

# Effect of Lipoteichoic Acid on Production and Activity of Streptolysin S

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**Abstract.** Bacterial lipoteichoic acid exhibited carrier activity for streptolysin S, in a resting streptococcal cell system, as well as in ordinary culture. The activity was lower than that of RNase core or polyguanylic acid, and yield of extracellular streptolysin S did not correlate with content of endogenous lipoteichoic acid, among several streptococcal strains. Like other carrier substances, lipoteichoic acid inhibited hemolytic activity of streptolysin S.

**Key words :** Streptolysin S, lipoteichoic acid, streptococci.

Streptolysin S(SLS), a serum-extractable and oxygen-stable streptococcal hemolysin, is produced extracellularly as active form, only when certain carrier substance is added exogenously. The active hemolysin is a complex composed of SLS peptide and the carrier such as RNase core of RNA, polyguanylic acid, albumin, bisazobenzidine dye or nonionic detergent ( 1 ). Except for serum components, such carrier substances are absent or scarce in natural environment for hemolytic streptococci. On the other hand, Theodore and Calandra have reported that lipoteichoic acid (LTA) extracted from staphylococci and streptococci activates membrane-bound SLS precursor in vitro ( 2 ). Although they have briefly mentioned that the amphiphile induced the formation of extracellular SLS, detailed study is not performed as yet.

In order to get clue to natural carrier for the hemolysin, effects of LTA were examined on production of extracellular SLS in whole cell system, and on the hemolytic activity of the toxin, in comparison with those of lipopolysaccharide (LPS), another amphiphile of bacterial outer membrane.

## Materials and Methods

**Chemicals and Enzymes.** RNase I core of yeast RNA, guanosine - 5'- monophosphate (GMP) and LTA of *Streptococcus pyogenes* and *Bacillus subtilis* were purchased from Sigma Chemical Co., USA. LPS of *Salmonella* (Ra, Rd<sub>1</sub> and Rd<sub>2</sub> and Re core) were obtained from BioCarb Chemicals, Lund, Sweden. Pronase was from Kaken Co., Tokyo and endoproteinase Glu-C (*Staphylococcus aureus* V8 protease) was from Seikagaku Kogyo Corp., Tokyo. Oligonucleotide-SLS complex was prepared as

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described previously ( 3 ). Titration of SLS, definition of hemolytic unit and determination of SLS-inhibiting activity were as described elsewhere (4 – 6). The carrier activity for SLS was determined as described previously (7, 8).

*Strains, Media and Culture Technique.* Strain Sa, an avirulent mutant of *Streptococcus pyogenes* ( 4 ) was from our laboratory stock. Strains T1 (low LTA content), M13 (low LTA content, focal distribution) and M53 (high LTA content) were generous gifts from Dr. M. Wagner of Lund University, whereas strains M34 (low LTA content) and M49 (high LTA content) were kindly supplied by Dr. H. Miörner of Zentralinstitut für Mikrobiologie und Experimentelle Therapie, Jena. Unless specified, the cocci were grown in a peptone-meat infusion broth (ordinary broth) or Todd-Hewitt broth (THB) at 37°C for 15 h, without shaking.

## Results

*Carrier Activity of Lipoteichoic Acid.* When washed cells of strain Sa were incubated with LTA, significant amount of hemolysin was produced extracellularly. Activity of this hemolysin was inhibited by Trypan blue, a specific inhibitor of SLS (data not shown). In the carrier activity for SLS, LTA from *Streptococcus pyogenes* was somewhat more potent than that from *Bacillus subtilis*. At the concentration range tested, however, LTA was evidently less active than RNase core of yeast RNA (Fig. 1). Similar results were obtained, when the carrier activity was tested in a growing culture of Sa (data not shown). Under the present conditions, the carrier activity of the RNase core reached a plateau at 150–200 µg/ml, whereas that of LTA further increased, albeit at a reduced rate.

If streptococcal LTA is endogenous carrier, content or distribution of this amphiphile might affect production of SLS. In order to test this possibility, SLS yield was compared in four streptococcal strains with different LTA content (10, 11, and personal communication). As shown in Table I, SLS yield was not related with cellular LTA content, regardless of the carrier species. In addition, the carrier activity of RNase core was distinctly higher than LTA, among the four strains. Besides the resting cell system, these strains were grown at 37°C in ordinary broth supplemented with 100 µg/ml of RNase core or 1% yeast RNA. Specific activity of SLS produced after the culture did not reflect LTA content of each strain (Table II).

*Effect of Protease Treatment.* It has been known that significant amount of surface LTA, potential endogenous carrier, is released by trypsin treatment of streptococcal cells ( 10 , 11 ). The protease treatment, therefore, might deprive the cocci of the capacity to produce the extracellular hemolysin in the resting cell system.

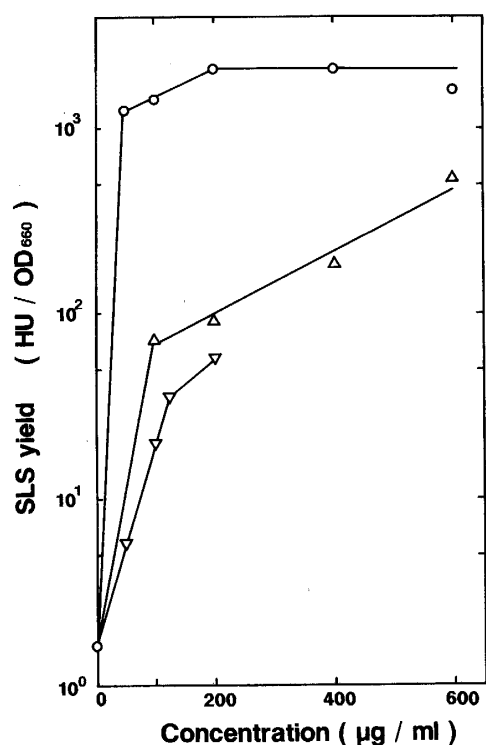


Fig. 1. Washed cells of strain Sa were incubated in BBM (9) containing the indicated substance at 37°C for 60 min, centrifuged, and SLS yield in the supernatant was determined. (○): RNase core of yeast RNA; (△): *Streptococcus pyogenes* LTA; (▽): *Bacillus subtilis* LTA.

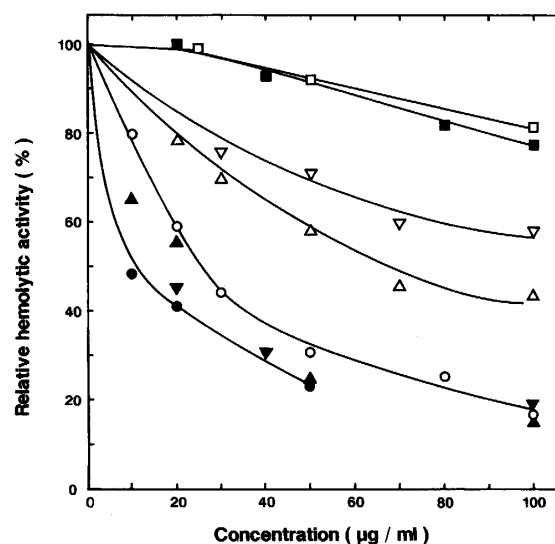


Fig. 2. Oligonucleotide-SLS ( $4.9 \times 10^3$  HU/ml) was diluted with 0.15 M saline containing the indicated substance, and incubated with erythrocytes, for determination of hemolytic activity. (○): RNase core; (△): *Streptococcus pyogenes* LTA; (▽): *Bacillus subtilis* LTA; (□): GMP; (●): Ra LPS (▲): Rd<sub>1</sub> LPS; (▼): Rd<sub>2</sub> LPS; (■): Re LPS.

Table I. The carrier activity of LTA and RNA core in four streptococcal strains

Strain (LTA content)	Broth			THB		
	LTA	RNA core	Ratio	LTA	RNA core	Ratio
T1 (low)	4.9	115.2	0.043	21.1*	26.5*	0.796*
M53 (high)	200.5	903.0	0.222	12.6*	13.8*	0.913*
M34 (low)	28.5	167.7	0.170	32.6	238.9	0.136
M49 (high)	11.8	155.0	0.076	11.8	529.9	0.022

Streptococcal cells grown at 37°C, for 15 h in ordinary broth, or for 3 h\* or 15 h in THB, were collected, washed 3 times with BBM, and suspended in BBM containing 100 μg/ml of streptococcal LTA or RNase core of yeast RNA. After incubation at 37°C for 60 min, each suspension was centrifuged, and hemolytic activity (HU/OD<sub>660</sub>) in the supernatant was determined. In the absence of the carrier, each strain did not produce active extracellular SLS.

Moreover, the treatment might release putative SLS-LTA complex into the surrounding medium (SLS per se is resistant to trypsin). For clarification of these points, cells of five streptococcal strains differing in LTA content were treated with trypsin, and then examined for the capacity to produce extracellular SLS, in the resting cell system. As shown in Table III, trypsin treatment rather enhanced the capacity for the hemolysin production, irrespective of the cellular LTA content. In addition, hemolytic activity of the supernatant, obtained from the coccal suspension treated with trypsin in the absence of exogenous carrier, was negligibly low. As shown in Table IV, CBH (12, 13) titer (cell-lyzing activity by direct contact) was also enhanced by pronase treatment.

*Absence of The Carrier Activity in LPS.* LPS of Gram-negative bacilli is, like LTA of Gram-positive bacteria, distributes on cell surface and amphiphilic. Several *Salmonella* LPS variously deficient in core oligosaccharide region were incubated with resting streptococci and hemolytic activity in the supernatant was assayed after centrifugation. Although polyguanylic acid was a potent carrier for SLS, its monomer unit GMP was inactive, and this nucleotide was included as a negative control. As seen in Table V, these LPS were devoid of the carrier activity for SLS.

*Inhibition of SLS activity by LTA and LPS.* Hemolytic activity of SLS is inhibited by polyguanylic acid ( 7 ). On the other hand, Congo red, an inhibitor of SLS, exhibits distinct carrier activity for hemolysin ( 6 ). It is thus interesting to examine the effect of LTA on hemolytic activity of SLS. As shown in Fig. 2, LTA from *Streptococcus pyogenes* and *Bacillus subtilis* were both suppressive to the hemolysin but, in accordance with the higher carrier activity, streptococcal LTA preparation was more efficient inhibitor to SLS. The RNase core of yeast RNA per se interfered with hemolytic action of SLS complex which contained the core as the carrier moiety, whereas GMP hardly affected the hemolysis. These results demonstrate that the carrier molecules have inherent dual functions on SLS: stabilization (activation) and inhibition of SLS activity. Interestingly, hemolytic activity of SLS was markedly inhibited by Ra, Rd<sub>1</sub> and Rd<sub>2</sub> LPS. but not particularly affected by Re core. This result indicated participation of proximal heptose-linked pyrophosphoryl ethanolamine in the inhibitory effect of LPS on the hemolysin. It is thus evident that a carrier substance acts as an inhibitor of the hemolysin, but an SLS inhibitor does not always work as the carrier.

*Protease-sensitivity of SLS.* SLS has been reported to have a relatively high content of glutamic acid ( 14 ). Hemolytic activity of SLS was not significantly affected by 6 hour treatment with 100 µg/ml of endoproteinase Glu-C (*Staphylococcus aureus* V8 protease, Ref.15 ) in ammonium bicarbonate buffer (pH 7.8) or in sodium phosphate buffer (pH 7.8) at 37°C. On the other hand, more than 94% of the hemolysin was destroyed within 30 min, by 100 µg/ml of pronase. It seems probable that glutamic acid and aspartic acid

Table II. SLS production in growing streptococci differing in LTA content.

Exp	Strain (LTA content)		HU/OD <sub>660</sub>
I	M34	(low)	2.1 X 10 <sup>4</sup>
	M49	(high)	2.4 X 10 <sup>4</sup>
II	T1	(low)	1.1 X 10 <sup>2</sup>
	M13	(low, focal)	9.6 X 10 <sup>2</sup>
	M53	(high)	2.9 X 10 <sup>2</sup>

Streptococci were grown at 37°C in peptone-meat infusion broth, supplemented with 100 µg/ml of RNase core of yeast RNA (Exp. I) or 1% yeast RNA (Exp. II). After 15 h (Exp. I) or 19 h (Exp. II), SLS activity in the supernatant was determined.

Table III. Effect of trypsin treatment on SLS production in streptococci differing in LTA content.

Strain (LTA content)	Treatment with	I		II	
		HU/OD <sub>660</sub>	%	HU/OD <sub>660</sub>	%
T1 (low)	None	61.4	100	148.8	100
	Trypsin	234.7	382	253.5	170
M13 (low, focal)	None	153.3	100	28.6	100
	Trypsin	217.6	142	39.5	138
M53 (high)	None	76.3	100	298.5	100
	Trypsin	556	142	986.0	330
M34 (high)	None	15.6	100	—	—
	Trypsin	22.2	142	—	—
M49 (low)	None	253	100	—	—
	Trypsin	419	166	—	—

Streptococci grown at 37°C, for 15 h in ordinary broth (I), or for 20 h in THB (II), were harvested, washed 3 times with BBM, and treated with 2 mg/ml trypsin in BBM at 37°C for 60 min. After washing 3 times with BBM, the cells were incubated at 37°C for 60 min in BBM containing 100 µg/ml of RNase core of yeast RNA, and SLS activity in the supernatant was determined.

Table IV. Effect of pronase treatment on CBH activity among streptococcal strains differing in LTA content.

Strain (LTA content)	Treatment with	CBH	
		HU/OD <sub>660</sub>	Ratio
Sa	None	90.6	1
	Pronase	174.8	1.93
T1 (low)	None	0.8	1
	Pronase	8.9	11.1
M13 (low, focal)	None	45.7	1
	Pronase	897.6	19.6
M34 (low)	None	23.6	1
	Pronase	340.7	14.4
M49 (high)	None	7.7	1
	Pronase	155.2	20.2

Streptococci were grown in ordinary broth at 37°C, for 15 h (Sa and M13) or 20 h (T1, M34 and M49), collected, washed 3 times with BBM, suspended in 1/30 volume of BBM and halved. To the one portion, pronase was added to 2 mg/ml. After incubation at 37°C for 60 min, the cells were washed 3 times with BBM, and CBH activity was determined.

Table V. Carrier activity of lipoteichoic acid and lipopolysaccharide for SLS, as compared with RNA core.

Addition	SLS formed (HU/OD <sub>660</sub> )
None	1.6
Streptococcal LTA	81.7
LPS Ra	2.2
Rd <sub>1</sub>	1.1
Rd <sub>2</sub>	0.8
Re	1.7
RNA core	1355.6
GMP	1.3

Washed cells of strain Sa grown in ordinary broth at 37°C for 15 h were suspended in BBM containing 100 µg/ml of the indicated compound. Each suspension was incubated at 37°C for 60 min, chilled, centrifuged and hemolytic activity in the supernatant was determined.

residues in SLS complex are masked to endoproteinase Glu-C, possibly by interaction with carrier.

## Discussion

Lipoteichoic acid (LTA) preparations extracted from *Streptococcus pyogenes* and *Bacillus subtilis* exerted the carrier activity for SLS, either in the resting cell system or growing culture. In addition, LTA exhibited inhibitory activity against hemolysis by SLS. In these respects, LTA is similar to, but less potent than RNase core, polyguanylic acid or Congo red. These carrier substances, belong to chemically different groups, tend to aggregate and form micelle in solution, by inter-molecular interaction. If endogenous streptococcal LTA serves as the moderate carrier and loosely retains SLS on the coccal surface, transfer of SLS peptide to such potent extracellular carrier as RNase core (16) may proceed rather easily. SLS peptide, unless complexed with the carrier, rapidly undergoes inactivation, probably through intra- and/or inter-molecular interaction. In a sense, the carrier substance is regarded as an extracellular chaperon for SLS. In infected host, serum albumin or lipoprotein, rather than poly- or oligo-nucleotide, probably functions as the natural carrier. Moreover, presence of intracellular SLS (ICH-S; 17) and cell-bound SLS (CBH) activities indicates that the nascent hemolysin molecule may directly be transferred from streptococcal surface to host cells. Dual effects of the carrier on SLS, activation (stabilization) and inhibition, are probably caused by competition between the carrier and the receptor around SLS peptide.

Regardless of the carrier function of extracellularly added LTA, whether ICH-S or CBH transiently forms active complex with endogenous LTA in streptococcal envelope is unknown. SLS yield did not correlate with LTA content, among different streptococcal strains. Trypsin treatment of streptococcal cells, which releases considerable portion of LTA, did not liberate active SLS complex. Moreover, yield of CBH and SLS complex in resting streptococci was distinctly enhanced by pretreatment with trypsin or pronase. It has also been reported that SLS production proceeds in streptococcal protoplasts (18) deficient in cell wall (hence surface LTA), in the presence of carrier oligonucleotide. These facts suggest that streptococcal endogenous LTA does not function as the natural carrier for SLS. In order to settle the issue, streptococcal strain totally deficient in LTA (null mutant) is required undoubtedly.

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