BIKEN JOURNAL Vol. 18, 187-192 1975

INTERACTION OF THERMOSTABLE DIRECT HEMOLYSIN OF *VIBRIO PARAHAEMOLYTICUS* WITH HUMAN ERYTHROCYTES

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(Received July 5, 1975)

SUMMARY The interaction between thermostable direct hemolysin produced by *Vibrio parahaemolyticus* WP-1 and human erythrocytes was studied. The lysis of human erythrocytes by the hemolysin was dependent of temperature and no hemolysis occurred at low temperature (0–4 C), but the hemolysin was adsorbed on human erythrocytes even at low temperature. No hemolysis was observed when antihemolysin antiserum was mixed with the hemolysin and human erythrocytes at zero time. On the other hand, lysis of the cells by hemolysin was not completely inhibited when the antiserum was added during the lag time and the inhibitory effect decreased with delay in the time of addition of antiserum. The inhibitory effect of the antiserum decreased with increase in the incubation temperature, increase in the concentration of divalent cations, and decrease in pH.

These results suggest that lysis of human erythrocytes by the hemolysin is at least a two-step process consisting of adsorption of the hemolysin to human erythrocytes and the step(s) following adsorption.

INTRODUCTION

Most pathogenic strains of *Vibrio parahaemolyticus* produce thermostable direct hemolysin, which causes a hemolytic ring on Wagatsuma medium (Miyamoto et al., 1969). The thermostable direct hemolysins produced by all Kanagawa phenomenon positive strains are found to be antigenically identical and no cross reactions are found between the hemolysins produced by Kanagawa phenomenon negative strains (Sakurai et al., 1974).

Previously we reported purification of thermostable direct hemolysin and some properties of the highly purified preparation (Miwatani et al., 1972; Sakurai et al., 1973). We also found that the crude hemolysin showed an Arrhenius effect like the α-toxin in *Staphylococcus aureus* (Arbuthnott, 1970; Miwatani et al., 1972; 1974). It seems important to study the mode of action of the hemolysin, but there have been no previous reports on this.

This report describes the interaction between thermostable direct hemolysin of *V.*

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parahaemolyticus and human erythrocytes.

MATERIALS AND METHODS

1. *Preparation of thermostable direct hemolysin*

The purified thermostable direct hemolysin of *Vibrio parahaemolyticus* WP-1 was prepared as described previously (Sakurai et al., 1973).

2. *Assay of hemolytic activity of the hemolysin*

Fresh human erythrocytes were washed three times with saline and then suspended in either 0.01 M phosphate buffer (pH 7.0) containing 0.9% NaCl or 0.01 M tris (hydroxymethyl) aminomethane buffer (pH 7.0) containing 0.9% NaCl at a final concentration of 1%. Suspensions were used within 8 hr after preparation.

One hemolytic unit (HU) is defined as the amount of thermostable direct hemolysin which causes 50% hemolysis of 1 ml of 1% suspension of human erythrocytes in 0.01 M phosphate buffer (pH 7.0) containing 0.9% NaCl on incubation at 37 C for 2 hr. The mixture were centrifuged at 3,000 rpm for 10 min and hemolysis was measured by determining the absorbance at 540 nm with a Coleman spectrophotometer. All assays of hemolysis were performed in a final volume of 2.0 ml. Complete hemolysis was achieved by suspending the same volume of human erythrocyte in 0.1% solution of Na_2CO_3.

3. *Preparation of antihemolysin antiserum*

Antihemolysin antiserum of the purified thermostable direct hemolysin was prepared by immunizing rabbits as described previously (Sakurai et al., 1973).

RESULTS

1. *Effect of temperature on the hemolytic activity and binding of the hemolysin*

As shown in Fig. 1, no hemolysis occurred when human erythrocytes were exposed to the hemolysin at 0–4 C for 2 hr in 0.01 M phosphate buffer (pH 7.0) containing 0.9% NaCl and maximal hemolysis was observed when they were incubated at 37 C.

To measure the hemolysin which was bound to human erythrocytes, the hemolysin and the cells were incubated at 37 C and the mixture was centrifuged before hemolysis occurred.

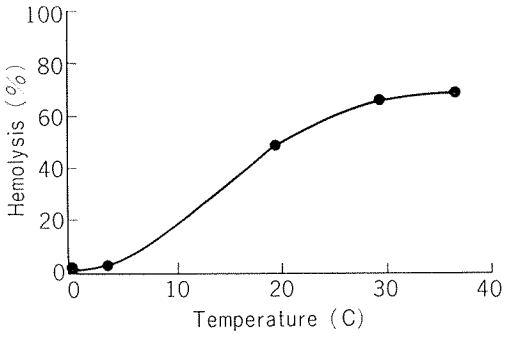

FIGURE 1. *Effect of temperature on hemolytic activity. 0.6 HU of hemolysin and 1% human erythrocytes in 0.01 M phosphate buffer (pH 7.0) containing 0.9% NaCl were incubated for 2 hr at the temperatures indicated. Hemolysis was measured by determining hemoglobin release at 540 nm after centrifugation (3,000 rpm, 10 min) at 4 C. Hemolysis (%) is expressed as a percentage of the hemolytic activity of 1.0 HU of hemolysin in 0.01 M phosphate buffer (pH 7.0) containing 0.9% NaCl at 37 C in 2 hr.*

However, little hemolysin was found to be bound to the cells. Thus, it seemed likely that the hemolysin dissociated from the cells during centrifugation. To avoid this dissociation, the binding of the hemolysin to human erythrocytes at 0 C was examined using the method described in the legend of Table 1. As shown in Table 1, human erythrocytes which had been precipitated through 5% sucrose solution after contact with hemolysin showed the same degree of hemolysis at 37 C in fresh phosphate buffer (0.01 M, pH 7.0) containing 0.9% NaCl in the presence and absence of antihemolysin antiserum sufficient to neutralize the hemolytic activity completely. On the other hand, no hemolysis was observed in 2 hr at 37 C when the antihemolysin antiserum was mixed with hemolysin and human erythrocytes which had been precipitated through 5% sucrose solution without contact with hemolysin. These results show that the hemolysin is bound to human erythrocytes at 0 C, but that it does not lyse the cells and that hemolysin which is bound to human erythrocytes is not neutralized by antihemolysin antiserum.

TABLE 1. *The adsorption of the hemolysin to human erythrocytes at 0 C*

Materials overlaid on 5% sucrose solution[a]		Materials added against human erythrocytes precipitated[b]		Hemolytic activity
Human erythrocytes	Hemolysin[c]	Hemolysin	Antiserum[d]	
+	−	−	−	0
+	−	+	+	0
+	−	+	−	0.430
+	+	−	−	0.120
+	+	−	+	0.130

[a] 1.0 ml of hemolysin (0.8 HU) or 1.0 ml of 0.01 M phosphate buffer (pH 7.0) containing 0.9% NaCl and 1.0 ml of 1% human erythrocytes were mixed and then the mixtures were gently overlaid on 2.5 ml of 0.01 M phosphate buffer (pH 7.0) containing 5% sucrose solution in a column (1.0×10 cm). The column was stood at 0 C for 16 hr and then the human erythrocytes precipitated through the sucrose solution were harvested and suspended in 1.0 ml of 0.01 M phosphate buffer (pH 7.0) containing 0.9% NaCl.

[b] The precipitated human erythrocytes and materials indicated were mixed and incubated at 37 C for 2 hr.

[c] Hemolytic activity 0.8 HU.

[d] Antihemolysin antiserum prepared against purified hemolysin as described in the text.

TABLE 2. *Effect of pH on the activity of thermostable direct hemolysin*[a]

pH	Hemolysis (%)[b]							
	Incubation time (min)							
	0	3	5	10	20	30	60	120
6.0	0	0	7.8	23.5	56.9	74.5	110	120
7.0	0	0	0	7.8	31.4	47.1	76.5	100
8.0	0	0	0	0	9.8	17.6	39.2	78.4

[a] 1.0 HU of hemolysin and 1% human erythrocyte suspension were incubated at 37 C in 0.01 M phosphate buffer containing 0.9% NaCl.

[b] Hemolysis (%) is expressed as a percentage of the hemolytic activity of 1.0 HU of hemolysin in 0.01 M phosphate buffer (pH 7.0) containing 0.9% NaCl in 2 hr at 37 C.

2. *Effect of temperature on the interaction between the hemolysin and human erythrocytes*

As shown in Table 2, hemolysis occurred after 10 min of incubation when the hemolysin and human erythrocytes were incubated in 0.01 M phosphate buffer (pH 7.0) containing 0.9% NaCl at 37 C. About 40% of the hemolysis was prevented when antiserum was added to the mixture (pH 7.0) after 3 min of incubation at 37 C. The inhibitory effect of the antiserum decreased with delay in the time of its addition (Table 3). The inhibitory effect also decreased with increase in the incubation temperature of the hemolysin and the cells. These results suggest that in the hemolytic process with hemolysin there was a lag time after the hemolysin was bound to human erythrocytes, and that there were several steps in the hemolytic process, only some which were prevented by the antiserum.

3. *Effect of pH on the interaction between the hemolysin and human erythrocytes*

As shown in Table 2, the hemolytic activity was between pH 6.0 and 8.0 and lower at pH 8.0. When the cells were incubated in the absence of the hemolysin under the same conditions, no lysis was observed.

TABLE 3. *Inhibitory effect of the antihemolysin antiserum on the activity of thermostable direct hemolysin under various conditions*[a]

pH	Temp (C)	Hemolysis (%)[b]						
		Preincubation time before adding the antiserum (min)						
		0	3	5	10	20	30	60
6.0	0	0	0	0	26.5	32.7	38.8	49.0
	4	0	40.8	91.8	98.0	100	106	112
	37	0	89.8	98.0	100	108	112	116
7.0	0	0	0	0	0	20.4	26.5	40.8
	4	0	24.5	53.1	61.2	67.3	75.5	77.6
	37	0	63.3	73.5	79.6	89.8	93.9	100
8.0	0	0	0	0	0	0	20.4	28.6
	4	0	12.2	32.7	44.9	51.0	55.1	59.2
	37	0	51.0	61.2	71.4	79.6	85.7	87.8

[a] The hemolysin (1.0 HU) and 1% human erythrocyte suspension were preincubated in 0.01 M phosphate buffer containing 0.9% NaCl at various temperatures and the pH's indicated and then 20 μliters of antihemolysin antiserum which completely inhibited the activity of the hemolysin was added and the mixtures were incubated at 37 C for 2 hr.

[b] Hemolysis (%) is expressed as a percentage of the hemolytic activity of 1.0 HU of hemolysin in phosphate buffer (0.01 M, pH 7.0) in 2 hr at 37 C.

The effect of pH on the interaction between the hemolysin and the cells was studied by exposing the cells to the hemolysin at various pH's and adding the antiserum to the mixtures at various times. As shown in Table 3, the hemolysis occurred when the mixtures were kept for 10 min at pH 6.0, 20 min at pH 7.0 or 30 min at pH 8.0 at 0 C. The inhibitory effect of the antiserum decreased with decrease in the pH from pH 6.0 to 8.0 and was independent of temperature.

4. *Effect of cations on the interaction between the hemolysin and human erythrocytes*

The effects of cations on the hemolytic activity and the interaction were studied by exposing human erythrocytes to the hemolysin in 0.01 M tris (hydroxymethyl) aminomethane buffer (pH 7.0) containing 0.9% NaCl in the presence of appropriate concentrations of cation. The hemolytic activity was enhanced by addition of 25 mM divalent cation but not 400 mM potassium ion (Fig. 2A). None of the cations tested caused hemolysis in the absence of the hemolysin under the present conditions. The inhibitory effect of the antiserum decreased in the presence of the divalent cations, Ca^{++}, Mn^{++}, and Mg^{++} (Fig. 2B) and with increase in the concentration of calcium ion (0–25 mM).

5. *Effect of horse erythrocytes on hemolytic activity*

Horse erythrocytes are resistant to the hemolysin, as described previously (Miwatani et al., 1972). If the hemolysin is adsorbed onto horse erythrocytes, then hemolysis of a constant amount of human erythrocytes should decrease on adding increasing amounts of horse erythrocytes. However, when the hemolysin was incubated with a constant amount of human erythrocytes and various amounts of horse erythrocytes at 37 C for 2 hr, it was found that the horse erythrocytes did not affect the lysis of human erythrocytes (Fig. 3). This result suggests that horse erythrocyte membranes do not have the specific site(s) found to which the hemolysin binds on the surface of human erythrocytes.

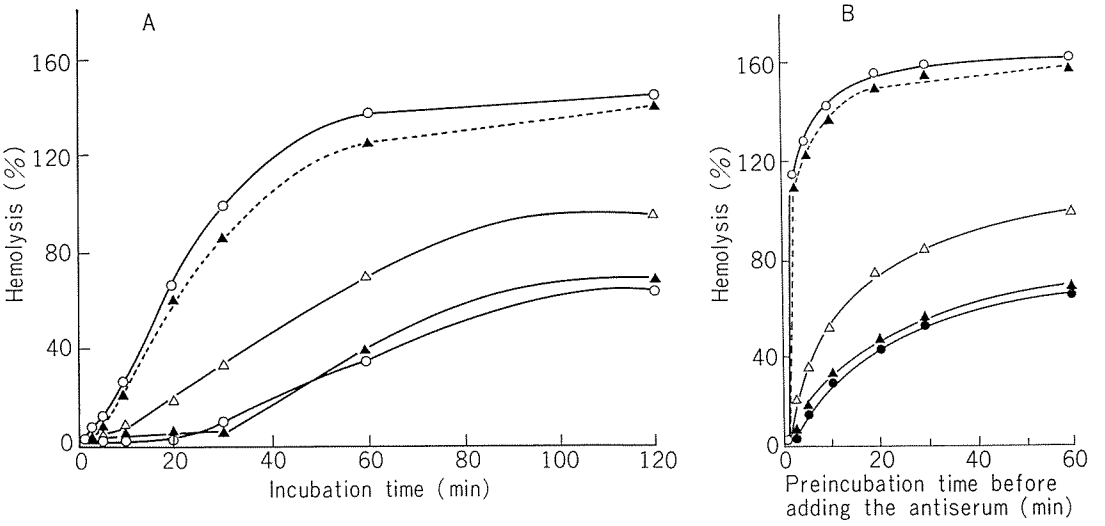

FIGURE 2. *Effects of cations on hemolytic activity. (A) Time course of hemolysis. 0.3 HU of the hemolysin and 1% human erythrocytes in 0.01* M *tris (hydroxymethyl) aminomethane buffer (pH 7.0) containing 0.9% NaCl were incubated in the presence of 25 mM divalent cations or 400 mM potassium ion at 37 C. (B) Time course of adsorption of the hemolysin to human erythrocytes. The hemolysin and 1% human erythrocyte suspension were preincubated under the same conditions for various times and then 20 μliters of the antihemolysin antiserum, which completely inhibited the activity of the hemolysin was added and the mixtures were incubated at 37 C for 2 hr. Hemolysis (%) was determined as described in the legend to Fig. 1. Symbols:* ○—○, Ca^{++}; ▲---▲, Mn^{++}; △—△, Mg^{++}; ▲—▲, K^{+}; ●—●, *Control.*

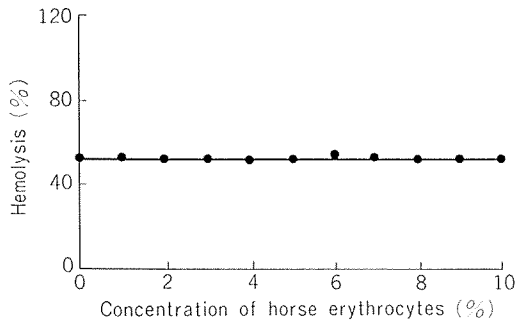

FIGURE 3. *Effect of horse erythrocytes on hemolytic activity. A 0.5 HU of the hemolysin, 1.0 ml of 0.5% human erythrocyte suspension and 0.5 ml volumes of various concentrations of horse erythrocyte suspension in 0.01* M *phosphate buffer (pH 7.0) containing 0.9% NaCl were incubated at 37 C for 2 hr. Hemolysis (%) was determined as described in the legend to Fig. 1.*

DISCUSSION

Thermostable direct hemolysin of *V. parahaemolyticus* was adsorbed to human erythrocytes at a low temperature, at which no hemolysis occurred. These results distinguished the process of adsorption from subsequent events leading to hemolysis. The initial interaction between the hemolysin and the cells appears to be adsorption of the hemolysin to the surfaces of the cells.

It is clear that free hemolysin which is not adsorbed to human erythrocytes is completely neutralized by antiserum, but we do not know whether hemolysins which are adsorbed to the cell surface are all neutralized by the antiserum. In this work we found that no hemolysis occurred when antiserum was mixed with hemolysin and human erythrocytes at zero time, and that the inhibitory effect of the antiserum decreased with delay in the time of its addition. These results suggest that the antiserum does not prevent all steps in the hemolytic process.

This work suggests that there are at least two steps in the lysis of human erythrocytes by

the thermostable direct hemolysin of *V. parahaemolyticus*: (i) interaction of the hemolysin with the target-cell membrane, (ii) a subsequent biochemical step(s) that results either directly or indirectly in changes of the membrane leading to cell lysis.

Bernheimer suggested that most cytolytic bacterial toxins interact with various lipids in membrane components (Bernheimer, 1968) and it was reported that staphylococcal alpha toxin (Arbuthnott, 1970) may become adsorbed to the cell surface by a hydrophobic interaction. Oberly and Duncan (1971, 1972) also reported that the interaction between streptolysin O and rabbit erythrocytes was independent of pH or ionic strength. On the other hand, in this work, we demonstrated that the interaction between the hemolysin of *V. parahaemolyticus* and human erythrocytes was dependent on pH and ionic strength. The activity of the hemolysin was inhibited by ganglioside but not by cholesterol, sphingomyelin (unpublished observation) or purified lecithin (Sakurai et al., 1973). Thus, hydrophilic interactions between the hemolysin and the cell may be important in this adsorption process. Polysaccharide in human erythrocyte membranes may be the component directly involved in the adsorption process of the hemolysin of *V. parahaemolyticus*.

This hemolysin may also be a valuable tool in studies of biological membrane structure, since its action is unique.

REFERENCES

Arhuthnott, J. P. 1970. Stahylococcal *a*-toxin, p. 189–236. *In* T. C. Montie, S. Kadis and S. J. Ajl [ed.], Microbial toxins, vol. 3. Academic Press Inc., New York.

Bernheimer, A. W. 1968. Cytolytic toxins of bacterial origin. Science 159: 847–851.

Miwatani, T., J. Sakurai, A. Yoshihara, and Y. Takeda. 1972. Isolation and partial purification of thermolabile direct hemolysin of *Vibrio parahaemolyticus*. Biken J. 15: 61–66.

Miwatani, T., Y. Takeda, J. Sakurai, A. Matsuzaki, and S. Taga. 1972. Effect of heat (Arrhenius effect) on crude hemolysin of *Vibrio parahaemolyticus*. Infect. Immun. 6: 1031–1033.

Miwatani, T., J. Sakurai, Y. Takeda, and S. Shinoda. 1974. Studies on direct hemolysin of *Vibrio parahaemolyticus*, p. 245–251. *In* T. Fujino, G. Sakaguchi, R. Sakazaki, and Y. Takeda [ed.] International Symposium on *Vibrio parahaemolyticus*. Saikon Publishing Co., Ltd., Tokyo.

Miyamoto, T., T. Kato, T. Obara, S. Akiyama, K. Takizawa, and S. Yamai. 1969. In vitro hemolytic characteristic of *Vibrio parahaemolyticus*; Its close correlation with human pathogenicity. J. Bacteriol. 100: 1147–1149.

Oberley, T. D., and J. L. Duncan. 1971. Characteristics of streptolysin O action. Infect. Immun. 4: 683–687.

Oberley, T. D., and J. L. Duncan. 1972. Streptolysin O induced alterations in rabbit erythrocyte membrane polypeptides. Biochem. Biophys. Res. Commun. 48: 1339–1347.

Sakurai, J., A. Matsuzaki, And T. Miwatani. 1973. Purification and characterization of thermostable direct hemolysin of *Vibrio parahaemolyticus*. Infect. Immun. 8: 775–780.

Sakurai, J., A. Matsuzaki, Y. Takeda, and T. Miwatani. 1974. Existence of two distinct hemolysins in *Vibrio parahaemolyticus*. Infect. Immun. 9: 777–780.