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

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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.

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 (V.P.I.) Anaerobe Laboratory. These strains were isolated from human clinical specimens and were sent to the V.P.I. 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 V.P.I. *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- to 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 Sorvall Inc., Norwalk, Conn.), sealing the tube with a size 0 rubber stopper, centrifuging at 2,000 × *g* (Sorvall 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 O_2-free CO_2. Immediately before injection of the bacterial culture, plastic

syringes were flushed several times with CO_2 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 20-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 O_2-free CO_2 using the techniques described in the V.P.I. *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 micromethod 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 fourfold 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 cultures. 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

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

[a] Ten mice per group.

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 (Fig. 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.

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.

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

[a] Number of viable cells/0.5 ml of inoculum.
[b] Twenty mice in each group.

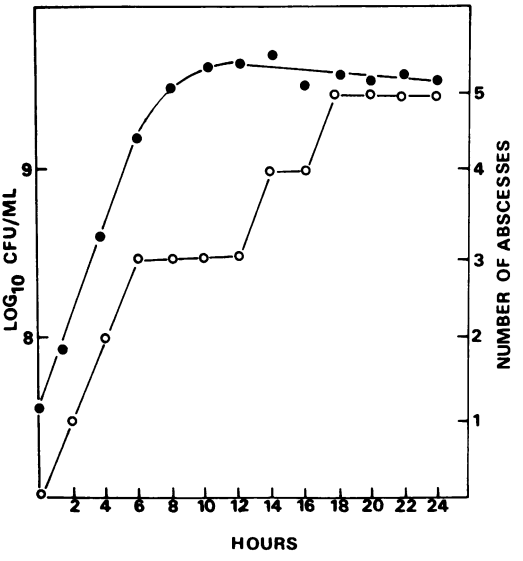

FIG. 1. *Determination of the culture age optimal for the establishment of B. fragilis subcutaneous abscesses in mice. Samples of the culture were taken at 2-h intervals, the number of viable bacteria per milliliter was determined, and five mice were injected from each sample. Symbols: (●) Viable cell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

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

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
B. distasonis	4243	49	48	98
B. ovatus	0038-1	49	44	90
B. thetaiotaomicron	5482	47	17	36
B. vulgatus	4245	47	36	77

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 gastic 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 infections, 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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