Anti-LPS factor in the horseshoe crab, *Tachypleus tridentatus*

Its hemolytic activity on the red blood cell sensitized with lipopolysaccharide

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Anti-LPS factor, which inhibits the endotoxin mediated coagulation system in the horseshoe crab, *Tachypleus tridentatus*, was found to lyse red blood cells sensitized with gram-negative bacterial LPS, but not to lyse unsensitized cells. This hemolysis occurred even at 0°C and was completed within 1 min. The binding of anti-LPS factor to LPS must be essential for the hemolysis, because free LPS inhibited the hemolytic action of anti-LPS factor.

Horseshoe crab *Lipopolysaccharide* *Hemolysis* *Red blood cell*

1. INTRODUCTION

Lipopolysaccharide (LPS) of gram-negative bacteria induces degranulation and blood coagulation of the amebocyte of the horseshoe crab. Amebocytes contain an LPS-mediated coagulation system consisting of several protein factors and an anticoagulant, named anti-LPS factor, which inhibits the initial stage of the coagulation cascade activated by LPS [1]. Anti-LPS factor has recently been purified and characterized as a basic protein with a molecular mass of 15 kDa [2,3]. As the other biological function, anti-LPS factor has antibacterial action against R-types of gram-negative bacteria [2]. In the course of these studies, we found that anti-LPS factor exerted hemolytic action on the red blood cells sensitized with LPS. This paper describes characteristics of the anti-LPS factor-mediated hemolysis.

Abbreviations: LPS, lipopolysaccharide; RBC, red blood cells

2. MATERIALS AND METHODS

Anti-LPS factor was isolated from the amebocyte lysate of *Tachypleus tridentatus* as in [1]. The final preparation was confirmed to show a single protein band on SDS–PAGE [4]. *Salmonella minnesota* R595 LPS (Re type) was prepared by the phenol–chloroform–petroleum ether method [5]. LPS of *S. minnesota* 1114W (S-type) and *E. coli* 0113 were hot-phenol extracts [6]. This LPS was dissolved in saline and sonicated at 20 kHz for 1 min before use. Native polysaccharide of *E. coli* 0113 was the O-antigenic polysaccharide moiety of *E. coli* 0113 LPS [7]. Red blood cells (RBS) were collected from human (O-type), chicken and horse blood with an addition of heparin. RBC were sensitized with the LPS derived from *S. minnesota* R595, 1114W and *E. coli* 0113. RBC were washed 3 times with 50 mM Tris-buffered saline (pH 7.2) and the concentration of the suspension was adjusted to 2.5%. One ml of this suspension was mixed with 0.5 ml LPS solution of 1 mg/ml and incubated at 37°C for 30 min. After the incubation,

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Table 1

Hemolytic activity of anti-LPS factor on RBC sensitized with LPS

RBC sensitized with	Dilution of anti-LPS factor											
	2	4	8	16	32	64	128	256	512	1024	2048	Control
S. minnesota R595 LPS (Human RBC)	+	+	+	+	+	+	+	+	+	+	−	−
S. minnesota 1114W LPS (Human RBC)	+	+	+	+	+	+	+	+	+	+	+	−
S. minnesota 1114W LPS (Chicken RBC)	+	+	+	+	+	+	+	+	+	+	−	−
S. minnesota 1114W LPS (Horse RBC)	+	+	+	+	+	+	+	+	+	+	+	−
E. coli 0113 LPS (Human RBC)	+	+	+	+	+	+	+	+	+	−	−	−
None (Human RBC)	−	−	−	−	−	−	−	−	−	−	−	−
None (Chicken RBC)	−	−	−	−	−	−	−	−	−	−	−	−
None (Horse RBC)	−	−	−	−	−	−	−	−	−	−	−	−

Fifty μl of 0.5% RBC sensitized with LPS was mixed with 50 μl of a 2-fold serial dilution of anti-LPS factor in a microtiter U-plate and incubated at 37°C for 30 min. The concentration of original solution of anti-LPS factor was 2 mg/ml. +, hemolysis-positive; −, hemolysis-negative

sensitized RBC were washed 3 times with the same buffer and finally the concentration of the suspension was adjusted to 0.5%. The RBC sensitized with LPS were checked to be agglutinated by the antiserum to the bacterium from which LPS was derived.

3. RESULTS

3.1. *Hemolytic activity of anti-LPS factor to the RBC sensitized with LPS*

The RBC sensitized with LPS were mixed with a 2-fold serial dilution of anti-LPS factor and incubated at 37°C for 30 min. After the incubation, unexpected hemolysis was observed in the wells in which the sensitized RBC and anti-LPS factor were mixed (table 1). However, anti-LPS factor did not cause any hemolysis of unsensitized RBC. Horse and chicken RBC sensitized with LPS were also hemolysed in the presence of anti-LPS factor. In the case of human and horse RBC sensitized with *S. minnesota* R595 LPS, the minimum concentration of anti-LPS factor for hemolysis was as low as 0.98 μg/ml (table 1). Although the hemolysis occurred in spite of the species of RBC, human RBC sensitized with LPS were used in the following experiments.

3.2. *Inhibition of anti-LPS factor-mediated hemolysis by free LPS*

The sensitized RBC were added to the mixtures in which anti-LPS factor and a 2-fold serial dilution of free LPS had been preincubated. As shown in table 2, *S. minnesota* R595 LPS completely inhibited the hemolysis of RBC sensitized with *S. minnesota* R595 LPS as in the case of the *S. minnesota* 1114W LPS system. But *E. coli* 0113 native polysaccharide did not inhibit the hemolysis of RBC sensitized with *S. minnesota* R595 LPS. The inhibitory effect of free LPS on the hemolysis caused by anti-LPS factor was also demonstrated by measuring hemoglobin liberated from the sensitized RBC in the presence of free LPS with various doses. As shown in fig.1, 0.31 μg *E. coli* 0113 LPS could block the hemolysis of the RBC sensitized with *S. minnesota* R595 LPS caused by

Table 2

Inhibition test of the hemolysis by free LPS

Endotoxin mixed with anti-LPS factor	Dilution of free LPS										
	4	8	16	32	64	128	256	512	1024	2048	Control
S. minnesota R595 LPS	−	−	−	−	−	−	−	−	−	−	+
S. minnesota 1114W LPS	−	−	−	−	−	−	−	−	−	−	+
E. coli 0113 native polysaccharide	+	+	+	+	+	+	+	+	+	+	+

Twenty-five μl of a 2-fold serial dilution of free LPS was mixed with 25 μl anti-LPS factor (final concentration 20 μg/ml); then 50 μl human RBC sensitized with the corresponding LPS was added. In the case of *E. coli* 0113 native polysaccharide, RBC sensitized with *S. minnesota* R595 LPS were used as target cells. The concentration of the original solution of free LPS was 1 mg/ml. +, hemolysis-positive; −, hemolysis-negative

0.5 μg of anti-LPS factor. In this case, the hemolysis was found to be inhibited by the free LPS (*E. coli* 0113) differently from the LPS (*S. minnesota* R595) used to sensitize RBC.

3.3. *Effect of temperature on the hemolysis*

The hemolysis of RBC sensitized with *S. minnesota* R595 LPS was carried out at 0°C and 37°C for 5 min with various concentrations of anti-LPS factor (fig.2). At 37°C, 0.625 μg/ml anti-LPS factor could complete the hemolysis. In the case of 0°C, 2.5–5.0 μg/ml anti-LPS factor was enough to complete the hemolysis.

3.4. *Time course of the hemolytic process*

The hemolytic process was chased by measuring the turbidity of the RBC sensitized with *S. minnesota* R595 LPS in the presence of anti-LPS factor with various concentrations (31.2, 125 and 250 ng/ml). As shown in fig.3, the hemolytic process was completed within1 min at 250 ng/ml.

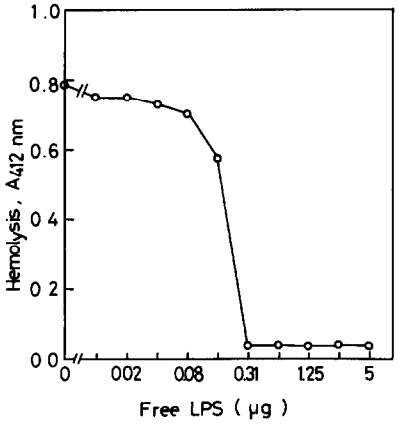

Fig. 1. Neutralization of the hemolysis by free LPS. Fifty μl *E. coli* 0113 LPS with various concentrations were added to 50 μl anti-LPS factor (10 μg/ml) and then 100 μl RBC sensitized with *S. minnesota* R595 LPS. After incubation at 37°C for 5 min, each mixture was centrifuged at 2000 rpm for 5 min. Liberated hemoglobin into supernatant was expressed as the absorbance at 412 nm. Final dose of anti-LPS factor was 0.5 μg.

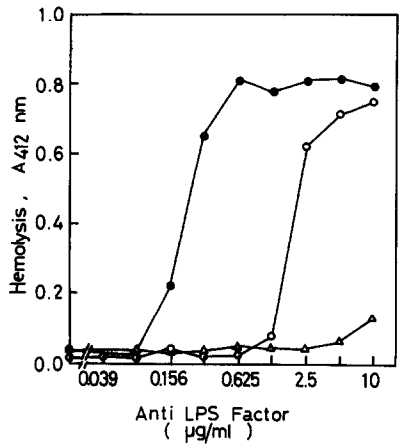

Fig. 2. Effect of temperature on the hemolysis. Reaction mixture containing 100 μl anti-LPS factor with various concentrations, 100 μl RBC sensitized with *S. minnesota* R595 LPS was incubated for 5 min at 0°C (○) and 37°C (●). Unsensitized RBC was also incubated with anti-LPS factor at 37°C as control (△). After the incubation, 800 μl of 50 mM Tris-buffered saline (pH 7.2) was added. Liberated hemoglobin was measured after centrifugation.

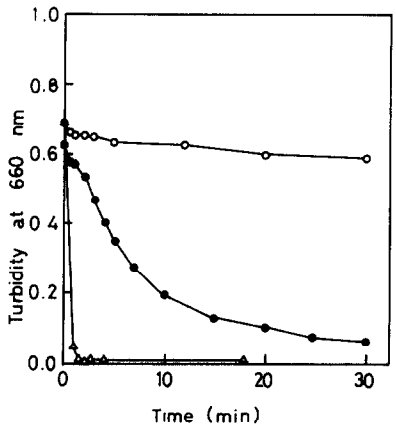

Fig. 3. Time course of the hemolytic process. Reaction mixture containing 100 μl RBC sensitized with *S. minnesota* R595 LPS, 50 μl anti-LPS factor with various concentrations - (○) 31.2 ng/ml, (●) 125 ng/ml, (△) 250 ng/ml as final concentration - and 850 μl of 50 mM Tris-buffered saline (pH 7.2) was made in the cell (light path, 1 cm) at 33°C. Turbidity (at 660 nm) was read at short intervals.

4. DISCUSSION

Anti-LPS factor, which has been identified to be an anticoagulant towards the LPS-mediated coagulation system in the horseshoe crab, *T. tridentatus,* was found to have a hemolytic function with respect to the RBC sensitized with various LPS. Anti-LPS factor seems to bind to the LPS, especially lipid A, embedded in the RBC membrane in that the hemolysis was inhibited by *S. minnesota* R595 LPS (Re type LPS) but not by *E. coli* 0113 native polysaccharide.

Since this hemolysis occurs even at 0°C, and is completed within 1 min at 33°C, the hemolysis induced by anti-LPS factor is likely to be a direct hemolytic reaction specific for LPS rather than an enzymatic reaction.

Binding of anti-LPS factor to lipid A has been suggested from the fact that it is antibacterial-specific for deep rough mutants of gram-negative bacteria and its activity is inhibited by the LPS of a deep rough mutant (unpublished). This binding must be essential for the hemolysis, the antibacterial action and the anticoagulant action of anti-LPS factor.

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