Inhibition of *Bacteroides fragilis* on Blood Agar Plates and Reversal of Inhibition by Added Hemin

TRACY D. WILKINS,* SARAH L. CHALGREN, F. JIMENEZ-ULATE, C. R. DRAKE, JR.,
AND J. L. JOHNSON

Anaerobe Laboratory, Virginia Polytechnic Institute and State University, Blacksburg, Virginia 24061

Received for publication 31 October 1975

Bacteroides fragilis strains formed much smaller colonies on most types of blood agar plates than they did on the same media without blood. Blood inhibited strains of *B. fragilis* subsp. *distasonis* the most and *B. fragilis* subsp. *fragilis* the least. The inhibition could be eliminated by adding hemin to the blood agar. The inhibitory component of the blood was inside the erythrocytes and appeared to be the hemin-free globin of hemoglobin.

During the course of experiments to determine which agar medium would best support growth of anaerobic bacteria, we found that *Bacteroides fragilis* strains grew better on agar medium without blood than on the same medium with 5% blood added. With some strains, there was complete inhibition of growth on the blood plates. Because of the clinical importance of *B. fragilis* and the common use of blood agar plates in clinical laboratories, we have investigated this phenomenon in greater detail. This communication reports that inhibition is dependent on the medium used, and describes the degree of inhibition of the different subspecies of *B. fragilis* and the reversal of the inhibitory activity of blood by added hemin.

MATERIALS AND METHODS

Blood. Defibrinated sheep blood was obtained from the Brown Laboratory, Topeka, Kan. Fresh blood from sheep, goats, horses, rabbits, and cows was collected aseptically and was defibrinated by gentle shaking with glass beads. Blood was stored at 5°C. For some experiments, the blood was laked (hemolyzed) by freezing and thawing.

Media. Media for plates were made aerobically and sterilized at 121°C for 15 min. Media were obtained from BBL, Baltimore, Md., except for brain heart infusion (BHI) agar (Difco, Detroit, Mich.) to which yeast extract (Difco) was added to give a final concentration of 0.5% in the medium.

Hemin solutions. For routine tests, we used hemin dissolved in NaOH (3), but for the more critical experiments reported in Table 1 and Fig. 2 we used the following alkaline-ethanolic solution A. Solution A was prepared by dissolving 200 mg of hemin (Sigma, St. Louis, Mo., equine type III) in 10 ml of 0.2 M KOH in 47.5% ethanol, adding 40 ml of water, and stirring at maximum speed with a magnetic stirrer for 2 h. This solution was filtered through membrane filters (Millipore Corp., Bedford, Mass.) of 0.8-, 0.65-, 0.4-, and 0.22- μ m pore size. The

final concentration of hemin remaining in solution was determined by using the extinction coefficient of the reduced alkaline pyridine hemochrome (2). All glassware was heated at 490°C for 4 h to destroy any residual hemin.

Bacterial strains. All strains were from the culture collection of the Virginia Polytechnic Institute (VPI) Anaerobe Laboratory. All subspecies designations of *B. fragilis* are according to the deoxyribonucleic acid (DNA) homology groups described by Johnson (4). A strain of *B. fragilis* subsp. *distasonis* (VPI B1-20) was used for tests of activity of blood fractions. The cultures were grown overnight in chopped meat (CM) broth (3) in CO₂-filled tubes.

Agar well tests for inhibition. Activity of blood fractions was determined by measuring zones of inhibition around wells cut into Schaedler agar plates. The wells were 5 mm in diameter and were cut with a sterile cork borer. The surface of the plate was swabbed with a 1:1,000 dilution of an overnight culture of strain B1-20 in CM broth, and the wells were filled with the material to be tested. The plate was incubated for 24 h in a GasPak anaerobe jar at 37°C. Diameters of zones of inhibition were measured.

Colony diameters. Isolated colonies were obtained either by spreading 0.1 ml of a 10⁻⁶ dilution of an overnight CM culture onto agar plates or by streaking cultures directly onto plates. Isolated colonies were measured with a dissection microscope equipped with an ocular micrometer. Five to ten isolated colonies on each plate were measured, and averages were reported.

Fractionation of blood. Defibrinated sheep blood (50 ml) was centrifuged for 10 min at 755 $\times g$, and the erythrocytes were washed four times with 0.85% NaCl. After the final wash, the cells were lysed by suspending them in 250 ml of distilled water. The lysed cells were incubated with ribonuclease (12 μ g/ml, bovine pancreatic type A, Sigma) for 30 min at room temperature, and the cell stroma were removed from the lysate by centrifuging it two times for 30 min at 16,300 $\times g$. The supernatant was then diluted to 1 liter with 0.02 M phosphate buffer (pH 7.5).

Solid ammonium sulfate was added to the solution at room temperature (22 C) to achieve successive concentrations of 50, 70, 80, and 100% saturation. The solution was allowed to sit for 30 min at each saturation level, and the precipitate was then removed by centrifuging for 30 min at $16,300 \times g$. The precipitates were dissolved in the 0.02 M phosphate buffer, dialyzed overnight against several changes of 2 liters of the same buffer, diluted to 50 ml with the buffer, and filter sterilized. These ammonium sulfate fractions were assayed for activity by the agar well method.

The material resulting from the 80% $(\text{NH}_4)_2\text{SO}_4$ saturation was further fractionated on a Sephadex G-100 column and on an isoelectric focusing column (LKB-Produkter AB; 8100-20). Two milliliters of the sample was applied to a Sephadex column (2.5 by 32 cm) that had been equilibrated with 0.03 M phosphate buffer at pH 7.0. Another 1 ml of the sample was fractionated on the isoelectric focusing column filled with a linear 0 to 50% sucrose gradient containing a pH 5 to 8 ampholyte (LKB) solution. The sample was incorporated in the gradient, and a voltage of 1,000 V was applied to the column for 48 h at 2 C. Isoelectric focusing concentrates each molecule at its isoelectric point; a detailed description of the method and apparatus has been written by Vestergberg (6). The column was emptied through a flow cell on a Gilford 2400 spectrophotometer, and fractions were assayed for activity with the agar well test.

Hemoglobin and native globin. Two-times-recrystallized sheep and bovine hemoglobins were obtained from Sigma. Hemin was removed from the sheep hemoglobin by extraction at 5 C with acid-acetone (5). The precipitate was washed with acetone, dissolved in water, and slowly titrated over a 2-h period with NaOH to neutrality (5). This material was tested for activity.

RESULTS

The inhibitory activity of blood for *B. fragilis* strains was demonstrated by swabbing the surface of agar plates with a sensitive strain and filling wells in the plates with whole blood. Zones of inhibition appeared, and with many of the sensitive strains there was a complete absence of growth near the blood (Fig. 1). The inhibition was also obvious when cultures were streaked on blood agar plates. Some sensitive strains did not form isolated colonies on BHI blood agar plates after 3 days of incubation, whereas colonies of 2-mm diameter occurred after overnight incubation on plain BHI plates.

Factors affecting extent of inhibition. The extent of inhibition was dependent upon the type of agar medium used for the blood agar plates (Table 1). Colonies of *B. fragilis* subsp. *distasonis* B1-20 were smallest on Schaedler and BHI blood agar plates, and intermediate in size on Trypticase soy, blood agar base, and

Columbia blood plates. There was no apparent inhibition on brucella blood agar.

The oxidation-reduction potential of the media did not seem to be a factor, because inhibition was observed with prereduced media in roll tubes as well as with aerobically prepared agar plates. The extent of inhibition varied with the subspecies (DNA homology group) of *B. fragilis* tested. *B. fragilis* subsp. *distasonis* was inhibited the most, and *B. fragilis* subsp. *fragilis* was inhibited the least (Table 2). Although there were strain variations within each homology group, none of the *B. fragilis* subsp. *fragilis* strains was inhibited as much as any of the *B. fragilis* subsp. *distasonis* strains. The other subspecies were intermediate in extent of inhibition (Table 2).

The type of animal blood used to make the blood agar plates also had an effect on the degree of inhibition. Blood from either sheep or cows was the most inhibitory, and rabbit blood was the least inhibitory (Table 3). Blood from different shipments and different animals also varied in the extent of inhibition.

Reversal of inhibition by added hemin. Hemin is a required growth factor for all *B. fragilis* subspecies, and it is routinely added to media for growing this organism. Because blood contains large amounts of hemin, it is often not added to blood agar plates; however, the addition of hemin to BHI blood plates eliminated the inhibiting effect of blood. There was a direct dose response in colony size to the amount of hemin added to the medium (Fig. 2). Considerably more hemin had to be added to the BHI blood agar than to the regular BHI agar to obtain the same size colonies, and with laked (lysed) blood plates, even larger amounts of hemin had to be added to obtain maximum growth (Fig. 2). Schaedler agar necessitated the addition of the largest amount of hemin to eliminate the inhibition. Although this medium contains 10 μg of hemin per ml when made from the commercial mixture, an additional 4 $\mu\text{g}/\text{ml}$ had to be added to obtain maximum colony size with whole blood, and 40 $\mu\text{g}/\text{ml}$ was necessary with laked blood. This medium supported good growth in the absence of blood, indicating that the hemin was available for growth.

Isolation of inhibitory component. The activity of blood fractions was measured by determining the diameter of zones of inhibition around wells in Schaedler agar plates. With this assay, we found that the inhibitory activity of whole blood was primarily inside the erythrocytes. The inhibitory activity was retained after the erythrocytes were washed with saline.

FIG. 1. Inhibition of *B. fragilis* subsp. *distasonis* strain B1-20 by whole sheep blood contained in a 5-mm-diameter well cut in a BHI agar plate supplemented with 5 μ g of hemin per ml.

When the erythrocytes were lysed with distilled water, the cell membranes were not inhibitory, but the supernatant was. The activity in the supernatant was not destroyed by heating at 56 C for 30 min, but was eliminated by heating

at 80 C for 10 min. Chocolate blood agar plates also were not inhibitory.

The inhibitory activity of the supernatant was retained by a 10,000-molecular-weight cut-off ultrafilter (Amicon, PM-10), was nondialyz-

TABLE 1. Colony diameters of *B. fragilis* subsp. *distasonis* strain B1-20 on six media with and without 5% sheep blood

Medium	Colony diam (mm)		
	Blood	No blood	
		No hemin	Hemin (10 μ g/ml)
BHI	0-0.1	0	1.9
Schaedler	0-0.1 ^a	2.0 ^a	2.0 ^a
Trypticase soy	0.3	0	1.2
Columbia	0.5	0	1.9
Blood agar base	0.6	0	1.0
Brucella	1.1	0	1.3

^a Schaedler medium contains 10 μ g of hemin per ml in the commercial mixture that was used for this experiment. Hemin concentrations refer to the amount of additional hemin added.

TABLE 2. Colony diameters of five DNA homology groups (subspecies) of *B. fragilis* on BHI agar with and without sheep blood

<i>B. fragilis</i> subsp.	Colony diam (mm) ^a	
	BHI + blood	BHI + hemin
<i>fragilis</i>	1.0 $\pm$ 0.5	1.9 $\pm$ 0.5
<i>ovatus</i>	0.5 $\pm$ 0.2	1.9 $\pm$ 0.6
<i>thetaiotaomicron</i>	0.3 $\pm$ 0.1	1.6 $\pm$ 0.3
<i>vulgatus</i>	0.3 $\pm$ 0.1	1.7 $\pm$ 0.3
<i>distasonis</i>	0.1 $\pm$ 0.1	1.5 $\pm$ 0.4

^a Colony diameters $\pm$ standard deviation, after 48 h of incubation. Average of 10 strains was calculated, except 9 strains were used with *B. fragilis* subsp. *vulgatus*.

TABLE 3. Colony diameters of strain B1-20 on BHI blood agar plates made with blood from several species

Type of blood	Colony diam (mm)
Sheep	<0.1
Cow	<0.1
Horse	0.1
Goat	0.2
Rabbit	0.4
None + 5 μ g of hemin per ml	1.5

able, and was precipitated at 80% saturation with ammonium sulfate. It was eluted from a G-100 Sephadex column in the same fractions as hemoglobin, and the fractions with the highest optical density at 280 nm also were most inhibitory. The supernatant was also fractionated on an isoelectric focusing column; the activity came off the column in the same fractions as the three red hemoglobin peaks. The first peak had the characteristic brown-red color of

FIG. 2. Colony diameters of strain B1-20 on BHI plates and BHI sheep blood plates as a function of amount of added hemin.

methemoglobin. The second and third peaks may have been oxyhemoglobin and reduced hemoglobin or possibly two genetic variants of sheep hemoglobin. The peaks showing activity produced a single band in disc electrophoresis, suggesting that hemoglobin was the only protein present.

Since erythrocytes are mostly hemoglobin and the activity followed hemoglobin in four different fractionation procedures, we tested commercial two-times-crystallized sheep and bovine hemoglobin. The pure hemoglobins were completely inactive, even at concentrations as high as 5%. We were at a loss to explain this result, until we considered that the inhibitory activity had always behaved as if it were due to a hemin-binding protein. Hemoglobin contains four hemin molecules per globin molecule and during crystallization any hemin-free globin might not be incorporated into the crystals. Therefore, we considered that the inhibitory activity of blood might be due to an excess of hemin-free globin inside the erythrocytes. To test the hypothesis that hemin-free globin is inhibitory, we extracted the hemin from the crystallized sheep hemoglobin with acid-acetone and renatured the hemin-free, native globin. This native globin produced clear zones of inhibition (17 to 21 mm) around the wells in Schaedler agar plates.

DISCUSSION

The inhibition of *B. fragilis* strains by blood appears to be due to the presence of hemin-free

hemoglobin (native globin) in erythrocytes. This would explain both the elimination of the inhibition by added hemin and the fact that the dose-response curves to hemin suggest saturation of some component of the blood. The fact that the phenomenon is dependent on the medium used is not so easily explained, but it may depend on such factors as ionic strength, pH, etc., that would affect solubility of hemin, and the affinity of the native globin for the hemin. The differences in the extent of inhibition of the various subspecies (DNA homology groups) suggest that the subspecies differ either in the amount of hemin required for growth or in their ability to compete with native globin for hemin. We have not found evidence of this inhibitory phenomenon during our routine testing of hundreds of strains of other anaerobes that comprised most of the species commonly found in human infections. This would be expected because these organisms did not require hemin.

The phenomenon could be of importance in the pathogenicity of *B. fragilis*. Several investigators suggested that the animal host may restrict the amount of iron available to pathogens, via transferrin and other iron-binding proteins (1). The same could be true of hemin. Hemin is a complex molecule that the body must synthesize. To conserve this molecule, the body may produce extra globin to bind any free hemin. Thus, it is interesting to speculate that a *B. fragilis* cell that has an absolute requirement for hemin for growth may have to compete with the body for this compound. In this regard, it is interesting that the subspecies *B. fragilis fragilis*, which is the most common subspecies isolated from infections, was also the

least inhibited by the hemin-binding capacity of blood.

The inhibition of strains of *B. fragilis* on BHI, Schaedler, Trypticase soy, Columbia, and blood agar base blood plates is naturally of concern to clinical laboratories. However, the inhibition can be eliminated by addition of 10 μ g hemin per ml to whole blood agar plates or 40 μ g/ml to laked blood plates. We have not detected inhibition of other organisms by 40 μ g of hemin per ml, but we have not tested a large enough number of strains to be certain of this point. Alternatively, brucella blood agar plates or chocolate blood plates could be used.

ACKNOWLEDGMENTS

This work was supported by Public Health Service grant no. GM 14604 from the National Institute of General Medical Sciences and by a grant from the Upjohn Co.

LITERATURE CITED

1. Bullen, J. J., H. J. Rodgers, and E. Griffiths. 1974. Bacterial iron metabolism in infection and immunity, p. 517-551. In *Microbial iron metabolism*. Academic Press Inc., New York.
2. Falk, J. E. 1964. Porphyrins and metalloporphyrins. Elsevier Publishing Co., New York.
3. Holdeman, L. V., and W. E. C. Moore (ed.). 1975. *Anaerobe laboratory manual*, 3rd ed. Virginia Polytechnic Institute and State University, Blacksburg, Va.
4. Johnson, J. L. 1973. Use of nucleic-acid homologies in the taxonomy of anaerobic bacteria. *Int. J. Syst. Bacteriol.* 23:308-315.
5. Lemberg, R., and J. W. Legge. 1949. Hematin compounds and bile pigments. Interscience Publishers, New York.
6. Vesterberg, O. 1971. Isoelectric focusing of proteins, p. 389-412. In W. B. Jakoby (ed.), *Methods in enzymology*, vol. 22. Academic Press Inc., New York.