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Anti-lipopolysaccharide activity of histatins, peptides from human saliva

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Abstract. Histatins are histidine-rich polypeptides secreted in human saliva. They were found to inhibit lipopolysaccharide (LPS)-mediated gelation of Limulus amoebocyte lysate, and to reverse the anti-complement action of LPS or lipid A. Histatins also gave precipitate bands in agarose gels with various LPS. The results indicate that histatins neutralized the activity of LPS by binding to the lipid A moiety of LPS.
Key words. Histatins; human saliva; LPS; Limulus test; hemolysis.

Histatins are a family of histidine-rich polypeptides secreted by human and subhuman primate salivary glands. Histatins 1–12 have been isolated from human parotid saliva and their amino acid sequences determined[1–3]. Histatins 1, 3 and 5 comprise 80–90% of the total histatins secreted. They are highly homologous polypeptides with a total of 38, 32 and 24 amino acid residues respectively, and all contain seven histidine residues. These polypeptides have antimicrobial activity[4,5] and inhibit hydroxyapatite crystal growth[6]. They also release histamine from mast cells[1]. However, a biological role of histatins in human saliva has not been defined in detail.

The present investigation describes the inhibitory action of histatins on the LPS-induced gelation of Limulus amoebocyte lysate, and the restoration of the anti-complement activity of LPS by the binding of histatins to its lipid A moiety.

Materials and methods

Histatins 1, 3 and 5 were isolated and purified from human saliva as described previously[7]. LPS from *E. Coli* 0111 B4, *Salmonella minnesota* Re 595 and *Pseudomonas aeruginosa*, and lipid A from *Salmonella minnesota* Re 595 were purchased from Sigma Chemical Co. (St. Louis, Mo.). Sheep red blood cells (SRBC) were sensitized with rabbit-antisheep erythrocyte antibody, and suspended in Veronal buffered saline (pH 7.3) containing 0.1% gelatin, 0.15 mM Ca^{2+} and 0.5 mM Mg^{2+} (ref. 8). The Limulus gelation test was performed with Limulus Test Wako (Wako Pure Chemicals Osaka, Japan), using an amoebocyte lysate prepared from *Limulus polyphemus*. Inhibition of the gelation was done as follows; 100 µl of LPS (0.1 ng/ml) was incubated with 10 µl of histatin at room temperature for 10 min, and the mixture was added to 100 µl of the amoebocyte lysate solution. After incubation for 1 h at 37 °C, the degree of gelation was observed.

The effect of LPS and lipid A on the hemolytic activity of complement was determined by the one-point method described previously[9]. The ability of histatins to prevent the anti-complement activity of LPS or lipid A was measured as follows: 100 µl of LPS (1 µg) or lipid A (0.1 µg) was incubated with histatins for 10 min at 37 °C. An equal volume of a 1/8 dilution of guinea pig serum was then added, and the mixture incubated for 30 min at 37 °C. After the incubation, 2 ml of sensitized SRBC (4×10^9 cells/ml) was added, and the mixture was further incubated for 1 h at 37 °C. Cells were then centrifuged and the percent of hemolysis was determined by the measurement of absorbance of the supernatant at 412 nm.

A double diffusion test was performed in a 0.8% agarose gel containing 10 mM Tris-HCl buffer (pH 7.2).

Results and discussion

The effect of histatins on the ability of *E. Coli* LPS to activate the clotting enzyme cascade of Limulus amoebocyte lysate was examined by the conventional Limulus test. Table 1 shows that the gelation induced by LPS (0.1 ng/ml) was weakly inhibited by 1.0 ng of histatin 5, and completely inhibited by 5.0 ng. The activity of histatin 3 was similar to that of histatin 5, whereas histatin 1 was less active (data not shown).

These results suggested that histatins could neutralize the ability of LPS. To examine the interaction of histatins with LPS, the effect of histatins on the anti-complement activity of LPS was investigated. Figure 1

Table 1. Inhibitory effect of histatin 5 on LPS activity in the Limulus gelation test

	Histatin 5 (ng/ml)	Gelation of amoebocyte lysate[a]
LPS[b] (0.1 ng/ml)	–	+
	0.5	+
	1.0	±
	5.0	–
	10.0	–

[a] +, firm gel formation; ±, soft gel formation; –, no gelation.
[b] LPS from *E. Coli* 0111 B4.

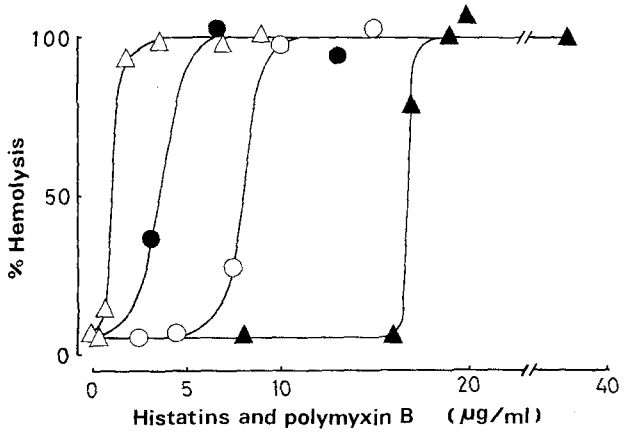

Figure 1. Inhibition of the hemolysis of sensitized sheep erythrocytes by LPS, and its reversal by histatins and polymyxin B. LPS (1 μg) from *Salmonella minnesota* Re 595 was used. The percentage of hemolysis was determined by the procedure described in the text. ●, histatin 5; ○, histatin 3; ▲, histatin 1; △, polymyxin B.

shows that the complement-dependent hemolysis of sensitized SRBC was inhibited by the LPS from *Salmonella minnesota* Re 595, and this inhibition was reversed by the addition of histatins. The order of the reversal effectiveness was histatin 5 > histatin 3 > histatin 1. This reversal effect on the anti-complement activity of LPS was also confirmed with polymyxin B (PB), which is known to abrogate the ability of LPS by binding to lipid A[8]. Table 2 shows that histatins also prevented the anti-complement activity of LPS when lipid A was used instead of LPS. Hemolysis of sensitized SRBC was not caused by the histatin or PB itself.

To provide further evidence that the histatins can interact with LPS, a double diffusion test was performed in an agarose gel. Figure 2A shows that histatin 5 and PB gave a precipitate band against the LPS of *E. Coli*. Histatin 5 also gave a precipitate band when tested against two different LPS (*Pseudomonas aeruginosa* and *Salmonella minnesota*). These precipitate bands were found to fuse with each other (fig. 2B). The data presented here indicate that histatins secreted in saliva bind to the lipid A region of the LPS molecule and neutralize the activity of LPS. Although the mechanism by which

Table 2. Reversibility of the inhibitory effects of the lipid A of *Salmonella minnesota* Re 595 on complement-dependent hemolysis by histatins.

Lipid A (μg)	Histatins (μg) 1	3	5	% Hemolysis
0	-	-	-	100
0.1	-	-	-	7.8
0.1	8.0	-	-	6.9
0.1	15.0	-	-	87.0
0.1	-	0.1	-	11.8
0.1	-	2.5	-	92.5
0.1	-	-	0.1	7.5
0.1	-	-	1.5	93.7

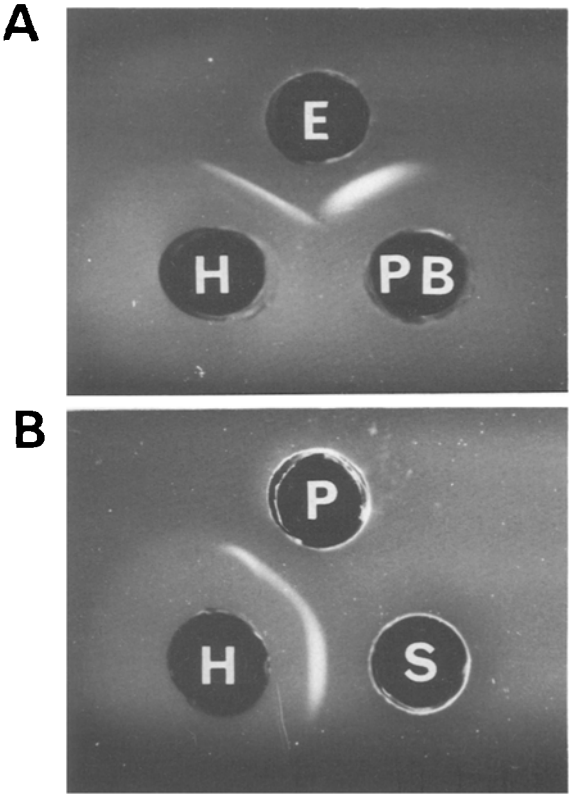

Figure 2. Precipitation of LPS by histatin 5 and polymyxin B in an agarose gel. H, histatin 5 (15 μg); PB, polymyxin B (20 μg); E, *E. Coli* LPS (5 μg); S, *Salmonella minnesota* LPS (5 μg); P, *Pseudomonas aeruginosa* LPS (5 μg).

histatins bind to the LPS is uncertain, positively charged amino acids in the histatins may play an important role. Histatins 1, 3 and 5 contain 36, 47 and 56% of basic amino acids in their molecules, respectively. The effectiveness of the anti-LPS activity of histatins seemed to increase with an increasing content of basic amino acids.

Endotoxic LPS is located in the outer membranes of gram-negative bacteria, and has a variety of biological and pathological activities[10]. The neutralization of LPS by histatins in the oral cavity is an important part of the host's defence system, in which the immune system is not involved. Antibacterial activities of histatins have been reported[4,5], but the details of the mechanism of action of histatins are not yet known. The binding of histatins to the bacterial outer membrane must be essential for their antibacterial action. Although the biological role of histatins in human saliva is unknown at present, it appears that binding of histatins to LPS alters the endotoxic properties of LPS in a way that could alter the host's response to gram-negative infection and endotoxemia.

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