plates were incubated at 37 C for 3 to 5 h until hemolysis of the blood had occurred. The assay medium was made 1 day prior to use and stored at 4 C. Quantitation of the amount of antibiotic present was determined by comparison with standard curves constructed by experimental regression with a computer. Clindamycin HCl was used to construct the standard curve for clindamycin. Whole sheep blood was used routinely for the standard curves. Preliminary trials indicated that no significant differences

Table 1. Susceptibilities of assay strains of *Clostridium perfringens* and test strains of Bacteroides to 7 antimicrobial agents.

Organism and strain	Minimal inhibitory concentration (mcg/ml)						
	Clindamycin	Penicillin G	Metronidazole	Rosamicin	Tetracycline	Doxycycline	Minocycline
<u>C. perfringens</u>							
ATCC 13124	0.015						
VPI 8050A			0.5				
SAL* 19		0.125		0.06	0.06	0.03	0.03
<u>B. fragilis</u>							
VPI 9032	0.015	32	1	0.5	0.5	0.06	0.03
VPI 8708	0.015	32	1	0.5	16-32	4	4

*SAL strain obtained from Dr. F. Tally, Tufts Medical Center

were detectible in the quantitation of the antibiotics regardless of whether measured in whole sheep blood, mouse blood, or pus from mouse abscesses. In some cases, pus without antibiotics did produce a small zone of unhemolyzed blood; however, the zone did not have a diameter exceeding the disk containing the sample. Therefore, quantitation was not affected.

Mice. Swiss white male mice, ICR strain, 18 to 20 g (Flow Laboratories, Dublin, Va.) were used for all experiments. The mice were caged in groups of 25 with a light-dark cycle of 12 h and were allowed to stabilize for 48-72 h before being used in any experiments.

RESULTS

Minimal inhibitory concentrations. The MICs of the antibiotics for the two B. fragilis strains are given in Table 1. B. fragilis VPI 9032 was considered to be relatively susceptible to all of the antibiotics with the exception of penicillin G. The other strain, VPI 8708, was considered susceptible to clindamycin, metronidazole, and rosamicin; intermediate to minocycline and doxycycline, and resistant to tetracycline and penicillin.

Clindamycin. Table 2 gives the concentrations of biologically active clindamycin present in blood and pus after a single intraperitoneal injection of 150 and 300 mg/kg doses. This was the only antibiotic tested that produced levels in the abscesses that were consistently higher than blood levels. Recommended peak blood levels for this drug in human therapy are generally considered to be within the range of 3 to 14 $\mu\text{g/ml}$ (10). The 150 mg/kg dose gave a peak blood

Table 2. Comparison of antibiotic concentrations in abscesses and in the blood of mice infected with *Bacteroides fragilis*

Antibiotic	Mg/Kg	Mcg drug per ml of abscess or blood \pm S.D.					
		1 ^a	2	4	6	8	
Clindamycin (i.p.) ^b	300	Abscess 12.3 \pm 3.3	5.6 \pm 3.2	2.2 \pm 1.2	1.6 \pm 0.5	1.7 \pm 0.2	
	150	Blood 5.5 \pm 2.0	0.6 \pm 0.2	0.2 \pm 0.02	0.2 \pm 0.04	0.2 \pm 0.1	
		Abscess 2.4 \pm 0.8	2.2 \pm 1.7	1.2 \pm 0.9	1.1 \pm 1.0	0.8 \pm 0.3	
Metronidazole (p.o.)	125	Blood 1.5 \pm 0.5	0.5 \pm 0.1	\sim 0.2	< 0.2	< 0.2	
		Abscess 17 \pm 10	10 \pm 2	6 \pm 1	7.6 \pm 1.8	7.0 \pm 2.0	
	62.5	Blood 22 \pm 8	19 \pm 13	\sim 5	8.0 \pm 2.7	7.4 \pm 2.0	
Abscess 8.5 \pm 0.6		6.0 \pm 1.2	6.0 \pm 1.1	5.3 \pm 0.4	5.3 \pm 0.5		
31.25	9 \pm 2	Blood 9 \pm 2	6.0 \pm 1.2	< 5	< 5	< 5	
		Abscess 2.4 \pm 0.8	2.4 \pm 0.5	2.4 \pm 0.6	3.4 \pm 1.1	< 1.7	
	Blood \sim 3.2	\sim 1.7	\sim 1.7	\sim 1.7	< 1.7		
Penicillin G (i.p.)	1000	Abscess 100 \pm 60	72 \pm 40	4 \pm 2	2.2 \pm 2.0	1.6 \pm 0.8	
	500	Blood 242 \pm 55	31 \pm 16	3.0 \pm 0.9	2.3 \pm 0.2	1.4 \pm 0.3	
		Abscess 48 \pm 23	10 \pm 8	4.0 \pm 2.1	0.2 \pm 0.1	< 0.1	
	250	Blood 110 \pm 60	22 \pm 11	1.9 \pm 1.1	< 0.1	< 0.1	
150	Abscess 16 \pm 10	Blood 9 \pm 4	3 \pm 2	0.1 \pm 0.04	< 0.1		
		Blood 25 \pm 4	5 \pm 3	2.0 \pm 1.9	< 0.1	< 0.1	
Rosamicin (p.o.)	150	Abscess 1.0 \pm 0.4	2.2 \pm 0.5	1.9 \pm 0.4	1.8 \pm 0.5	2.1 \pm 0.6	
	Blood 0.9 \pm 0.3	0.9 \pm 0.2	0.9 \pm 0.2	1.3 \pm 0.5	1.0 \pm 0.5		
Tetracycline (p.o.)	500	Abscess 5.4 \pm 4.0	6.8 \pm 3.9	4.2 \pm 1.1	5.8 \pm 3.7	1.5 \pm 0.5	
	250	Blood 10.9 \pm 4.0	3.4 \pm 1.1	2.4 \pm 0.7	1.3 \pm 0.8	\sim 0.6	
		Abscess 2.8 \pm 0.7	2.3 \pm 0.7	2.2 \pm 0.2	1.7 \pm 0.5	1.7 \pm 0.2	
150	Blood 6.9 \pm 3.1	2.4 \pm 0.5	1.7 \pm 1.0	\sim 1.2	1.4 \pm 0.4		
Doxycycline (p.o.)	150	Abscess 1.2 \pm 0.4	2.2 \pm 0.4	3.4 \pm 1.5	2.0 \pm 0.6	2.1 \pm 0.5	
	75	Blood 3.6 \pm 1.2	4.0 \pm 0.9	3.9 \pm 1.1	2.3 \pm 0.7	2.0 \pm 0.9	
		Abscess 1.0 \pm 0.4	1.3 \pm 0.6	2.2 \pm 0.7	2.4 \pm 0.9	1.3 \pm 0.4	
150	Blood 3.3 \pm 0.4	3.5 \pm 1.6	2.1 \pm 0.6	1.6 \pm 0.1	1.2 \pm 0.4		
Minocycline (p.o.)	150	Abscess 0.6 \pm 0.3	1.9 \pm 1.0	1.5 \pm 1.1	1.7 \pm 0.2	0.8 \pm 0.3	
	75	Blood 4.8 \pm 3.2	4.3 \pm 2.4	1.5 \pm 0.6	2.3 \pm 2.1	0.4 \pm 0.2	
		Abscess 0.7 \pm 0.05	0.6 \pm 0.1	1.0 \pm 0.4	0.5 \pm 0.1	0.6 \pm 0.1	
75	Blood 2.8 \pm 0.8	1.5 \pm 0.4	0.9 \pm 0.4	0.7 \pm 0.4	0.5 \pm 0.4		

^aTime in hours after drug administration^bi.p., intraperitoneal; p.o. per os

level in mice of 11 $\mu\text{g/ml}$ and a 300 mg/kg dose gave a peak concentration of 29 $\mu\text{g/ml}$. At the first sampling period, 1 h after the drug was given, clindamycin concentrations in the abscesses were about double blood levels for both the 150 and 300 mg/kg doses. The ratio of abscess concentration to blood level continued to increase for 4 to 6 h after the drug was given (Table 3). Even after 8 h, concentrations in the abscesses were 4 to 8 times the concentrations present in the blood.

Metronidazole. Concentrations of metronidazole in blood and pus were determined following single oral doses of 31.25, 25, and 125 mg/kg (Table 2). Each dose produced concentrations in the abscesses that were approximately the same as obtained in the blood (Table 3). The only exception occurred with the 125 mg/kg dose which gave slightly higher concentrations in the blood than in the abscesses for 2 hours after dosage. By the 4th hour, however, blood and abscess levels were approximately the same for this dose as well, and remained so for the remaining sample periods.

Penicillin G. Penicillin G, given intraperitoneally as single doses of 250, 500, and 1000 mg/kg, gave blood levels that exceeded the concentrations obtained in the abscesses 1 h after dosage (Table 2). Although the 500 mg/kg dose continued to give higher levels in the blood than in the abscesses for the second hour, both the 250 and 1000 mg/kg doses produced concentrations in the abscesses in excess of simultaneous blood levels. After this period, penicillin concentrations in both blood and abscess were below 5 $\mu\text{g/ml}$ for all three doses. Due to the low levels present and normal variation from

Table 3. Abscess to blood ratio of the antibiotic concentrations in mice infected with Bacteroides fragilis

Drug administered		Abscess/blood ratio of drug concentration				
Antibiotic	Mg/Kg	1 ^a	2	4	6	8
Clindamycin (i.p.) ^b	300	2/1	9/1	10/1	8/1	8/1
	150	2/1	4/1	6/1	--- ^c	---
Metronidazole (p.o.)	125	1/1	1/2	1/1	1/1	1/1
	62.5	1/1	1/1	1/1	1/1	1/1
	31.25	1/1	1/1	1/1	---	---
Penicillin G (i.p.)	1000	1/2	2/1	---	---	---
	500	1/2	1/2	---	---	---
	250	1/1	2/1	---	---	---
Rosamicin (p.o.)	150	1/1	2/1	2/1	2/1	2/1
Tetracycline (p.o.)	500	1/2	2/1	2/1	3/1	2/1
	250	1/2	1/1	1/1	1/1	1/1
Doxycycline (p.o.)	150	1/3	1/2	1/1	1/1	1/1
	75	1/3	1/2	1/1	1/1	1/1
Minocycline (p.o.)	150	1/8	1/2	1/1	1/1	2/1
	75	1/4	1/2	1/1	1/1	1/1

^aTime in hours after drug administration

^bi.p., intraperitoneal; p.o., per os

^cConcentration of drug present too low for comparison

mouse to mouse, meaningful comparisons could not be made for the 4th through the 8th h.

Rosamicin. Rosamicin, a new macrolide antibiotic, was tested as a single dosage of 150 mg/kg given orally. Recommended blood levels for man have not been published for rosamicin. Single oral dosages of 150 mg/kg have been given to mice with no adverse effects (unpublished data, Schering Corp.). The 150 mg/kg dosage gave concentration of approximately 1 $\mu\text{g/ml}$ in both blood and pus 1 h after administration (Table 2). From the 2nd through the 8th h, blood concentrations remained around 1 $\mu\text{g/ml}$. Abscess concentrations for these periods was approximately double the blood levels (Table 3).

Tetracycline, minocycline, and doxycycline. Two dosages of tetracycline, minocycline, and doxycycline each were given orally and the resulting concentrations in blood and pus were determined (Table 2). Each antibiotic was tested at a dosage that gave a peak blood level in mice comparable to that recommended for man and at a higher dosage. Peak blood levels in recommended dosages for man are about 6 $\mu\text{g/ml}$ for tetracycline (6) and 3 $\mu\text{g/ml}$ for minocycline and doxycycline (7, 8). In our experiments, each of these drugs were present in higher concentrations in the blood than in the abscesses for the first or second hour following administration. After this, blood levels and abscess levels were about the same. The only exception occurred with the 500 mg/kg dose of tetracycline. This dose gave blood levels that were two-fold higher than abscess levels at the first hour; but, for the remaining sample periods, concentrations were roughly double blood concentrations (Table 3).

Although minocycline and doxycycline gave similar peak blood levels, doxycycline maintained higher blood levels than minocycline and produced higher concentrations in the abscesses (Table 2). Each drug was present at higher concentrations in the blood than in the abscesses for 2 h after administration. After this period, blood and abscess concentrations were very similar. Doses of doxycycline, however, gave blood and abscess concentrations that were double those obtained with similar doses of minocycline for the 4th through the 8th hour after administration of the drug.

DISCUSSION

Of the seven antibiotics tested in the experiments reported here, all, with the exception of clindamycin, produced concentrations in the blood that exceeded or were the same as concentrations present in the abscesses during the first hour following administration. In a number of instances blood levels exceeded abscess concentrations for up to two hours as well. This may explain a popular idea that antibiotic levels in the blood generally exceed the levels obtainable in abscesses. However, we did not find significantly higher blood levels than abscess levels for any of the antibiotics later than 2 h after dosage.

Generally different dosages of the same antibiotic gave similar results in the relation of blood levels to abscess levels of the drug, but, different antibiotics gave different results. Clindamycin gave the greatest difference between blood and abscess levels. In these experiments, clindamycin produced abscess levels that were several times higher than the corresponding blood levels. Santoro, *et al.* (14)

reported that 22 of 29 strains of B. fragilis were killed by 1.6 $\mu\text{g/ml}$ of clindamycin and all of these strains were inhibited by 0.8 $\mu\text{g/ml}$ or less. In our experiments, even though blood levels were not maintained at levels of 0.8 $\mu\text{g/ml}$ past the 1st hour, abscess levels were equal to or above the concentration for 8 h and in many instances were equal to or above bacteriocidal concentrations. Presently, we do not know why clindamycin gave higher abscess levels than blood levels; nor do we know if the antibiotic behaves in a similar fashion in abscesses initiated with other bacteria or in other animal species.

The concentrations of metronidazole in the abscesses was practically the same as the blood level regardless of dosage or time sampled. In work reported previously, metronidazole, at dosages of either 31.25 or 62.5 mg/kg completely protected mice from infection with the same strains of B. fragilis as used for these experiments (manuscript in preparation). Both strains had an MIC of 1 $\mu\text{g/ml}$ for metronidazole which is less than the concentrations of this drug in the abscesses. Since this drug, like clindamycin, was present in the abscesses at levels in excess of the MIC for most strains of B. fragilis, this may help explain the effectiveness of metronidazole against B. fragilis infections in man (17).

Most B. fragilis strains are considered to be relatively resistant to penicillin G. The two test strains used by us each had an MIC of 32 $\mu\text{g/ml}$. Only the highest dose, 1000 mg/kg, tested gave levels in either blood or abscesses in excess or equal to the MIC for two hours after administration. Within 4 h after dosage, less than 5 $\mu\text{g/ml}$ of the drug was present. We do not know if the rapid disappearance of

penicillin from both blood and abscesses was due to normal excretion of the drug from the body or if the drug was hydrolyzed by bacterial enzymes such as β -lactamase. Penicillin is known to be excreted rapidly by the kidneys while the presence of β -lactamase in B. fragilis and the role that it may have in the resistance of this organism to β -lactam antibiotics is still unclear (11, 12, 20).

Rosamicin is a macrolide antibiotic somewhat similar to erythromycin. Erythromycin gives higher tissue concentrations than serum concentrations and is reported to be retained in tissue longer than in the blood (6). Rosamicin, in our experiments, was initially equally distributed in blood and abscesses within 1 h after dosage. Blood concentrations remained at about the same level for 8 h; concentrations in the abscesses 2 h after administration were approximately double blood levels and remained at this level for the rest of the sampling periods. Rosamicin has been reported to have similar bactericidal activity to clindamycin. Rosamicin, at 1.6 $\mu\text{g}/\text{ml}$ killed 24 of 29 strains of B. fragilis in vitro (14). In our study, a single 150 mg/kg dose gave concentrations in the abscesses of about 2 $\mu\text{g}/\text{ml}$ within 2 h after dosage. Therefore, this drug may prove useful in the treatment of B. fragilis infections in man.

We have noted previously that minocycline had a greater efficacy than either tetracycline or doxycycline against B. fragilis infection in this mouse model (manuscript in preparation). Due to the lower MIC required, it is understandable that minocycline might confer greater protection than tetracycline; however, in vitro results show minocycline and doxycycline to be similar (15). We found in previous ex-

periments as well as the ones reported here that equal doses of doxycycline gave higher sustained blood levels than did minocycline, even though the peak blood levels achieved are similar (manuscript in preparation). Since minocycline was more effective in therapy of the experimental infection, we thought that this drug might reach higher concentrations in the abscesses than doxycycline. However, the opposite was true. The same weight to weight dose of doxycycline gave concentrations in the abscess that were about twice that obtained with minocycline. It would appear that the greater efficacy associated with minocycline was not due to increased abscess levels.

The antimicrobial agents reported here represent several different classes of antibiotics. We found that none of these antibiotics were present in the abscesses at concentrations significantly lower than blood levels later than 2 h after administration. In the case of clindamycin, we found bacteriocidal concentrations were often present in the abscesses even when in the blood the drug was at concentrations less than the MIC. Although these data cannot be extrapolated to humans or even to other experimental animal infections, studies such as this may be beneficial in understanding the effectiveness and the ineffectiveness of antimicrobial agents in the treatment of anaerobic infections as well as in the evaluation of new antibiotics. Additional studies involving the concentrations of a drug achieved in abscesses in humans and in other animal models are required in order to determine if results such as those reported here are specific for the drug or for the animal.

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PART V

The Pathogenic Components of the Microbiota of the Gingival Crevice Area of Man

SUMMARY

The predominant cultivatable microorganisms of the gingival crevice of man were isolated from 9 individuals. Of a total of 82 isolates recovered, 53 (70%) were anaerobes and 24 (30%) were facultatives. Pure cultures and recombined mixed cultures were examined for the ability to produce infection in mice. The pathogenicity of each pure culture was tested with approximately 20 mice and recombined mixed cultures were tested with 20-30 mice. The minimum combination of organisms required to reproduce an infection similar to the original mixed flora infection was determined with 3 of the samples. Streptococcus intermedius was an essential component in all 3 samples for the production of an infection similar to that obtained with the gingival scrapings. Other organisms required in combination with S. intermedius included Veillonella parvula, V. parvula and Fusobacterium nucleatum, or Eubacterium saburreum and Bacteroides ruminicola. Bacteroides melaninogenicus was neither required as an essential component for infection nor did its addition to any combination result in an increase in pathogenicity. Pure cultures of S. intermedius, F. nucleatum, B. ruminicola, B. melaninogenicus, V. parvula, and E. saburreum all produced infection in the mice. The incidence and severity of infection produced, however, was much lower than that obtained with recombined mixed cultures.

INTRODUCTION

The microflora of the gingival crevice area in man is capable of producing infection when inoculated into experimental animals. Similar infections occur in man in such conditions as human bite wounds (2), noma (13), and dental infections (Williams, et al., Absts. Ann. Meet. Am. Soc. Microbiol. 1977. Abst. B13, p. 17). The microflora recovered from such infections in both man and animals is similar to that found in periodontal disease (5, 13, 15, 22, 26). Microorganisms recovered from these infections are predominantly anaerobic bacteria and often include spirochetes, fusobacteria, bacteroides, vibrios, actinomyces, and gram positive and gram negative cocci (10, 15, 16, 18).

Individual isolates from the gingival crevice are usually non-infective for experimental animals. Combinations of different isolates, however, have been found by a number of investigators to be pathogenic for animals (8, 14, 20, 23, 24, 28). In most instances, the minimum number of organisms required to produce the infection were not determined. MacDonal, et al. (16) however, found that only 4 organisms were required to reproduce a typical "fusospirochetal" infection in guinea pigs. Bacteroides melaninogenicus was considered to be the primary pathogen in this mixture since deletion of this organism from mixtures of oral or intestinal bacteria resulted in loss of infectivity (31). Spirochetes, vibrios, fusobacteria, and streptococci were considered to be non-essential for the production of infection with the oral flora.

Anaerobic culture techniques have been greatly improved and new experimental animal models have been developed in the last few years. Due to these advances, we wished to determine whether or not micro-

organisms previously thought to be non-pathogenic either in pure culture or in recombined mixed cultures might now be shown to have pathogenic potential. In this study, we identified the predominant microorganisms recovered from infections in mice initiated with scrapings from the gingival crevice area of 9 individuals, determined which of the isolates from 6 of these samples were infective in pure culture for mice, and, from three of these specimens, determined the minimum combination of isolates required to reproduce the original type of infection.

MATERIALS AND METHODS

Sampling. Samples were taken from single sites of the gingival crevice from a total of 9 males and females aged 25-40 years using a sterile dental scaler. Periodontal tissue adjacent to the sample site varied from that being clinically healthy to that showing moderate gingivitis. None of the individuals had received antimicrobial agents within three months prior to sampling.

Selection of potential pathogens. Gingival scrapings were inoculated into chopped meat (CM) broth (11) immediately after sampling. The CM broth culture was incubated overnight and then 0.1 ml of this culture was used to inoculate a 10 ml volume of chopped meat carbohydrate semisolid (CMC-SS) medium (35). The CMC-SS cultures were incubated for 18-20 h, transferred to sterile serum vials, and 0.5 ml was injected subcutaneously into each of 10 mice as previously described (35). Each sample produced abscesses at the point of injection within 3-5 days. Pus was removed aseptically and 0.1 ml was injected subcutaneously into fresh mice. Serial animal passage was repeated

three more times. Following the final passage, 0.1 ml of pus was removed aseptically and immediately dispersed into pre-reduced salt solution (11) which was gassed under a stream of O_2 -free CO_2 . Ten-fold serial dilutions were made using the same diluent. One-tenth ml of the appropriate dilutions was spread onto plate media or inoculated into melted agar roll tubes (11). Plates were examined after 24 and 48 h incubation and each different colony type was picked into CM broth or chopped meat carbohydrate (CMC) broth (11). Roll tubes were examined after 48 h incubation with the exception of laked blood roll tubes (11) which were examined from 5-14 days for the appearance of black pigmented colonies characteristic of Bacteroides melaninogenicus.

Media. Plate media consisted of brain heart infusion agar (Difco) supplemented with 0.5% yeast extract (w/w) (Difco), hemin (5 mcg/ml), and vitamin K (0.5 mcg/ml) to which either 5% whole sheep blood (v/v) or 0.5% pyruvate (v/v) was added. Pyruvate plates were used in duplicate with blood agar plates to ensure the recovery of organisms such as Veillonella which cannot utilize glucose. Roll tube media consisted of supplemented brain heart infusion agar (BHIA) prepared as described in the VPI Anaerobe Laboratory Manual (11). Laked blood roll tubes were made by adding 4-5% (v/v) laked sheep blood to molten BHIA roll tubes cooled to about 55 C (11). Pyruvate roll tubes consisted of BHIA medium to which 0.5% pyruvate (v/v) was added before autoclaving.

Characterization of bacterial isolates. Each pure isolate was classified according to the criteria of gram stain, fermentation and and biochemical reactions, and analysis of fermentation end-products

by gas chromatography. Classification was based on the scheme given in the VPI Anaerobe Laboratory Manual (11). Fermentation and biochemical reactions were performed using the micromethod described by Wilkins and Walker (36). Tests for confirmation of biochemical reactions as well as for additional biochemical tests were performed as described in the VPI Anaerobe Laboratory Manual (11). Presumptive identification of facultative streptococci were made based on the scheme of Deibel and Seeley (3).

Pathogenicity of pure cultures. Each pure isolate was maintained as a stock culture in CM broth, kept at room temperature and transferred weekly. The stock culture was used to inoculate CM broth, which was grown overnight at 37 C and then was used to inoculate CMC-SS medium. The CMC-SS culture was incubated at 37 C overnight, 16-20 h, transferred to sterile serum vials, gassed with O₂-free CO₂ and used as inoculum as previously described (35). Initially, 0.5 ml of CMC-SS culture was injected subcutaneously under the loose skin of the groin of mice. A minimum of 5 mice per trial were used on at least two separate occasions for each isolate. Isolates implicated as being required for infection in recombined mixed cultures were also tested on a minimum of 10 mice per trial on two separate occasions by injecting a total volume of 0.5 ml containing equal volumes of CMC-SS culture and sterile CMC-SS medium.

Pathogenicity of recombined mixed cultures. Recombined mixed cultures were made by combining equal volumes of pure cultures, grown in CMC-SS medium as described above. Initially, all of the different isolates obtained after serial animal passage were recombined and

injected subcutaneously in 0.5 ml amounts in the groin area of mice. Isolates were deleted one at a time until the original infection could no longer be produced. Each recombined mixed culture was tested in two separate trials of 10-15 mice each. Microbiological examination of pus from the resulting abscesses was routinely performed to determine if all organisms injected were still present and if contamination had occurred. In the few instances where contamination occurred, the experiments were repeated.

Determination of infectivity. The infectivity of pure cultures or recombined mixed cultures was expressed as the percent of mice tested which developed abscesses or necrotic lesions. Mice were examined daily, beginning on the third day, for up to 14 days following the bacterial injection. Evidence of infectivity was death, development of a spreading necrotic lesion or development of a localized pus filled abscess. Mild inflammation and swelling was considered to be noninfective.

Animals. White Swiss male mice ICR strain, 18-20 g, (Flow Laboratories, Dublin, Va.) were used for all experiments. The mice were caged in groups of 25 with a light-dark cycle of 12 h and were allowed to stabilize for at least 48 h before being used in any experiment.

Anaerobic techniques. All broth media were prepared pre-reduced (11), and all culture manipulations were performed as described in the VPI Anaerobe Laboratory Manual (11). Manipulation of anaerobic plates were performed in an anaerobic chamber containing an atmosphere of 85% N₂, 10% H₂ and 5% CO₂ similar to that described by Aranki and Freter (1).

RESULTS

Predominant microorganisms recovered. A total of 82 isolates were recovered after animal passage from abscesses in mice that were initiated with the gingival scrapings from 9 individuals. Of these isolates, 58 (70%) were anaerobic and 24 (30%) were facultative. Six to eleven different organisms were recovered from each abscess. Isolates from individual samples that were phenotypically identical were considered to represent one strain. The predominant microorganisms recovered from each abscess sample are listed in Table 1. The majority of the isolates were anaerobic and facultative gram positive cocci and gram negative anaerobic rods. The gram positive cocci, primarily streptococci, were isolated from each of the 9 specimens. Streptococcus intermedius was the only organism recovered from all samples. Other streptococci included S. constellatus, S. morbillorum, S. anginosus, and β -hemolytic streptococci. Other gram positive cocci isolated were Peptococcus magnus and Peptostreptococcus anaerobius. Gram negative cocci were exclusively Veillonella parvula.

Gram negative anaerobic rods were isolated from 7 of the 9 abscess specimens and were predominantly bacteroides and fusobacteria. Bacteroides melaninogenicus was the most frequently encountered. Of the 6 strains of this organism we isolated, 4 were B. melaninogenicus subsp. intermedius and 2 were subsp. asaccharolyticus. The other bacteroides were isolated from 3 samples and were identified as B. ruminicola. Fusobacteria were recovered from 7 samples. Four of these were F. nucleatum and 3 were F. naviforme.

Table 1. Predominant cultivatable microorganisms recovered from abscesses in mice initiated with mixed flora from the gingival crevice area of man

Microorganisms	Sample Number								
	1	2	3	4	5	6	7	8	9
<u>Streptococcus intermedius</u>	x	x	x	x	x	x	x	x	x
<u>Bacteroides melaninogenicus</u>		x	x	x			x	x	x
<u>Bacteroides ruminicola</u>		x				x			
<u>Fusobacterium nucleatum</u>		x		x				x	x
<u>Fusobacterium naviforme</u>			x				x		x
<u>Veillonella parvula</u>		x	x		x		x		
<u>Eubacterium saburreum</u>		x					x		
<u>Peptostreptococcus anaerobius</u>	x			x					x
<u>Peptococcus magnus</u>				x	x	x			
Other streptococci ^a	x		x	x	x	x	x	x	x

^aIncludes S. constellatus, S. morbillorum, S. anginosus, and beta-hemolytic streptococci.

Gram positive rods were isolated only occasionally, generally as single isolates of eubacteria, lactobacilli, actinomyces, or propionic bacteria.

Infectivity of pure cultures. Each isolate from 6 of the 9 abscess samples was tested for infectivity in a minimum of 10-20 mice by subcutaneous injection. Listed in Table 2 are the organisms found to be pathogenic and the incidence of infection. With the exception of a single strain of Staphylococcus aureus, none of the pure cultures produced infection in more than 50-60% of the mice. The incidence of infection by each strain was relatively consistent; but, variation occurred among strains of the same species.

The S. intermedius isolates consisted of both anaerobic and facultative strains. The majority of these produced infection in 40-50% of the mice. The infections were predominately spreading necrotic lesions, although some localized abscesses also occurred. The necrotic lesions spread from the site of injection in the groin across the abdominal wall and often resulted in sloughing of the skin. In most instances, the infection healed rapidly following the loss of skin. The other streptococci were either non-infective or else produced a low grade infection that only persisted for about 7 days. None of the P. magnus or P. anaerobius strains were infective for mice.

All 6 strains of B. melaninogenicus were tested for the ability to produce infection as pure cultures. Three of these produced a mild localized infection in 10-30% of the mice. A small abscess was produced at the site of injection that was about 0.5 cm in diameter 7 to 10 days after bacterial injection. These abscesses did not persist

Table 2. Percent infectivity of pure cultures isolated from abscesses in mice following serial animal passage (20-30 mice per group)

Microorganisms	Sample Number					
	1	2	3	4	5	6
<i>Streptococcus intermedius</i>	45	50	45	10	10-50 ^a	10-40
<i>Bacteroides melaninogenicus</i>	-- ^b	30	10	0	--	--
<i>Bacteroides ruminicola</i>	--	30	--	--	--	10
<i>Fusobacterium nucleatum</i>	--	55	--	55	--	--
<i>Fusobacterium naviforme</i>	--	--	20	--	--	--
<i>Eubacterium saburreum</i>	--	20	--	--	--	50
<i>Peptostreptococcus anaerobius</i>	0	--	--	0	--	--
<i>Peptococcus magnus</i>	--	--	--	0	0	0
<i>Veillonella parvula</i>	--	45	20	--	45	--
<i>Staphylococcus aureus</i>	--	--	--	100	--	--
Other streptococci ^c	30-40	--	25-40	--	0	10

^aTwo or more strains from same sample

^bNot present in this sample

^cIncludes *S. constellatus*, *S. morbillorum*, *S. anginosus* and beta-hemolytic streptococci.

for longer than 14 days after injection and usually broke through the skin and drained within 10 days. Bacteroides ruminicola gave a similar type and incidence of infection.

The two strains of F. nucleatum both infected 55% of the mice. This organism, as well as the other species of fusobacterium tested, produced a localized subcutaneous abscess at the site of injection within 3-5 days. The abscesses remained relatively small, 0.5-1.0 cm in diameter, and generally persisted for at least 14 days. All of the fusobacteria strains produced an effect in the mice similar to endotoxin shock. The mice appeared ill, had a ruffled coat, their eyes were partially closed, and their respiratory rate was rapid. This condition occurred within 24-48 h after bacterial injection but did not persist past 72 h.

The three strains of V. parvula were infective in pure culture and produced small localized abscesses in 20-40% of the mice. The abscesses were similar to those described for the fusobacteria except that these were usually not visible until after 5 days following the bacterial injection. Endotoxin-like shock was not observed in any of these mice.

Infectivity of recombined mixed cultures. Three samples, numbers 2, 5, and 6, were tested as recombined mixed cultures. We first tested a combination which contained all of the isolates recovered from that sample, to determine that the organisms recovered were able to reproduce the original infection. We then tested recombined mixed cultures from which single organisms were omitted. This process was continued with the elimination of individual isolates until the original infec-

tion could no longer be produced. We found that only 3 or 4 organisms from each of the three samples were required to produce an incidence and severity of infection that appeared to be similar to that produced with the original gingival crevice scrapings. These organisms were then tested in a number of recombined mixtures to determine which combination of the fewest isolates would reproduce the original infection. The 3 or 4 organisms involved were also tested as pure cultures, as controls for the recombined mixed cultures by injecting an inoculum containing equal volumes of CMC-SS culture and sterile CMC-SS medium. Each pure culture was tested in a total of 20 mice and each recombined mixed culture in 20 to 30 mice.

The infectivity of recombined mixed cultures and of the pure cultures are given in Table 3 for sample 2. Although B. melaninogenicus was originally recovered from this sample, it was deleted from the recombined mixed cultures without affecting either the incidence or the course of the infections produced. None of the four isolates (Table 3) produced pure culture infections in more than 50% of the mice. In contrast, injection of a combination of equal volumes of S. intermedius, F. nucleatum, and V. parvula cultures produced infection in 100% of the mice and killed approximately 70%. Deletion of any one of these three organisms or replacement of any one with B. ruminicola resulted in a decrease of 25-50% in the incidence of infection.

The infection produced by the combination of these three organisms appeared identical to that produced by the original mixed flora. Both

Table 3. Infectivity of different combinations and of pure cultures from gingival sample 2

	Percent of Mice Infected ^a
<u>Pure Cultures</u>	
<i>Streptococcus intermedius</i>	50
<i>Fusobacterium nucleatum</i>	40
<i>Veillonella parvula</i>	30
<i>Bacteroides ruminicola</i>	30
<i>Bacteroides melaninogenicus</i>	20
<i>Eubacterium saburreum</i>	20
<u>Recombined Mixed Cultures</u>	
All isolates	100
<i>S. intermedius</i> - <i>F. nucleatum</i> - <i>V. parvula</i> - <i>B. ruminicola</i> - <i>B. melaninogenicus</i> - <i>E. saburreum</i>	100
<i>S. intermedius</i> - <i>F. nucleatum</i> - <i>V. parvula</i>	100
<i>S. intermedius</i> - <i>F. nucleatum</i> - <i>B. ruminicola</i>	76
<i>S. intermedius</i> - <i>F. nucleatum</i> - <i>B. melaninogenicus</i>	64
<i>S. intermedius</i> - <i>V. parvula</i> - <i>B. ruminicola</i>	70
<i>S. intermedius</i> - <i>V. parvula</i> - <i>B. melaninogenicus</i>	72
<i>S. intermedius</i> - <i>F. nucleatum</i>	68
<i>S. intermedius</i> - <i>V. parvula</i>	76
<i>F. nucleatum</i> - <i>B. ruminicola</i>	64
<i>F. nucleatum</i> - <i>B. melaninogenicus</i>	40
<i>F. nucleatum</i> - <i>V. parvula</i>	50

^aEach pure culture was tested with 20 mice; each recombined mixed culture was tested with 25 mice.

infections were characterized by a foul smelling necrotic lesion that began at the site of infection and rapidly progressed across the abdominal cavity. Death appeared to be due to invasion of the infection into the peritoneal cavity and usually occurred 7 to 10 days after bacterial injection. Mice that did not die during this period usually lost the skin over the necrotic area. Once the necrotic area was open, it healed rapidly and the mice recovered.

Sample 5 was of interest since it contained only gram positive and negative cocci. Neither bacteroides nor fusobacteria were recovered from this specimen. Although 8 strains were isolated only three produced infection in mice. These three strains included one strain of V. parvula and two strains of S. intermedius. Although we generally considered all isolates of a particular species from one specimen to be the same strain, there appeared to be sufficient differences between the two isolates of S. intermedius to justify differentiation between the two. One strain was anaerobic and remained so on continual transfers, was infective for 40% of the mice, and gave a positive reaction on bile-esculin medium (3). The other strain was facultative, produced infection in 10% or less of the mice, and gave a negative reaction on bile-esculin medium. The infectivity of these three organisms as pure cultures and as recombined mixed cultures is given in Table 4. A recombined mixed culture consisting of equal volumes of all 8 isolates produced infection in 100% of the mice and killed 50-60%. Combinations in which single isolates were omitted produced no change in the incidence of infectivity unless either the anaerobic strain of S. intermedius or V. parvula were omitted. Deletion of either of these resulted in a decrease in infection from

Table 4. Infectivity of combination and of pathogenic pure cultures from gingival sample 5

	Percent of Mice Infected ^a
<u>Pure Cultures</u>	
<i>Streptococcus intermedius</i> A ^b	40
<i>Streptococcus intermedius</i> B	10
<i>Veillonella parvula</i>	10
<u>Recombined Mixed Cultures</u>	
All 8 isolates	100
<i>S. intermedius</i> A - <i>S. intermedius</i> B - <i>V. parvula</i>	92
<i>S. intermedius</i> A - <i>V. parvula</i>	92
<i>S. intermedius</i> B - <i>V. parvula</i>	50
<i>S. intermedius</i> A - <i>S. intermedius</i> B	45

^a20-25 mice were tested with each pure culture or recombined mixed culture.

^b*S. intermedius* A, anaerobic strain; *S. intermedius* B, facultative strain.

100% to approximately 60%. A dual combination of only the anaerobic S. intermedius and V. parvula produced infection in approximately 90% of the mice. This dual mixture produced an infection similar to but not exactly like the infection produced by all 8 isolates together. Although the incidence of infection was not drastically different, the infection was milder, not as progressive, and rarely resulted in death. Addition of individual isolates back to this mixture did not produce a noticeable change in the course of the disease until all of the original 8 isolates were recombined.

The other sample that was subjected to extensive testing contained gram positive cocci, fusobacteria, and Bacteroides oralis. This sample was very similar to sample 2 except that B. melaninogenicus and Veillonella were not recovered. The incidence of infection produced by various pure cultures and recombined mixes of sample 6 are given in Table 5. The predominant pathogenic organisms recovered from this sample were S. intermedius, B. ruminicola and E. saburreum. Like the previous sample, sample 6 also contained two isolates of S. intermedius that differed from each other in regard to oxygen tolerance and infectivity. In this case, the facultative S. intermedius was slightly more infective in pure culture. This strain produced an infection in 30% of the mice compared to only 10% for the anaerobic strain.

A total of 9 isolates were originally recovered from this sample following serial animal passage. An infection was produced using a recombined mixed culture consisting of equal volumes of all 9 isolates that was similar to that produced with the original gingival samples. Five of these isolates could be deleted without affecting either the

Table 5. Infectivity of different recombined mixed cultures and of pure cultures used in these mixtures from gingival sample 6

	Percent of Mice Infected ^a
<u>Pure Cultures</u>	
Eubacterium saburreum	50
Streptococcus intermedius A ^b	10
Streptococcus intermedius B	30
Bacteroides ruminicola	0
<u>Recombined Mixed Cultures</u>	
All isolates	100
B. ruminicola - E. saburreum - S. intermedius A - S. intermedius B	100
B. ruminicola - E. saburreum - S. intermedius A	65
B. ruminicola - E. saburreum - S. intermedius B	70
B. ruminicola - S. intermedius A - S. intermedius B	45
E. saburreum - S. intermedius A - S. intermedius B	40
S. intermedius A. - S. intermedius B	30
B. ruminicola - S. intermedius A	30
B. ruminicola - S. intermedius B	35
E. saburreum - S. intermedius A	40
E. saburreum - S. intermedius B	45
B. ruminicola - E. saburreum	40

^a20 mice tested with each group.

^bS. intermedius A, anaerobic strain; S. intermedius B, facultative strain.

incidence or the nature of the infection. A recombined mixture of equal volumes of E. saburreum, B. ruminicola, and the two strains of S. intermedius reproduced the original infection. Elimination of any one of these organisms, however, resulted in a decrease in the incidence of infection. Deletion of either of the S. intermedius strains resulted in a decrease in infectivity from 100% to 65-70%. Recombined mixed cultures containing both S. intermedius strains but in which either E. saburreum or B. ruminicola was deleted produced infection in only 40-45% of the mice. Mixed cultures containing only two of these four isolates produced infection in 30-40% of the mice.

The nature of the infection produced varied with different combinations of the 4 organisms. All 4 together produced an infection similar to that obtained with all 9 isolates given together. In both cases, a localized abscess was produced at the site of infection within 3 to 4 days following the bacterial injection. This progressed to a more necrotic type of lesion, within a few days that spread across the abdominal wall. The necrotic area was filled with thick pus from which all four organisms could be recovered. The skin covering this area gradually cracked and was often sloughed within 7 to 10 days. In a few instances, the necrotic area healed once the skin was lost. Generally the area remained necrotic and appeared as an open sore. Often the infection penetrated the peritoneal cavity and resulted in death. Deletion of any one of the four organisms involved resulted in a much milder form of infection that rarely resulted in the death of the animal.

DISCUSSION

The experimental approach used in this study was designed to recover the predominant cultivatable microorganisms from the gingival crevice which had pathogenic potential. The organisms present at this site have been the subject of extensive investigations by Gibbons and Socransky as well as others (4, 6, 7, 30, 33, 37). MacDonald, *et al.*, (16) have shown that only a small fraction of the complex mixed flora associated with this region is required to produce typical infections and that the majority of the organisms do not have an essential role. We thought that serial animal passage might result in the elimination of most of the non-pathogenic organisms that were not essential for infection. The pathogens, as well as organisms involved synergistically with these components, should persist through the serial animal passage. We realize that the experimental approach used may have resulted either in the elimination of some organisms that have pathogenic capabilities or in the elimination of helper organisms that might be required for certain synergistic mixed infection. No attempt was made to recover spirochetes or actinomyces. The organisms recovered, however, reproduced the same type of infections that were initially obtained with the scrapings from the gingival crevice.

The predominant cultivatable microorganisms recovered from the abscess consisted primarily of bacteroides, fusobacteria, and streptococci. These organisms have been reported by several investigators to be frequently recovered from periodontal disease and other oral infections (4, 7, 26, 30, 37). The most comprehensive studies of the predominant cultivatable organisms recovered from the gingival crevice

were done by Socransky, et al., (32) and Gibbons, et al., (6). In these studies, fusobacteria, bacteroides, spirochetes, and facultative streptococci were predominant. Helderman found fusobacteria, selenomonas, bacteroides and veillonellae to be present in samples from inflamed and non-inflamed gingiva (9). Gram negative anaerobic rods were found to increase in proportion to the increase in inflammation. Slots (29) used strictly anaerobic culture techniques and found that anaerobes accounted for approximately 90% of 475 isolates from 8 patients with advanced periodontal disease. Fusobacteria and bacteroides accounted for 74% of the isolates. Williams, et al., (Absts. Ann. Meet. Am. Soc. Microbiol. 1977. Abst. B13, p. 17) found that 75% of the isolates from odontogenic infections were either anaerobic or microaerophilic. Predominant organisms were F. nucleatum, B. melaninogenicus, other bacteroides including B. ruminicola and B. oralis, anaerobic diphtheroids, and streptococci. Our data on the predominant organisms recovered following animal passage of gingival scrapings agrees very well with data reported by others in regard to the predominant organisms recovered from the gingival crevice, advanced periodontitis, and other oral infections.

Unlike previous investigators, we successfully produced pure culture infections with a number of the individual isolates. The success of our method appears to be due to adherence to strict anaerobic techniques, the use of pre-reduced medium, and the presence of a physical localizing factor (agar). Mergenhagen (18) has shown that in order to establish infections in rabbits with anaerobic streptococci that either a reducing substance or a localizing factor or both were required. We were unable to establish infection, particularly with anaerobic gram

negative bacilli, in preliminary experiments when we injected cultures grown in pre-reduced media without agar.

With the exception of a single strain of Staphylococcus aureus none of the isolates in pure culture produced infection in more than 55% of the mice. We found, however, in each of the 6 specimens, that 3-6 isolates were recovered that were pathogenic at least to some extent in pure culture.

With 3 of the specimens, we determined the minimum number of organisms required to reproduce the original type of infection. We purposely chose 3 specimens from which different types of microflora were recovered. Streptococcus intermedius was the only organism common to all 3 specimens that was required in recombined mixed cultures to produce the original infections. This organism appeared to be responsible for a spreading gangrenous type of lesion. The fact that pure cultures of this organism and a number of recombined mixed cultures which contained S. intermedius produced this type of infection supports this idea. Also, deletion of this organism often changed the course of infection to that of a localized abscess. This spreading type of infection may be due to hyaluronidase production. Both an increase in hyaluronidase activity in the saliva of individuals with periodontal disease (17, 25) and an increase in the incidence of hyaluronidase producing streptococci in patients with gingivitis (27) have been reported.

Although no other single organism appeared necessary for infection in all 3 specimens, V. parvula was required in two. In one case, a two culture mixture of S. intermedius and V. parvula was infective for approximately 90% of the mice. In another, this mixture produced

infection in 76%. In both instances, the incidence of infection with the mixed combination was significantly higher than with either alone. Although a symbiotic relationship has been demonstrated with a streptococcus and V. parvula in vitro (19), this is the first instance, to our knowledge, that a synergistic infection has been shown to be produced by these two organisms. A synergistic relationship between these two organisms might be explained on the basis of the production of lactic acid by S. intermedius as a major end-product which could be used by V. parvula as a substrate. The fermentation of lactate by veillonella is well documented (21). In turn, the removal of the lactate by V. parvula may enhance the growth of S. intermedius. In addition, S. intermedius may produce enzymes such as hyaluronidase which would contribute to the infective process. We do not know what pathogenic mechanisms V. parvula may have. We also do not know if S. intermedius could be replaced in this synergistic infection by another streptococci or lactic acid producing organism.

Although B. ruminicola and E. saburreum were both recovered from two of the three samples, the requirement for these two organisms to reproduce the original infection differed. In sample 2, neither of these organisms were necessary to reproduce the original infection, although both had limited pathogenicity in pure culture. In sample 6, however, both organisms were required and deletion of either greatly reduced the incidence of infection.

Bacteroides ruminicola has been isolated from oral abscesses (Williams, B. L., G. McCann, and F. Schoenknecht. Abst. Ann. Meet. Am. Soc. Microbiol. 1977. Abst. B13, p. 17) and is very similar to B. oralis

which is frequently isolated from the gingiva. Theilade and Gilmour described 19 strains of Eubacterium saburreum (formerly Leptotrichia aerogenes) that had been isolated from the human oral cavity (34). This organism is a gram positive rod but often stains gram negative and is easily confused with fusobacteria. We do not know what significance this organism may have in human oral infections.

In sample 2, a combination of Fusobacterium nucleatum, V. parvula, and S. intermedius was required to reproduce the original infection. Fusobacterium nucleatum has been frequently isolated from various diseases of the human oral cavity including gingivitis, periodontosis and advanced periodontal disease (9, 29, 37). Slots (29) found that this organism and B. melaninogenicus constituted the majority of the isolates recovered from 7 of 8 patients with advanced periodontal disease. This organism has also been implicated in acute necrotizing ulcerative gingivitis (12). We have found that a combination of F. nucleatum and Treponema denticola produced a synergistic infection in mice in which the treponemes persisted for up to 10 days (Walker, C. B., B. J. Veltri, Jr., B. Laughon, and T. D. Wilkins. 17th Intersci. Conf. Antimicrob. Agents Chemother. 1977). The relationship between these two organisms appears to be specific and neither of the organisms could be replaced by another organism. Fusobacteria and spirochetes are found together in acute necrotizing ulcerative gingivitis and circumstantial evidence appears sufficient to implicate these organisms as etiologic agents in this disease (12).

Bacteroides melaninogenicus was recovered from 6 of the 9 samples. This organism was not required as an essential component for the pro-

duction of infection in mice with gingival crevice flora. In sample 2, we demonstrated that the original infection could be reproduced by a minimum of 3 organisms and that B. melaninogenicus was not required. We were able to establish pure culture infections with several of these strains of B. melaninogenicus although the incidence of infection was 30% or less. Socransky and Gibbons (31) found that this organism was an essential component in recombined mixed cultures for the production of experimental "fusospirochetal" type infections in guinea pigs. These investigators stressed, however, that theirs was not the only combination that might be capable of producing such infections. Our work confirms this idea. It is possible that the use of a different animal model or the use of cultures in semisolid medium for inoculation or both may have had some influence on the results.

The results of this study have confirmed that organisms frequently encountered in the gingival crevice area of man, have pathogenic potential. We have found that a minimum of 3-4 organisms, which are frequently encountered in oral diseases, combined together are capable of producing infection in mice that mimic the infection produced by scrapings from the human gingiva. The difference in results from each of the 3 samples supports the views expressed by MacDonald (16) that mixed infections produced with oral bacteria may be nonspecific in a bacteriological sense but that a biochemical specificity - a requirement for particular combination of metabolites and enzymes not produced by a single organism - may exist.

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DISCUSSION AND SUMMARY

Experimental Animal Infection

In 1975, when this investigation was begun, there were no satisfactory methods for producing a pure culture infection in an experimental animal with B. fragilis. Hill et al. were the only investigators that had been successful in demonstrating the infectivity of B. fragilis for a laboratory animal. These investigators could only achieve infection in 20 to 25% of the mice inoculated, however, and necropsy was required to determine the presence of infection. In May, 1975, Renz et al. described the production of a pure B. fragilis infection in mice. This model infection did not differ significantly from that of Hill et al. except that infection developed in approximately 50% of the mice. Both infections were dependent on the use of mucin as an adjuvant for the initiation of infection, required necropsy to determine the presence of infection, and resulted in a relatively low incidence of infection. All of these were serious drawbacks that limited the usefulness of these model infections for large scale experiments such as would be involved in the evaluation of antimicrobial agents. Mucin is derived from the gastric contents of swine and the composition varies widely from batch to batch. Very often infection may be obtained with one particular batch of mucin but not with similar batches. This can cause a problem for other investigators who attempt to reproduce the original work. The disadvantages associated with a low incidence of infection along with the necessity to perform necropsy to determine infection are evident, particularly where studies involving a large number of animals are concerned.

Later in 1975, the model infection described in part I of the dissertation was presented in part at the 15th Interscience Conference for Antimicrobial Agents and Chemotherapy. This model infection was the first instance of a pure B. fragilis infection in experimental animals in which the majority of the animals developed infection. The infection resulted in a localized subcutaneous abscess that was readily visible without requiring necropsy. Approximately 90% of the mice reproducibly developed infection within 5 days post-injection. The only adjuvant used was commercially available agar which was incorporated into the growth medium. The model infection has since been reproduced by a number of other investigators with different strains of B. fragilis and is currently in routine use by several pharmaceutical companies for the evaluation of antimicrobial agents. Other investigators are using the model infection in studies involving the pathogenic mechanisms of B. fragilis. The model has been shown to be usable in large scale studies and has been used in this laboratory in experiments involving the simultaneous use of up to 1000 mice. As described in part V, the technique used to establish infection is not limited to B. fragilis but is also applicable to other anaerobic bacteria as well. Pure culture infections have been established with various species of oral bacteria including B. melaninogenicus, B. ruminicola, several species of fusobacteria, anaerobic gram positive cocci, Eubacterium saburreum and Veillonella parvula.

Other investigators have since developed additional experimental animal infections with pure cultures of B. fragilis. In these models, however, initiation of infection requires either specialized techniques

such as surgical implantation or a pre-existing disease condition such as atherosclerosis. The infection produced is not readily apparent and necropsy is often necessary. The animals used, either rabbits or rats, are more expensive and require more space for housing. These factors all have an effect on the number of animals that can be used in a particular experiment as well as on the actual number and types of experiments that can be performed.

In vivo Evaluation of Antimicrobial Agents:

Determination of Efficacy

B. fragilis is one of the most resistant anaerobes to antimicrobial agents. Most clinical isolates are considered resistant to the penicillins and to most of the cephalosporins. Approximately 60% of these isolates are also resistant to achievable levels of tetracycline and its analoges. The organism is relatively resistant to the macrolide antibiotics such as erythromycin and appears to be totally resistant to the aminoglycoside family of antibiotics. Of the drugs approved for treatment of anaerobic infections, clindamycin and chloramphenicol are the only antibiotics routinely recommended for treatment of B. fragilis infections. Some question has even been raised about the in vivo efficacy of chloramphenicol. Although in vitro susceptibility tests indicate that B. fragilis should be susceptible to clinically achievable concentrations of this drug, there have been several instances where antimicrobial therapy with this antibiotic have not been successful. Results of both in vitro and in vivo testing of clindamycin, as well as clinical experience, indicate that this antibiotic is effective against B. fragilis. Due to the rare occurrence of pseudomembraneous colitis and the more commonly encountered

diarrhea that has been associated with clindamycin, many clinicians are more hesitant to use this drug than they once were.

The resistance of B. fragilis to many antimicrobial agents, coupled with the frequency of recovery of this organism from clinical specimens, has contributed to an increased interest in new and experimental drugs that might be effective against this organism. One of the primary objectives in obtaining a pure culture infection with B. fragilis was that a model infection would provide a means for evaluating such antibiotics prior to use in clinical trials in man. Information concerning various pharmacodynamic parameters of a drug such as toxicity, distribution, elimination and/or inactivation, and the length of time therapeutic levels are maintained can only be determined by in vivo methods. Such information is clearly desirable prior to the use of the drug in clinical trials. In vitro susceptibility testing is at best an artificial situation. Minimal inhibitory concentrations of an antibacterial agent may be influenced by the media used, pH, incubation conditions, and the number of organisms present. In addition, the minimal inhibitory concentration determined in vitro may not reflect the concentration of the drug required to inhibit the organism in vivo.

The data presented in parts II and III represent the most complete in vivo testing of both commonly used and experimental antimicrobial agents against B. fragilis to date. Part II is concerned with antibiotics that have been in use for several years. Although extensive data have been collected through clinical use of these agents, these drugs have not been evaluated previously under controlled conditions in vivo against B. fragilis. The results obtained in this study (Part II) agree

with the data obtained from clinical usage of these drugs. While this does not indicate that data obtained from the model infection can be directly extrapolated to man, it does indicate that data obtained with experimental antimicrobial agents may give an indication of what to expect of these agents in clinical trials.

In part III, several experimental antibiotics were tested in this model infection. The methods used were the same as were used in part II. Each antibiotic was tested at concentrations which produced blood levels in the test animals similar to what would be clinically achievable in man. This is an important concept. Previous investigators have compared the efficacies of antibiotics purely on the basis of weight. This is often misleading. In clinical practice, each antibiotic is usually given at a particular dosage which differs from drug to drug. The dosage given is determined by the concentration of the antibiotic that can be safely tolerated in the blood. Since the pharmacodynamics of different antibiotics are different, the quantity of these drugs that can be safely tolerated also differs. The differences in the amounts of different antibiotics given in clinical practice may be taken into account by two methods. One method is to give the amount required to produce blood levels of the drug in the experimental animal similar to those recommended in human therapy. Another technique is to adjust the normal human dosage to correspond to the body weight and surface area of the experimental animal. Of these two, the former probably gives a more realistic evaluation. The latter requires taking into consideration not just a weight-to-weight adjustment, but also the differences in the body surface of man and the experimental animal. Difficulties are often en-

countered in such an adjustment in dosage amounts when very small animals such as mice are used. For this reason, the results presented in parts II and III are based on the quantity of the drug required to give blood levels comparable to what is or should be clinically achievable. An attractive feature of this method is that it allows for comparison with similar experiments conducted with different experimental animals.

With some of the antibiotics tested, dosages were also tested that gave blood concentrations above and below the recommended level. This was done in particular with the experimental antibiotics to determine what effect increases or decreases would have on the efficacy of the drug.

In vivo Evaluation of Antimicrobial Agents:
Concentration Within Abscess

In part IV, the concentrations of biologically active antibiotic within the abscesses were determined for several of the antibiotics previously tested in parts II and III. There has been a great deal of recent interest in this area by clinicians. For some time the prevailing theory has been that antibiotic concentrations within localized abscesses were lower than blood concentrations. Unfortunately there has been almost no work done in this area to prove or disprove this concept. Since infections with anaerobic bacteria often result in the formation of abscesses, it is important to know how much of the antibiotic present in the blood is available at the loci of infection. The reason for this is clear. If the abscess level of a drug is significantly less than the corresponding blood concentration and the blood concentration is only slightly higher than the minimal inhibitory concentration, therapy

with such a drug would not be advisable unless blood concentrations could be safely increased. Research in this area has also been hindered by the lack of a suitable model infection. The nature of such an investigation requires a model infection in which the locus of infection is both available for sampling and is of sufficient size to yield an adequate sample volume. In addition, due to the number of samples required, a high incidence of infection is also desirable. The B. fragilis model infection described in part I fulfilled all of these requirements. Several antibiotics were tested for their ability to achieve adequate concentrations within abscesses. Concentrations achieved with the abscesses were compared to simultaneously occurring blood concentrations. The results obtained indicate that abscess concentrations are not significantly lower than blood concentrations for most antibiotics and, in some cases, may even be higher.

Generally, treatment of infections involving abscesses consists of surgical intervention and drainage followed by antimicrobial therapy. However, sometimes the location of the abscess may make surgical intervention unfeasible or the abscesses may be so small that all cannot be located. Although additional research needs to be done in this area, the findings that certain antibiotics are maintained at higher levels in the abscesses than in the blood may have application for therapy of such infections.

Experimental Animal Infections with Anaerobic Bacteria
From the Human Gingiva Crevice

In part V, pure cultures and recombined mixed cultures of bacteria from the gingival crevice of man were used to produce infection in mice. The purpose of this investigation was to determine if the previously described technique used to establish a pure B. fragilis infection was applicable to other anaerobic bacteria and to determine which organisms commonly found in the gingival crevice had pathogenic potential. As previously discussed, elegant work has been done in this area by MacDonald, Socransky, and Gibbons. These investigators defined the necessary organisms required to produce a typical fusospirocetal infection with bacteria from the gingiva crevice. They concluded that B. melaninogenicus was the primary pathogenic organism involved. However, due to the requirement of most strains of this organism for an exogeneous source of vitamin K, helper organisms were required along with B. melaninogenicus to obtain infection in guinea pigs. Deletion of B. melaninogenicus from a number of recombined mixed cultures always resulted in loss of infectivity. It was also found that only strains of B. melaninogenicus which did not require vitamin K were pathogenic in pure culture.

In the work presented in part V, several species of anaerobic bacteria isolated from the gingival crevice were found to produce infection in pure culture. Pure culture infections were obtained with B. melaninogenicus, B. ruminicola, F. nucleatum, anaerobic streptococci, Eubacterium saburreum and V. parvula. It was also found that the infection produced with the polymicrobial mixture taken directly from the gingival crevice could be reproduced with a minimum of two to four organisms in a recom-

bined mixed culture. The particular organisms required were different in different samples. In no case was B. melaninogenicus required to reproduce the original infection. Addition or deletion of this organism from any recombined culture had no effect on either the incidence or the severity of the infection.

These findings are in contrast to the earlier work of MacDonald, Socransky, and Gibbons. The differences noted, however, may be due to both the different experimental animals used and to the differences in techniques used to establish infection.

The results in part V demonstrate that the technique described for producing infection is not limited to only B. fragilis. Pure culture infections were produced with several species of anaerobic gram-negative rods which have generally been considered to be non-pathogenic for laboratory animals. In addition, pure culture infections were established with anaerobic gram-positive cocci, gram-negative cocci, and a gram-positive rod. Several synergistic relationships were also discovered that produced a more severe infection than did the individual components alone.

Future Research Areas

The criteria previously stated for a satisfactory experimental infection with B. fragilis have all been met. The model infection developed produces a visible infection in the majority of the animals, the infection is amendable to antimicrobial therapy, can be reproduced by other investigators with other strains as well as the same strains, and the technique used is applicable to other species of anaerobic bacteria.

As is often the case, a research problem is never successfully completed but instead leads to other areas that require investigation. Al-

though the initial criteria for this problem have all been satisfied, there are now even more areas that require investigation. A method has been developed that allows for the establishment of pure culture infections with B. fragilis, B. melaninogenicus, F. nucleatum, and anaerobic gram-positive cocci. These organisms are four of the five most frequently encountered anaerobic bacteria in human clinical specimens. Very little is known concerning the mechanisms of pathogenicity of any of these organisms. Although a number of antimicrobial agents were evaluated against the B. fragilis infection, there are still more potentially effective antibiotics that should be evaluated against this organism as well as other species of anaerobes. Further work should be done to determine the concentrations of antimicrobial agents that are present within the abscess during therapy. This, in particular, should be done with other clinically important organisms to determine if the concentrations of a drug achieved within an abscess bears any dependence on the infecting organism.

The findings that mixed cultures of anaerobic bacteria often produce a more severe infection in experimental animals than do the individual components alone should be studied further. Most anaerobic bacteria isolated from clinical specimens occur in mixed culture. The possible synergistic relationships that may occur in such infections are of interest both to the clinician and to the microbiologist.

Just as the results of the research presented here were needed ten years ago, so are the results in these other areas needed today. Hopefully, the work included in this dissertation will be helpful to other investigators in obtaining these results.

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EXPERIMENTAL ANAEROBIC BACTERIAL INFECTIONS IN MICE

by

Clay B. Walker

(ABSTRACT)

The development of a technique for producing a pure Bacteroides fragilis infection in mice is described. The infection produces large subcutaneous abscesses at the site of bacterial injection in approximately 90% of the mice. The abscess can be seen as an obvious swelling within 5-7 days post-injection. The infection was initiated by injection of pure cultures grown in a semisolid medium. Similar infections were also produced with pure cultures of B. distasonis, B. ovatus, B. thetaiotaomicron, and B. vulgatus.

The efficacies of several antimicrobial agents were determined for the B. fragilis infection. The antibiotics tested were clindamycin, chloramphenicol, carbenicillin, cefoxitin, erythromycin, penicillin, metronidazole, cephalothin, rosamicin, minocycline, doxycycline, and tetracycline. Minocycline, doxycycline, and tetracycline were each tested against a tetracycline resistant strain and a tetracycline susceptible strain of B. fragilis. Chemotherapy was begun 4 h after bacterial injection and was given every 8 h thereafter for 5 days. Blood concentrations were determined over an 8 h period for each concentration of antibiotic tested. Minocycline was more effective than either doxycycline or tetracycline regardless of which strain of B. fragilis was used. Metronidazole was the most effective antibiotic tested, protecting up to 100% of the mice from infection. Clindamycin protected up to 84% and rosamicin protected approximately 50%. Carben-

icillin, penicillin, cephalothin, chloramphenicol and erythromycin each protected less than 40% of the mice from infection.

The concentrations of biologically active antibiotic achieved in B. fragilis abscesses were determined and compared with blood concentrations obtained following administration of clindamycin, penicillin, metronidazole, rosamicin, tetracycline, doxycycline, and minocycline. Concentration of each drug were determined in blood and in abscesses at 1, 2, 4, 6, and 8 h after administration of the drug. Concentrations in the abscesses generally were lower than blood levels for the first 1-2 h. From 2-8 h, abscess concentrations were equal to or slightly higher than blood concentrations. Clindamycin was the only major exception to this. This antibiotic was consistently at higher concentrations in the abscesses than in the blood.

Pure culture and recombined mixed culture infections were also produced in mice with anaerobic bacteria from the gingival crevice of man. Pure culture infections were produced with Bacteroides melaninogenicus, Bacteroides ruminicola, Fusobacterium nucleatum, Veillonella parvula, Eubacterium saburreum, and anaerobic gram-positive cocci. Several synergistic infections were produced with recombined mixed cultures which resulted in a more severe infection and a higher incidence of infection than that produced by pure cultures alone. The minimum combination of organisms required to reproduce the infection obtained with the polymicrobial flora from three of the gingival crevice samples was also determined.