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Inactivation of a Streptococcal Bacteriocin (Viridin B) by Mammalian Hemoglobin (39829)[1]

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Viridin B, a bacteriocin produced by *Streptococcus mitis* (*S. mitior*), has been described recently (1, 2). This bacteriocin has several unusual characteristics. It is heat labile, has a broad activity spectrum, and does not adsorb readily to susceptible bacteria. Viridin B was shown to be bactericidal to *Neisseria sicca* but only bacteriostatic to a coagulase-negative staphylococcus (1). This dual effect was substantiated and expanded by studying ultrastructural modifications and some biochemical alterations in these two indicator strains (2).

While investigating viridin B properties, we fortuitously discovered that its inhibitory capacity is completely absent when tested on sheep blood agar plates. This feature has not been described previously for any other bacteriocin (3). The purpose of this report is to describe the inactivation of viridin B by mammalian hemoglobin and to discuss implications of such inactivation.

Materials and methods. The bacteriocin-producing organism is a *Streptococcus mitis* strain 42885. The indicators used were *Neisseria sicca* strain 15362, which is killed by the bacteriocin, and a coagulase-negative staphylococcus strain 23709, which is only inhibited by the bacteriocin. A detailed description of these strains has been reported previously (1).

Viridin B preparations were made from an ammonium sulfate precipitate of mechanically disrupted *S. mitis* cells, as reported earlier (1). Preparations were stored at −20° until use. The concentration of viridin B ranged between 80 and 640 arbitrary units (A.U.), determined according to a described procedure (1). Partially purified preparations were obtained by gel filtration on a Sephadex G-200 column (1, 2).

Human erythrocytes were obtained from healthy donors. Ovine, bovine, and rabbit cells were obtained commercially (Flow Laboratories, Rockville, Md.). All cells were suspended in Alsever's solution. Prior to use, erythrocytes were washed three times in phosphate-buffered saline (PBS), pH 7.2. Equal volumes of packed erythrocytes and bacteriocin preparations, dialyzed previously in PBS, were mixed and incubated at 25° for varying times. Twofold dilutions of the mixtures were made in PBS, and the titer of the bacteriocin was determined as described previously (1). Controls consisted of equal volumes of bacteriocin and PBS treated as above.

Lysates from sheep erythrocytes were obtained by suspending them in sterile distilled water for 15 min at 25°. To 1 ml of packed cells, 10 ml of distilled water was added. The supernatant fluid, after centrifugation at 2000*g* for 20 min, was sterilized by filtration through 0.45-μm Millipore filters. Equal volumes of lysate and viridin B were mixed and tested as above to determine bacteriocin titer.

Sheep erythrocyte lysate was fractionated by gel filtration on a Bio-Rad P-100 column. Five milliliters of lysate was placed on the 1.5 × 90-cm column, and 6-ml aliquots were collected. Inactivation of viridin B was tested by mixing equal volumes of twofold dilutions from each aliquot to a bacteriocin preparation containing 40–80 A.U. The neutralizing activity was expressed as the reciprocal of the highest dilution that totally abolished the bacteriocin activity.

Heme was obtained from sheep erythrocyte lysate according to the method of Kan *et al.* (4). Hemoglobin was precipitated by zinc acetate and was solubilized in cold acid acetone. Additional acid acetone precipi-

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tated the globin. The acid acetone in the supernatant was evaporated under vacuum suction, and the remaining solution was dialyzed against PBS and was used as the heme preparation.

Globin was obtained by the methyl ethyl ketone extraction method (5). To acidified hemoglobin, ice-cold methyl ethyl ketone was added separating heme in the ketone layer and globin in the aqueous layer. The latter was dialyzed against PBS and was used as the globin preparation.

Cytochrome c (Sigma Chemical Co., St. Louis, Mo.) was tested at concentrations of 2, 1, and 0.2 mg/ml. Bilirubin (Pfanstiehl Laboratories, Waukegan, Ill.) was used at concentrations from 0.002 to 0.33 mg/ml. It was solubilized in 0.75 *M* NaOH, the pH was adjusted to 6.9 with 5 *N* HCl, and the solution was dialyzed against PBS. Imferon (Lakeside Laboratories, Milwaukee, Wis.) was used as a ferric-dextran complex at concentrations of 0.005 to 5 mg/ml.

The effect of various substances on viridin B activity was tested by mixing with equal volumes of the bacteriocin preparation containing 80 A.U. After incubation at 25° for 1 hr, twofold dilutions of the mixtures and controls were made in PBS, and 0.05 ml from each dilution was spotted onto a lawn of the indicator strain. The bacteriocin titer was the reciprocal of the highest dilution that caused inhibition of indicator strain growth.

Results. Tryptic soy agar and sheep blood agar plates were seeded with the *Neisseria sicca* indicator strain. Twofold dilutions of a viridin B preparation were made, and 0.05 ml of varying dilutions was spotted onto the plates. The results are shown in Fig. 1. Inhibition of the indicator strain by the viridin B preparation can be noted on the tryptic soy agar plate, but no inhibition can be observed on blood agar. Also, when chocolate agar was used, no inhibition could be detected. Similar results were obtained when the coagulase-negative staphylococcus was used as the indicator strain.

Since the only difference among the above media is the presence of erythrocytes or their products in the blood and chocolate agar plates, the effect of sheep erythrocytes on viridin B activity was investigated. When a partially purified bacteriocin preparation was added to washed and packed sheep erythrocytes, immediate lysis of the cells occurred. Assay for viridin B activity in the mixture or in the supernatant fluid revealed no bactericidal activity. Whole erythrocytes, red cell fragments, or filtered supernatant fluid of erythrocyte lysate were all effective in abolishing the bactericidal activity.

Lysates of human, ovine, bovine, or rabbit erythrocytes destroyed the bactericidal effect of viridin B. Some of the physical and chemical properties of the neutralizing activity contained in ovine erythrocyte lysate were investigated. The neutralizing activity was nondialyzable but did pass through a 0.45-μm filter (Millipore). Activity was not affected by heating at 80° for 15 min, but was destroyed when boiled for 5 min. Treatment with 0.5 mg/ml of trypsin or protease did not alter the activity.

Erythrocyte lysate, filtered, and dialyzed against PBS, was fractionated by gel filtration on a Bio-Rad P-100 column calibrated for molecular weight determination. A narrow single peak was obtained that contained all the neutralizing activity against the bacteriocin (Fig. 2). This peak of activity corresponded to a molecular weight of approximately 60,000, the established molecular weight of hemoglobin.

The suggestion that the activity resides in hemoglobin prompted the investigation as to the component of this substance responsible for the neutralizing activity. Heme and globin were obtained from sheep erythrocyte lysate preparation as outlined under Materials and methods. The effect of heme

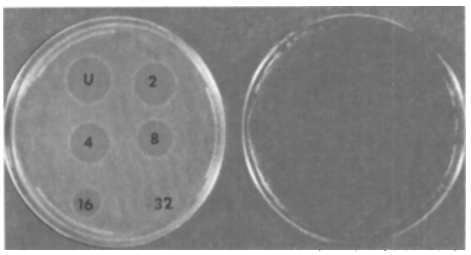

FIG. 1. Inhibition of *Neisseria sicca* (clear areas) by various concentrations of viridin B on tryptic soy agar (left). Numbers are reciprocals of twofold dilutions of the bacteriocin. No inhibition can be detected on sheep blood agar (right) using the same concentrations spotted on corresponding areas.

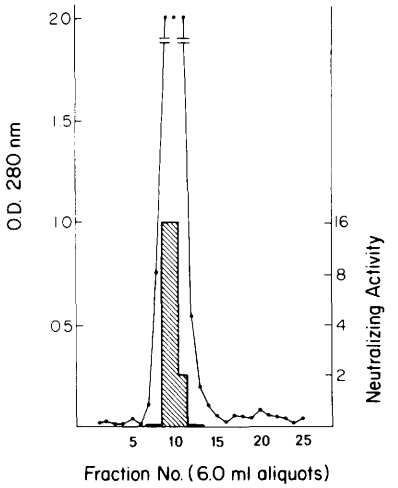

FIG. 2. Fractionation of sheep erythrocyte lysate on a Bio-Rad P-100 column (1.5 × 90 cm). Each fraction was assayed for bacteriocin neutralizing activity (shaded area).

and globin preparations on viridin B activity was tested as illustrated in Fig. 3. A tryptic soy agar plate devoid of serum was seeded with the indicator strain. Uniformly sized wells were made in the agar, and the wells were filled subsequently with various preparations. The plates were incubated at 37° and were checked after 18–24 hr for inhibition zones around the wells. Globin had no effect on the bacteriocin activity. Heme, on the other hand, totally abolished the bactericidal effect of viridin B when heme was put in wells 15 min before or simultaneously with the bacteriocin. Addition of heme 15 min after bacteriocin did not inhibit the bactericidal effect.

Viridin B inactivation by heme was tested in a checkerboard titration using varying concentrations of the two substances. The inactivation was dependent on both bacteriocin and heme concentrations. It was determined that 0.08 ng/ml of heme inactivated 1 A.U. of viridin B.

It has been described for other bacteriocins that certain substances, such as serum or albumin, may block receptors on the surface of indicator cells to which bacteriocins adsorb, thus rendering the cells resistant to the lethal effects of the bacteriocin (3). This possibility was investigated in the present system. Indicator cells of *Neisseria sicca* were preincubated with the heme preparation for various periods of time, and the cells were then washed free of any excess heme. When such cells were treated with viridin B they were still susceptible to its lethal effect and in a manner similar to control cells not previously incubated with heme. This suggests that the effect of heme is not due to blockage of receptors, but rather to a direct effect on the bacteriocin itself.

Substances chemically related to heme were also tested for their capacity to inactivate viridin B. Cytochrome c, bilirubin, ferrous sulfate, and ferric-dextran complex at various concentrations had no effect on the bacteriocin. Attempts were made to study the effect of protoporphyrin IX (dimethyl ester); however, this substance was soluble only in organic solvents that destroyed the bacteriocin or were inhibitory to the indicator strain.

The effect of other substances that have been reported to inhibit certain other bacteriocins was investigated (Table 1). Among the cations tested, only Ca^{2+} exhibited an inhibitory effect. Urea was also inhibitory but only in high concentrations.

Discussion. Bacteriocins, substances produced by bacteria that inhibit other bacteria, are common among many species (3). Bacteriocinogeny is a mechanism of bacterial interference; however, a definitive role for bacteriocins *in vivo* has not been established. Bacteriocin inactivation by sub-

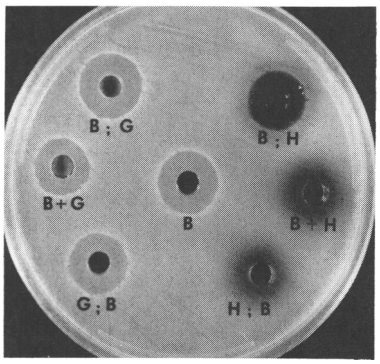

FIG. 3. Effect of globin and heme on viridin B bactericidal activity. Center well (B) contains bacteriocin and equal volumes of buffer. Set of three wells on left contain bacteriocin followed 15 min later by globin (B;G), bacteriocin and globin premixed (B+G), or globin followed 15 min later by bacteriocin (G;B). Heme (H) was substituted for globin in wells on right.

TABLE 1. EFFECT OF VARIOUS SUBSTANCES ON VIRIDIN B[a]

Substance	Concentration (*M*)	Effect
NaCl	0.01; 0.1; 1	None
KH_2PO_4	0.01; 0.1; 1	None
NaH_2PO_4	0.01; 0.1; 1	None
$MgSO_4$	0.01; 0.1; 1	None
$MnCl_2$[b]	0.01; 0.1	None
$CaCl_2$	0.01; 0.1	None
	1	Total inactivation
Urea	0.01; 1	None
	7	Total inactivation

[a] Amount of viridin B: 80 A.U.

[b] $MnCl_2$ at 1 *M* inhibited the indicator strain and therefore could not be tested.

stances naturally found in a particular ecosystem is an important consideration, since the presence of such substances may not favor the expression of the bacteriocin effect *in vivo*.

Several bacteriocin inhibitors have been described. Bacteriocins of gram-positive bacteria are susceptible *in vitro* to certain proteolytic enzymes (3). Other substances, including certain body fluids, have also been shown to inactivate certain bacteriocins. Normal human sera neutralized the effect of staphylococcin C55. This neutralizing activity was heat labile and distinct from antibody (6). Some streptococcal bacteriocins have been shown to be inactivated by saliva, presumably due to the presence of proteases (7). Bacteria normally susceptible to a *Streptococcus mutans* bacteriocin *in vitro* become resistant in the presence of sucrose, due to elaboration of dextran and levan polysaccharides (8). These substances are major components of dental plaque matrix and are believed to be responsible for the inhibitory effect of dental plaque against some bacteriocins (7).

The observations made in this report are unique since inactivation of bacteriocins by heme or other erythrocyte contents has not been described previously. In screening organisms for bacteriocinogeny, care must be exercised, therefore, to avoid the use of inhibitory media. Viridin B was shown to be inactivated by heme but not by some chemically related substances. Heme seemed to act directly on the bacteriocin. The mechanism of such inactivation was not investigated in the present report.

The possibility that biological substances in blood other than erythrocytes may inactivate viridin B deserve exploration. Recent studies (Dajani, A. S. and Veres, C.: data presented at the annual meeting of the American Society for Microbiology, May 13, 1977) indicate that lysates of human leukocytes and unheated human and rabbit sera also inactivate viridin B. Bovine serum albumin had no effect on the bacteriocin.

Lysis of erythrocytes was noted promptly after addition of the bacteriocin preparation. The preparation used was only partially purified (1), and it is possible that other biologically active substances may be present in it. It is also possible that the bacteriocin and hemolysin produced by *S. mitis* may reside on the same molecule. A bacteriocin produced by *Streptococcus faecalis* subsp. *zymogenes* (9) has been shown to be identical to the hemolysin produced by this strain (10). Further studies are in progress to assess the possible identity of viridin B with the *S. mitis* hemolysin.

Summary. Viridin B, a bacteriocin of *Streptococcus mitis* (*S. mitior*), had no bactericidal effect on indicator strains grown on blood agar, whereas activity was readily demonstrable on the same agar devoid of blood. Addition of viridin B to human, ovine, bovine, or rabbit erythrocytes caused immediate lysis of the cells and total inactivation of the bacteriocin. Inactivation was effected also by erythrocyte lysates. Activity in the lysates was heat resistant, nondialyzable, and insensitive to proteolytic enzymes. Chromatography on a Bio-Rad P-100 column indicated that the inhibitory activity resided exclusively in hemoglobin. Fractionation of hemoglobin showed that heme and not globin was totally responsible for this inactivation. The effect of heme appeared to be directly on viridin B rather than on blockage of indicator cell receptors. Cytochrome c, bilirubin, and ferrous and ferric ions had no effect on the bacteriocin. Among various other ions tested, only Ca^{2+} inhibited viridin B. Urea was also inhibitory but only in high concentrations.

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