BIKEN JOURNAL Vol. 21 1-8, 1978

COMPARISON OF BACTERIAL CARDIOTOXINS: THERMOSTABLE DIRECT HEMOLYSIN FROM *VIBRIO PARAHAEMOLYTICUS*, STREPTOLYSIN O AND HEMOLYSIN FROM *LISTERIA MONOCYTOGENES*

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(Received November 7, 1977)

SUMMARY Some properties of the bacterial cardiotoxins, thermostable direct hemolysin from *Vibrio parahaemolyticus* (vibriolysin), and streptolysin O and hemolysin from *Listeria monocytogenes* (listeriolysin), were compared. These toxins had rapid lethal effects on mice when injected intravenously. The electrocardiographic changes of rats after intravenous injections of these toxins were very similar, showing bradycardia and inhibition of atrio-ventricular conduction. These toxins also caused cessation of the spontaneous beating and degeneration of cultured foetal mouse heart cells. When equal hemolytic units of these three toxins were administered, vibriolysin had the most potent effects on mice and cultured mouse heart cells. Differences in the kinetics of the hemolysis by each toxin and in the effects of cholesterol of their hemolytic actions suggest that the mode of action of vibriolysin is different from those of streptolysin O and listeriolysin.

INTRODUCTION

In previous papers we reported that purified thermostable direct hemolysin from *Vibrio parahaemolyticus* (vibriolysin) showed cardiotoxic activity (Honda et al., 1976a and 1976b; Goshima et al., 1977; Seyama et al., 1977). Intravenous injection of 5 to 10 μg of vibriolysin into either mice or rats killed the animals within 1 to 2 min. Electrocardiograms of rats after intravenous administration of vibriolysin showed significant changes, such as depression of intra-atrial and intra-ventricular conduction of electrical activation, while electroencephalograms traced simultaneously remained normal for quite a long time after the heart of the animals had stopped beating. Beating of isolated perfused hearts of rabbits and rats was also stopped by vibriolysin. Moreover, vibriolysin was also toxic to cultured heart cells of rats and mice and the beating of cultured cells was stopped by addition of low concentrations of vibriolysin to the medium.

The bacterial exotoxins, streptolysio O (Cantoni and Bernheimer, 1945; Keller et al., 1956; Halbert et al., 1961; Halbert, 1971) and hemolysin from *Listeria monocytogenes* (listeriolysin) (McIlwain et al., 1964; Kingdon and Sword, 1971; Sword and Kingdon, 1971) have also been reported to show cardiotoxicity; both toxins caused significant changes in the electrocardiograms of experimental animals such as mice and rabbits when injected intravenously.

In this work we compared the properteis of these bacterial cardiotoxins and discussed the significance of the cardiotoxic activity of vibriolysin in *V. parahaemolyticus* infection.

MATERIALS AND METHODS

1. *Preparation of purified vibriolysin*

Vibriolysin was isolated from the culture filtrate of *V. parahaemolyticus* WP-1 (RIMD 2210086) and purified by ammonium sulfate fractionation and successive column chromatographies on diethylaminoethyl-cellulose, hydroxylapatite and Sephadex G-200, as reported previously (Honda et al., 1976b). The purified vibriolysin was demonstrated to be a single protein by sodium dodecyl sulfate-polyacrylamide gel disc electrophoresis, analytical ultracentrifugation and immunoelectrophoresis. The specific activity of the purified vibriolysin was 4,000 HU/mg protein.

2. *Preparation of partially purified streptolysin O*

Streptococcus pyogenes RIMD 3126114 (group A, type 3) was cultured without shaking in Todd-Hewitt medium at 37 C for 16 hr. The culture filtrate was mixed with solid ammonium sulfate (39.0 g per 100 ml) and the resulting precipitate was dissolved in a small amount of 0.1 M phosphate buffer (Na_2HPO_4-KH_2PO_4, pH 7.0) containing 3 mg/ml of cysteine hydrochloride and dialysed against the same buffer. Then the preparation was applied to a hydroxylapatite colum (2.2 by 30 cm) previously equilibrated with 0.1 M phosphate buffer (pH 7.0) containing 3 mg/ml of cysteine hydrochloride. The column was washed with the same buffer, and then eluted with a linear gradient of 0.1 to 0.3 M phosphate buffer containing 3 mg/ml of cysteine hydrochloride. Fractions with hemolytic activity were dialyzed against 0.1 M phosphate buffer (pH 7.0) containing 3 mg/ml of cysteine hydrochloride and used as partially purified streptolysin O in this study. Before use, streptolysin O was preincubated with an equal volume of 0.1 M phosphate buffer (pH 7.0) containing 3 mg/ml of cysteine hydrochloride at 37 C for 30 min.

3. *Preparation of partially purified listeriolysin*

Listeria monocytogenes RIMD 1205020 (Furuse strain provided by Dr. T. Nagai, Sapporo Medical College) was cultured without shaking in Brain Heart Infusion Broth at 37 C for 24 hr. The same procedures were used for fractionation of the culture filtrate with ammonium sulfate and hydroxylapatite column chromatography of the preparation as those for streptolysin O, except that the phosphate buffer did not contain cysteine hydrochloide. Fractions with hemolytic activity were dialyzed against 0.1 M phosphate buffer and used as partially purified listeriolysin in this study. Listeriolysin was preincubated with an equal volume of 0.1 M phosphate buffer (pH 7.0) containing 3 mg/ml of cysteine hydrochloride at 37 C for 30 min.

4. *Assay of the lethal toxicity of the toxin*

Lethal toxicity was assayed by intravenous injection of the toxins into 4- to 6-week-old mice (ddO strain) and measurement of the survival time of the animals.

5. *Electrocardiography*

Electrocardiograms of rats after injection of the toxins under anesthesia with 800 mg of urethane per kg body weight were recorded with a Nihon-Denko polygraph. Leads from the electrodes were placed in the right foreleg and left hind leg.

6. *Culture of mouse heart cells*

Mouse heart cells from 14- to 16-day-old fetal mice were cultured essentially as described previously (Goshima, 1969 and 1975; Honda et al., 1976a; Goshima et al., 1977). After incubation at 37 C for 24 hr all the cells beat spontaneously, synchronously and regularly at 100 to 180 beats/min, and the beat was maintained stably for at least 24 hr when the cells were kept at 37 C in the standard medium described previously (Honda et al., 1976a). The beating rate of cell clusters changes in response changes of temperature and potassium and calcium and calcium concentrations in the medium, in a

manner similar to that of normal heart. A volume of less than 0.1 ml of concentrated toxin solution that had been passed through a membrane filter (Millipore Corp.) was added to 1.8 to 2 ml of the medium with gentle shaking.

7. *Assay of hemolytic activity*

Hemolytic activity was assayed essentially as described previously (Takeda et al., 1974). The standard reaction mixture (1.0 ml) contained 0.9% NaCl, about 8×10^7 washed mouse erythrocytes per ml and the indicated amount of either vibriolysin, streptolysin O or listeriolysin. The reaction mixture was incubated at 37 C for 20 min and then centrifuged at 3,000 rpm for 5 min, and hemolytic activity was determined by measuring the absorbance of the resulting supernatant fluid at 540 nm. In this study, the amount of hemolytic agent which lysed 50% of the mouse erythrocytes (about 8×10^7 cells) in 1.0 ml of reaction mixture on incubation at 37 C for 20 min was defined as 1 hemolytic unit (HU).

RESULTS

1. *Lethal toxicities of vibriolysin, streptolysin O and listeriolysin*

The lethal toxicities of vibriolysin, streptolysin O and listeriolysin to mice were measured and the results are shown in Table 1. As reported previously (Honda et al., 1976), intravenous injection of 4 HU (1 μg) of vibriolysin killed all the animals in about 20 min. Intravenous injections of streptolysin O and listeriolysin also killed the animals, but more hemolytic units of these compounds were needed to kill all the animals. The minimum lethal doses of streptolysin O and listeriolysin were 24–34 HU and 160–240 HU, respectively. From the data shown in Table 1, the LD_{50} of streptolysin O and listeriolysin were calculated to be 16 HU and 110 HU, respectively, these values being much greater than that of vibriolysin.

TABLE 1. *Lethal effects of various bacterial cardiotoxins on mice*

Amount administered intravenously (HU)	No. of deaths/ No. of mice tested	Survival time (Mean±S.D.) (min)
Vibriolysin		
4	10/10	18.7±4.8
2	0/10	—
Streptolysin O		
34	5/5	17.1±8.6
24	5/6	20.3±2.4
17	3/5	24.9±8.2
12	1/5	35.0
6	0/5	—
Listeriolysin		
240	7/7	15.2±5.7
160	5/7	16.2±7.6
80	2/7	28.3±1.8
40	0/7	—

2. *Electrocardiographic changes of rats after intravenous injections of streptolysin O and listeriolysin*

Typical electrocardiograms of rats after intravenous injections of streptolysin O and listeriolysin are shown in Fig. 1 (streptolysin O) and Fig. 2 (listeriolysin). In each case, the significant changes in the electrocardiograms indicated the cardiotoxicity of the toxin. Bradycardia was observed a few minutes after the injection followed by inhibition of atrioventricular conduction. These changes in the electrocardiograms were very similar to those observed after injection of vibriolysin (Honda et al., 1976a).

3. *Effects of vibriolysin, streptolysin O and listeriolysin on the beating of cultured mouse heart cells*

Cessation of the beating and degeneration of cultured mouse heart cells after addition of vibriolysin have been reported (Honda et al., 1976a). The effects of streptolysin O and listeriolysin on cultured mouse heart cells are summarized in Table 2. Like vibriolysin, low concentrations of streptolysin O and listeriolysin stopped the beating of cultured mouse heart cells, the minimum effective concentra-

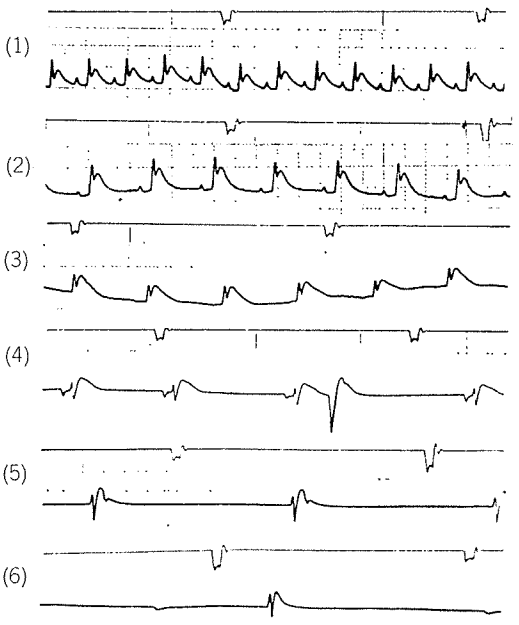

FIGURE 1.

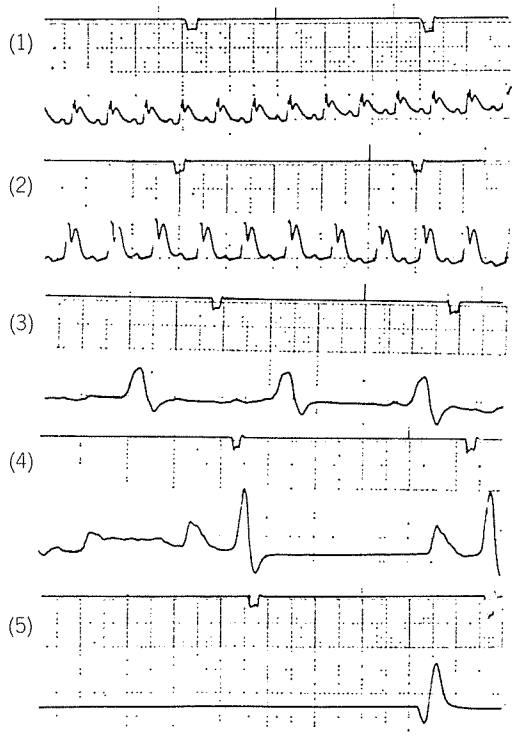

FIGURE 2.

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FIGURE 1. Electrocardiograms of a rat injected with streptolysin O. 1800 HU of streptolysin O was injected intravenously into a rat weighing 300 g. Electrocardiograms were recorded as described in the text. Panel (1) shows electrocardiograms before toxin administration and panels (2), (3), (4), (5) and (6) show those 3 min, 23 min, 52 min, 56 min and 60 min, respectively, after the injection. The interval between spikes in the upper line in each panel represents 1 sec.

◀

FIGURE 2. Electrocardiograms of a rat injected with listeriolysin. 1200 HU of listeriolysin was injected intravenously into a rat weighing 280 g. Electrocardiograms were recorded as described in the text. Panel (1) shows electrocardiograms before toxin administration and panels (2), (3), (4) and (5) show those 1 min, 15 min, 23 min and 36 min, respectively, after the injection. The interval between spikes in the upper lines of each panel represents 1 sec.

TABLE 2. *Effects of various bacterial cardiotoxins on cultured mouse heart cells*

Amount of toxin added to medium (HU/ml)	No. of cells stopped/No. of cells examined	No. of degenerated cells/No. of cells examined
Vibriolysin		
2	12/12	10/12
1	16/16	0/16
0.4	15/18	0/18
0.2	9/10	0/10
0.1	0/10	0/10
Streptolysin O		
4	3/3	3/3
2	7/10	0/10
1	1/5	0/5
0.5	0/5	0/5
Listeriolysin		
80	5/5	5/5
20	3/3	0/3
16	2/2	0/2
10	2/3	0/3
8	0/4	0/4

tions of vibriolysin, streptolysin O and listerolysin being 0.2 HU, 1 HU and 10 HU, respectively. When higher concentrations of these toxins were added to the medium, the cells degenerated.

4. *Comparison of the kinetics of hemolysis by vibriolysin, streptolysin O and listeriolysin*

Figure 3 shows the kinetics of hemolysis of mouse erythrocytes induced by vibriolysin, streptolysin O and listeriolysin. The kinetics of hemolysis by streptolysin O (Fig. 3B) and listeriolysin (Fig. 3C) were quite similar: hemolysis started immediately after addition of the toxins and reached a plateau after about 10 to 30 min on incubation at 37 C; moreover the degree of hemolysis was proportional to the amount of the toxin added to the reaction mixture. On the other hand, with vibriolysin (Fig. 3A) a certain lag time was observed before hemolysis started, the time required to reach the plateau was much longer, and the action continued until all the erythrocytes in the reaction mixture had been lysed, even when only a small amount of toxin was added.

5. *Effect of cholesterol on the hemolysis by vibriolysin, streptolysin O and listeriolysin*

It has been found that hemolysis by streptolysin O is inhibited by cholesterol (Halbert, 1971). As shown in Fig. 4, hemolysis by listeriolysin was also inhibited by cholesterol, but hemolysis by vibriolysin was not affected by cholesterol even at high concentration.

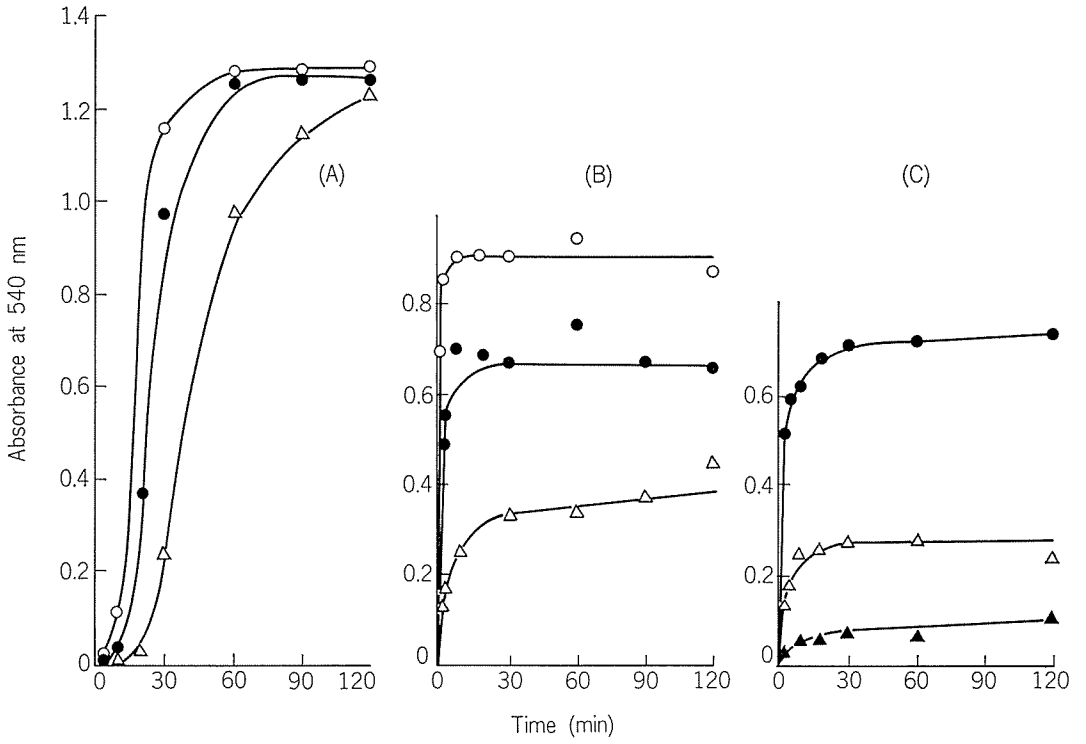

FIGURE 3. Kinetics of hemolysis induced by vibriolysin, streptolysin O and listeriolysin. Hemolysis was assayed as described in the text except that the time of incubation was as indicated. (A) Vibriolysin; 1.5 HU (○), 1.0 HU (●), 0.5 HU (△). (B) Streptolysin O; 1.5 HU (○), 1.0 HU (●), 0.5 HU (△). (C) Listeriolysin; 1.0 HU (●), 0.5 HU (△), 0.25 HU (▲).

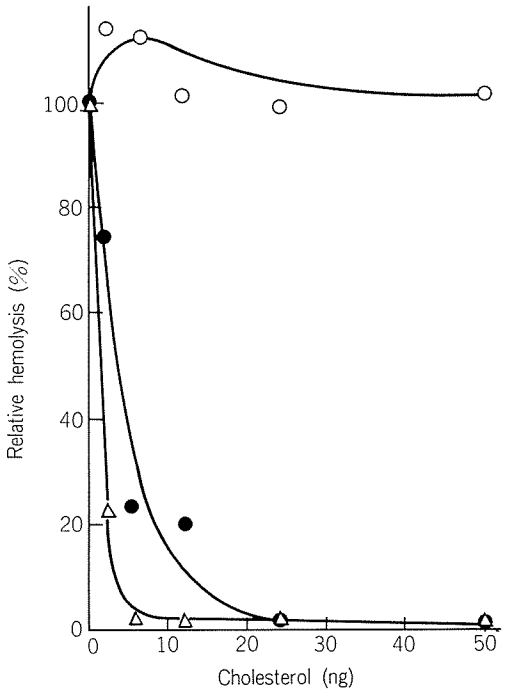

FIGURE 4. Effect of cholesterol on hemolysis induced by vibriolysin, streptolysin O and listeriolysin. The indicated amounts of cholesterol and 1 HU of either vibriolysin, streptolysin O or listeriolysin were incubated at 37 C for 30 min and then the hemolytic activity of each reaction mixture was assayed as described in the text. Cholesterol was dissolved in methanol and a small amount of the solution was used for the incubation. Hemolytic activity in the presence of cholesterol was expressed as a percentage of that in the absence of cholesterol. ○, vibriolysin; ●, streptolysin O; △, listeriolysin.

DISCUSSION

In this work the cardiotoxicities and some other properties of thermostable direct hemolysin from *V. parahaemolyticus* (vibriolysin), streptolysin O and hemolysin from *Listeria monocytogenes* (listeriolysin) were compared. On intravenous administration, these toxins killed mice within a short time due to their cardiotoxicity, which was demonstrated as changes in the electrocardiograms and inhibition of the beating of cultured mouse heart cells. Comparison of the cardiotoxicities of these three toxins on cultured mouse heart cells showed that the amount (HU) of vibriolysin necessary to stop the beating of cultured mouse heart cells and cause their degeneration was less than that of streptolysin O or listeriolysin.

Various other purified bacterial exotoxins studied in this laboratory did not show cardiotoxicity on cultured mouse heart cells; these exotoxins were cholera enterotoxin, staphylococcal enterotoxin, diphtheria toxin, tetanus neurotoxin, *Clostridium botulinum* type E toxin and *Clostridium perfringens* enterotoxin (Goshima et al., 1977). Hardegree et al. (1971) reported the cardiotoxicity of tetanolysin, an oxygen-labile toxin produced by *Clostridium tetani*. Thus it seems probable that other oxygen-labile toxins, such as pneumolysin and cereolysin have cardiotoxic activity.

We reported recently that the cardiotoxic action of vibriolysin is due to depolarize the membrane without affecting the mechanism for generating an action potential (Goshima et al., 1977; Seyama et al., 1977). However, little is known about the mechanisms of the cardiotoxic actions of streptolysio O and listeriolysin. As shown in Fig. 3, their hemolytic actions were quite different from that of vibriolysin. These two toxins are both oxygen-labile (Halbert, 1971; Sword and Kingdon, 1971), whereas vibriolysin is not. Moreover they are heat-labile, whereas vibriolysin is stable on heating at 100 C for 10 min (Miwatani and Takeda, 1976). Another difference is that their hemolytic actions are inhibited by cholesterol, whereas that of vibriolysin is not (Fig. 4). Thus, their actions are probably different from that of vibriolysin.

Since the main symptoms of *V. parahaemolyticus* infection are diarrhea and abdominal cramp (Miwatani and Takeda, 1976), little attention has been paid to the cardiotoxin produced by *V. parahaemolyticus*. Recently, we demonstrated that the titer of antibody against

vibriolysin was increased in sera of patients suffering from gastroenteritis due to *V. parahaemolyticus* (Honda et al., 1976c; Miwatani et al., 1976). We also demonstrated that electrocardiograms of patients with *V. parahaemolyticus* infection showed significant changes in the T wave and sometimes the P wave also (Honda et al., 1976c). Moreover, in Japan, several deaths due to *V. parahaemolyticus* infection have been reported (Fujino et al., 1953; Miwatani and Takeda, 1976). These observations, together with the data presented in this paper, suggest that the cardiotoxin produced by *V. parahaemolyticus* may contribute to the clinical symptoms of *V. parahaemolyticus* infection.

ACKNOWLEDGMENTS

The authors express their thanks to Dr. K. Goshima for his interest and discussion of this study. Thanks are also due to Dr. T. Nagai for kindly providing a strain of *Listeria monocytogenes*.

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