

# Decreased Production of RNA-Streptolysin S in Streptococci Devoid of Extracellular RNase Activity

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In cultures of certain streptococcal strains, RNA added as a carrier for streptolysin S (SLS) was hardly degraded, owing to deficiency of extracellular RNase activity. Production of RNA-SLS into culture supernatant was markedly reduced in the RNase-deficient streptococci. Even in these RNase-less strains, guanylic-acid rich oligonucleotides, polyG or trypan blue effectively induced SLS production, as in RNase-positive cells. These results demonstrate involvement of the streptococcal nuclease in manifestation of SLS-inducing effect of exogenous RNA. Additional data indicated that cellular growth was promoted by supplementation of RNA, in the nuclease-producing streptococci.

## Introduction

Production of SLS, an oxygen-stable exotoxin of hemolytic streptococci, is markedly enhanced by supplementation of yeast RNA into culture medium [1]. This "RNA effect" is increased by digestion of the polynucleotide with RNase I and the SLS-inducing activity is exclusively found in the RNase-resistant core fraction [2]. (The term "induction" or "inducer" has widely been used, in an operational sense, to refer to promotion of SLS production by exogenous RNA, although there is no proof of increased synthesis of SLS messenger RNA.) Active principle in the core is composed of oligonucleotides rich in guanylyl residue [3], whereas polyG per se exhibits notable SLS-inducing effect [4]. Through complex formation (which is thought to occur on streptococcal surface) with nascent SLS, these oligo- or poly-nucleotides serve as an external carrier for SLS and stabilize the toxin peptide released extracellularly [5]. Removal of the carrier moiety from SLS complex inevitably causes inactivation of the hemolysin.

Although RNase I is useful for preparation *in vitro* of the carrier oligonucleotides, this pancreatic enzyme is unavailable for streptococci in their natural host tissues. Previously, we have demonstrated that digestion of the carrier RNA with

streptococcal extracellular nuclease yields core oligonucleotides with potent SLS-inducing activity [4, 6]. In order to clarify role of the streptococcal nuclease in SLS production *in vivo*, RNA effect was compared between the nuclease-positive and -negative streptococci.

## Materials and Methods

### Chemicals

Yeast RNA was obtained from Kohjin Co., Tokyo. PolyG was purchased from Boehringer Mannheim GmbH. AF (guanylic-acid rich oligonucleotide fraction with potent carrier activity for SLS) was prepared from RNase I core of yeast RNA, by DEAE cellulose chromatography [7, 8].

### Streptococcal culture and resting cell system

Strains of hemolytic streptococci used were Sa, Su, Sv, C203S and Blackmore. The bacteria were cultured in peptone-meat infusion broth at 37 °C without aeration and their growth was monitored by measuring turbidity at 660 nm, using a Bausch & Lomb Spectronic 20A spectrophotometer. For resting cell experiments, the bacteria grown for 15 h at 37 °C were collected, washed three times with chilled 0.15 M saline and suspended in Bernheimer's basal medium [9]. When RNA degradation or SLS synthesis was to be followed, indicated amount of

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yeast RNA, polyG, or AF was added to each medium.

#### Titration of SLS

Titration of SLS and definition of hemolytic unit (HU) were the same as those described previously [7, 8]: one ml of diluted SLS sample was mixed with one ml of 3% rabbit-erythrocyte suspension in 0.15 M saline, and incubated at 37 °C for 60 min. After addition of 2 ml of chilled saline, the mixture was centrifuged and hemoglobin released into the supernatant was determined by the optical density at 541 nm. One unit (HU) of the hemolytic activity was defined as the amount causing the lysis of half the erythrocytes contained, in 60 min.

#### Assay of acid-soluble fraction

Streptococcal culture was chilled, centrifuged at 12 000 r.p.m. for 10 min at 0 °C and the supernatant was removed. A portion of the chilled supernatant was mixed with four volumes of ice-cold 0.5 N perchloric acid and kept at 0 °C for 30 min. The acidified mixture was centrifuged at 12 000 r.p.m. for 10 min and optical density at 260 nm of the acid soluble supernatant was measured with a Hitachi UV-VIS spectrophotometer, after suitable dilution.

#### Determination of nuclease activity

Both DNase activity and RNase activity were assayed by measuring the optical density at 260 nm of the acid-soluble oligonucleotides liberated from

calf thymus DNA or yeast RNA [6]. Owing to heterogeneity in nucleotide composition and chain length of the oligonucleotides, the digestion product could not be determined in micromols. Therefore, an increase in the optical density at 260 nm of 1.0, in 10 min, was defined as one unit of enzyme activity [6].

## Results

#### Survey of nuclease activity in hemolytic streptococci

First, nuclease activities were surveyed in the culture supernatants of various streptococcal strains, using calf-thymus DNA and yeast RNA as the substrates. Although several  $\beta$ -hemolytic streptococci were found to be deficient in RNase activity, strain Su was studied in detail, taking advantage of its attenuated character. As shown in Fig. 1, culture supernatant of strain Su exhibited no RNase activity, whereas the enzyme activity was easily detected in the spent medium where strain Sa was grown. Both strains, however, produced significant amount of DNase extracellularly. In these experiments, nuclease assay was performed at an alkaline pH, leaving possibility to miss RNase with acidic pH optimum. Therefore, nuclease activities were determined at various pH, using extracellular protein fraction concentrated by precipitation with ammonium sulfate. As presented in Table I (Exp. I), RNase was hardly detected in the concentrated protein fraction from Su culture supernatant, though the fraction contained distinct DNase activ-

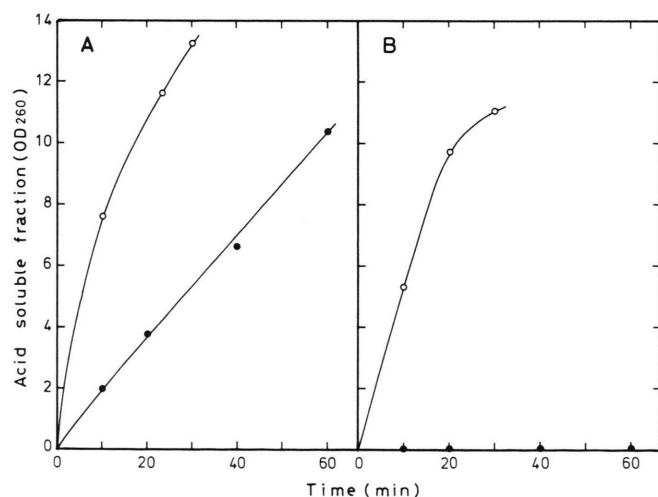


Fig. 1. Nuclease activities in culture supernatants of streptococcal strains Sa and Su. Streptococci were grown in peptone-meat infusion broth, at 37 °C for 15 h, and the culture supernatants were obtained by centrifugation. To compare nuclease activities produced extracellularly, the culture supernatant was mixed with calf thymus DNA (○) or yeast RNA (●), in 50 mM glycine-KOH buffer (pH 9.0) containing 1 mM MgCl<sub>2</sub> and 1 mM CaCl<sub>2</sub>, and time course of liberation of acid soluble nucleotides (expressed in OD<sub>260</sub>) was followed at 37 °C. A: strain Sa; B: strain Su.

Table I. Difference in extracellular nuclease activity between *Streptococcus hemolyticus* strain Sa and Su. In Exp. I, protein fraction collected from the culture supernatant by ammonium sulfate precipitation [6] was dissolved in deionized water, and its nuclease activity was determined at different pH, using buffers containing 1 mM MgCl<sub>2</sub> and 1 mM CaCl<sub>2</sub>. The buffers (50 mM) used were sodium acetate (pH 4.0–6.5), Tris · HCl (pH 7.5 and 8.0) and glycine-KOH (pH 7.0, 8.0–10). In Exp. II, streptococci grown in peptone-meat infusion broth at 37°C for 15 h were collected, washed and suspended in Bernheimer's basal medium [9]. The suspension was incubated at 37°C and, at the indicated time, aliquot was removed, centrifuged and nuclease activity in the supernatant was determined.

Exp.	pH or Time [min]	Nuclease activity (unit/ml)			
		Strain Sa		Strain Su	
		DNase	RNase	DNase	RNase
I	4.0	3.1	2.9	3.6	< 0.1
	5.0	34.9	23.1	7.1	0.2
	5.5	135.7	90.4	50.5	0.4
	6.5	348.7	179.0	184.6	0.9
	7.0	376.2	201.1	243.8	0.7
	7.5	336.2	178.2	—	1.7
	8.0	333.8 <sup>a</sup>	177.7 <sup>a</sup>	—	2.4 <sup>a</sup>
		360.0 <sup>b</sup>	203.1 <sup>b</sup>	235.4 <sup>b</sup>	1.2 <sup>b</sup>
	9.0	334.3	211.4	130.1	2.7
	10	266.3	185.5	27.0	2.9
II	0	0	0	0	0
	10	0.54	0.14	0.16	0
	20	1.40	0.37	0.79	0
	40	3.12	0.80	1.18	0.04
	60	3.84	1.10	1.16	0.10

<sup>a</sup> in Tris · HCl buffer.

<sup>b</sup> in glycine-KOH buffer.

ity with optimal pH ranging 7 to 8 and sensitive to EDTA. On the other hand, the extracellular fraction of strain Sa showed Mg<sup>2+</sup>- and Ca<sup>2+</sup>-dependent RNase activity as well as DNase activity, at a broad alkaline pH range. When washed cells of strain Sa were incubated in Bernheimer's basal medium [9], both DNase and RNase activities were produced extracellularly (Table I, Exp. II). In the resting cell system, strain Su also released DNase, but RNase activity was not detectable in the surrounding medium. These results indicate that absence of the RNase production in Su culture is not due to repression by peptone-meat infusion broth.

#### Degradation of carrier RNA and RNA-SLS production in streptococci differing in RNase activity

Degradation of carrier RNA for SLS and the RNA-dependent toxin production were followed in

cultures of hemolytic streptococci with or without extracellular RNase activity. As demonstrated in Fig. 2, extensive degradation of carrier RNA occurred in RNase-positive strain Sa, when the culture entered into stationary phase. The toxin production became evident, about two hours after onset of the RNA degradation. In culture of Su lacking in extracellular RNase, both rate and extent of RNA degradation were negligibly low. In this strain, RNA was totally inactive as the carrier or inducer for SLS. These results strongly indicate participation of the streptococcal extracellular RNase activity in manifestation of RNA effect for SLS production.

#### AF- or polyG-dependent SLS production in RNase-deficient streptococci

Even when high concentration of RNA was added as the carrier, SLS production was not promoted in the RNase-deficient Su cells. When, however, AF was used, SLS production occurred in Su culture, as efficiently as in the nuclease-positive Sa cocci (Fig. 3). In addition, SLS-inducing effect of polyG did not differ significantly, between Su and Sa.

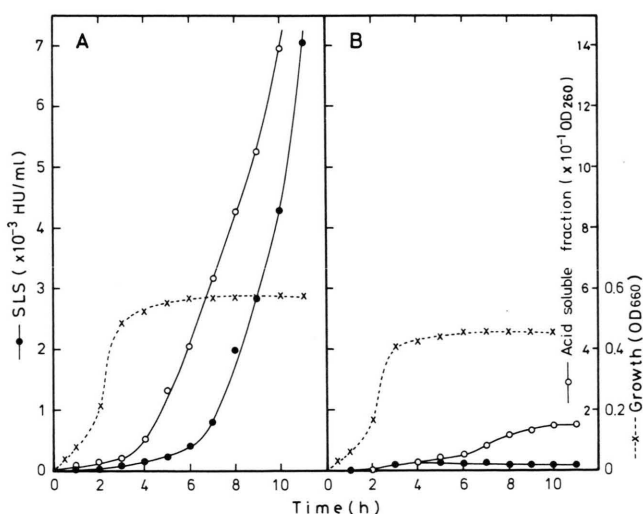


Fig. 2. Degradation of carrier RNA and production of SLS in streptococcal cultures. Hemolytic streptococci strain Sa (A) or Su (B) were cultured at 37°C, in peptone-meat infusion broth supplemented with 10 mg/ml of yeast RNA, and their growth was followed by measuring turbidity (OD<sub>660</sub>). At the time indicated, aliquot was removed, centrifuged at 0°C and amounts of SLS (HU/ml) and acid-soluble fraction (OD<sub>260</sub>) liberated into the culture supernatant were determined as described under Materials and Methods.

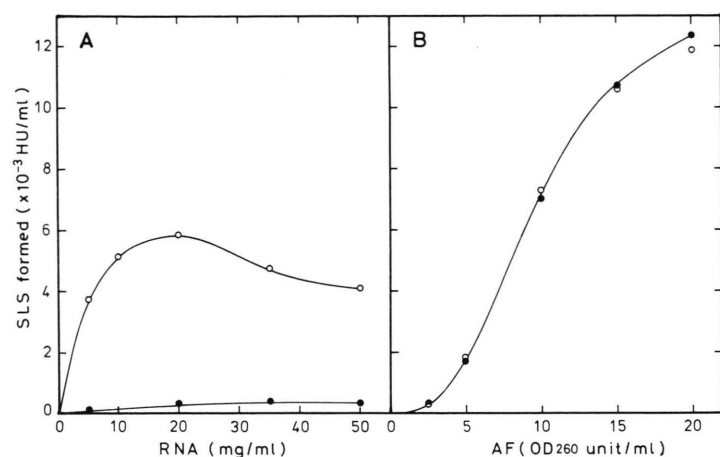


Fig. 3. Effects of RNA and AF on SLS production in streptococcal cultures. Cells of strain Sa (○) or Su (●) were grown at 37 °C, in peptone-meat infusion broth supplemented with indicated amount of yeast RNA (A) or AF (B). After 15 h, each culture was chilled, centrifuged and SLS activity in the culture supernatant was titrated.

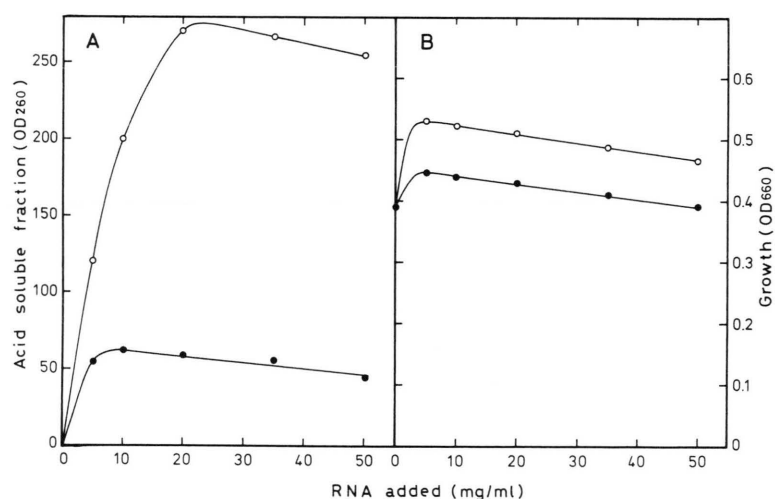


Fig. 4. Degradation of RNA and extent of streptococcal growth. Streptococci strain Sa (○) and Su (●) were cultured at 37 °C for 15 h, in peptone-meat infusion broth containing the indicated amount of yeast RNA. After measuring the cellular growth (expressed in OD<sub>660</sub>), the culture was centrifuged and amount of acid-soluble fraction (OD<sub>260</sub>) in the culture supernatant was determined. A: acid-soluble fraction; B: cellular growth.

Table II. SLS production and degradation of carrier RNA in growing streptococci. Each streptococcal strain was inoculated into peptone-meat-infusion broth supplemented with 20 mg/ml of yeast RNA or 1 mg/ml of polyG and incubated at 37 °C for 15 h. After measuring cellular growth (turbidity at 660 nm), the culture was centrifuged and amounts of SLS and acid-soluble fraction in the supernatant were determined.

Strain	SLS (HU/ml)		Acid-soluble fr. <sup>a</sup>		Growth (OD <sub>660</sub> )	
	RNA	PolyG	RNA	PolyG	RNA	PolyG
Sv	1974	3120	140	5.2	0.507	0.368
Sa	8934	4261	213	4.6	0.765	0.540
Su	136	4001	36	2.2	0.470	0.465
C203S	2924	2400	269	3.0	—	0.413
Blackmore	244	5569	33	4.6	0.417	0.353

<sup>a</sup> OD<sub>260</sub> unit/ml.

As seen in Table II, SLS synthesis in strain Blackmore was also promoted by polyG, but not by RNA which was hardly acid-solubilized by the bacteria. In contrast with strain Su and Blackmore, cells of Sv and C203S, like Sa, degraded the carrier RNA into acid-soluble fraction and yielded considerable amount of RNA-SLS into the culture supernatant. Irrespective of presence or absence of extracellular nuclease, polyG was effective in SLS induction. Moreover, addition of 10 µg/ml of trypan blue resulted in production of about 900 HU of SLS per ml of Su culture. Similarly, vital red exerted distinct carrier effect on the nuclease-negative strain as well (data not shown).

*Growth promoting effect of RNA  
on nuclease-positive strains*

The data presented in Table II also indicated that growth of streptococcal strains such as Sv, Sa and C203S was significantly promoted by supplementation of RNA. Under the conditions employed, streptococcal growth was not affected by polyG. Cellular yield of such nuclease-deficient strains as Su and Blackmore was, however, not specially increased in the RNA-containing medium. As seen in Fig. 4, growth of streptococcal cells was promoted nearly in parallel with acid-solubilization of RNA. Degradation products of RNA such as nucleosides and bases might serve as nutrients for this luxurious microorganism.

## Discussion

As demonstrated above, RNA-dependent SLS production is markedly reduced in hemolytic streptococci deficient in extracellular RNase activity, although the bacterial toxin formation is still induced efficiently by AF, polyG or trypan blue. In addition, treatment of RNA with the purified nuclease yields oligonucleotides having potent carrier activity for SLS [6]. These results clearly show that extracellular RNase activity is essential for exertion of RNA effect for SLS production.

Present data also prove involvement of the extracellular nuclease in promotion of streptococcal growth caused by RNA. In tissues injured by streptococci, the nuclease, together with host RNase(s) other than pancreatic RNase I, may degrade liberated RNA into core and acid-soluble fraction. Possibly, the core serves as the carrier for SLS, whereas acid-soluble nucleotides may be converted into nucleosides and bases, and utilized in streptococcal salvage pathway. In accordance with this scheme, hemolytic streptococci possess phosphomono- and di-esterases [10] and enzymatic activities required for nucleoside metabolism [11]. In this sense, occurrence of extracellular RNase activity in SLS-negative strain C203U [4, and unpublished observation] is not always wasteful.

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