

# Effects of Membrane-Acting Drugs and Aerobiosis on Production of Streptolysin S and Nuclease in Hemolytic Streptococci

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Yield of streptolysin S (SLS) in streptococcal culture was considerably reduced by procaine, dibucaine, atropine or chlorpromazine at concentrations which scarcely affected production of an extracellular nuclease as well as the bacterial growth. Cerulenin was also inhibitory to SLS formation, but its effect was more pronounced on the nuclease production. By aerobiosis, amount of SLS produced into culture supernatant was increased significantly, whereas yield of the nuclease was rather unaffected.

## Introduction

SLS is an oxygen-stable cytolytic exotoxin produced by *Streptococcus pyogenes* in the presence of certain carrier substance such as RNA [1]. Although several aspects of SLS production have been elucidated [2], mechanism of synthesis and secretion of this peculiar toxin is only fragmentary known. For further characterization of SLS formation, comparative studies with production of conventional extracellular enzymes or exotoxins seem to be essential. In this report, effects of several membrane-acting drugs and aerobiosis have been tested on production of SLS and an extracellular nuclease [3], in hemolytic streptococci. This nuclease is, like SLS, produced into surrounding media by growing streptococci and by the washed resting cells, but carrier substance is unnecessary for its production. Experimental results have been obtained suggesting existence of different secretory mechanisms for the two extracellular proteins.

## Materials and Methods

### Chemicals

Calf thymus DNA, cerulenin, procaine hydrochloride and dibucaine hydrochloride were pur-

chased from Sigma Chemical Co., St. Louis. Chlorpromazine HCl was a product from Yoshitomi Pharmaceutical Ind. Ltd., Osaka. Yeast RNA was obtained from Kohjin Co., Tokyo. 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 [4, 5].

### Strain, media and culture technique

An avirulent mutant of hemolytic streptococci, strain Sa [4] was used. The bacteria were grown in a peptone-meat infusion broth, at 37 °C. In conventional culture, the medium in a flask was inoculated with streptococci, cotton-plugged, and incubated aerobically, but without forced aeration. In the "aerobic" culture, the seeded medium was spreaded out thin (less than 2 mm in thickness), in a flat-bottomed flask. In the "anaerobic" culture, each test tube was filled with the medium to the top, boiled to expel out air and promptly cooled. After inoculation with the seed bacteria, the tube was tightly closed with a silicon-rubber stopper, and incubated without shaking. When SLS production was to be investigated, yeast RNA (1%) or AF (2 OD<sub>260</sub> units/ml) was supplemented. SLS production in a resting cell system [4] was also studied aerobically or anaerobically as above, in Bernheimer's basal medium [6] containing AF.

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### Others

Measurement of bacterial growth, titration of SLS activity, assay of nuclease activities and degradation of the carrier RNA were performed as previously described [3, 7].

## Results

### Effect of membrane-acting drugs on production of SLS and nuclease

Experiments in this section were performed in the conventional culture which was nearly aerobic. Production of SLS and the extracellular nuclease in the growing streptococcal culture was affected by cerulenin, a specific inhibitor of fatty acid synthesis

in various bacteria [8]. As shown in Fig. 1A, the nuclease production was extremely sensitive to cerulenin, whereas the antibiotic exerted less marked effect on the streptococcal growth (and hence on the synthesis of cellular bulk proteins). As a consequence of decreased production of the extracellular nuclease, degradation of the carrier RNA was considerably reduced and the yield of RNA-SLS was markedly lowered in the cerulenin-containing culture, as in mutants deficient in the nuclease activity [7]. AF-dependent SLS production in growing streptococci was intermediately sensitive to cerulenin. In the resting cell system, considerably higher concentration of cerulenin was required to inhibit the production of the nuclease and SLS (Fig. 1B).

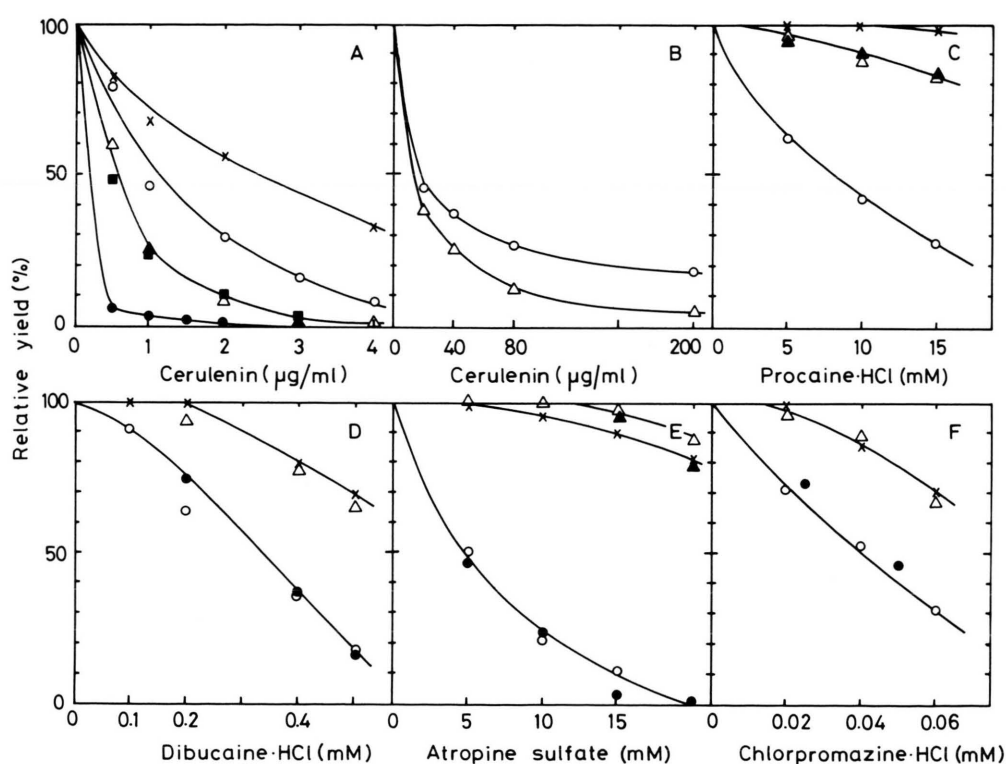


Fig. 1. Effects of cerulenin and membrane-active amphiphilic drugs on production of SLS and extracellular nuclease in hemolytic streptococci. In A, C, D, E and F, cells of *Streptococcus pyogenes* strain Sa were grown at 37 °C, in peptone-meat infusion broth containing the indicated amount of the drug and yeast RNA (1%) or AF (2 OD<sub>260</sub> units/ml). When effect on the nuclease production was to be tested, the SLS carrier (RNA or AF) was omitted from the medium. After 16 h-incubation, extent of the bacterial growth was determined and each culture was centrifuged at 0 °C. The supernatant was removed and amounts of SLS, acid-solubilized fraction of the carrier RNA and extracellular nuclease were determined. In B, cells of strain Sa, grown at 37 °C for 16 h in peptone-meat infusion broth, were collected, washed three times with 0.15 M saline and suspended in BBM containing the indicated amount of cerulenin. For SLS production, 2 OD<sub>260</sub> units/ml of AF were further added. After incubation at 37 °C for 1 h, each mixture was centrifuged and the resulting supernatant was assayed for SLS or the nuclease activity. ×, growth; ○, AF-SLS; ●, RNA-SLS; Δ, DNase activity; ▲, RNase activity; ■, acid-soluble fraction.

Procaine is known to inhibit post-translational processing of certain bacterial proteins such as alkaline phosphatase and outer-membrane protein of *Escherichia coli* [9, 10]. This narcotic, unlike cerulenin, rather preferentially inhibited SLS production in growing streptococci (Fig. 1 C). Thus, by addition of 10 mM procaine · HCl, the relative yield of SLS was reduced to less than 40%, whereas that of the nuclease (as measured by both DNase and RNase activities) amounted to nearly 90% of the unadded control. Growth of streptococcal cells was not significantly affected, even in the presence of 15 mM procaine · HCl. In the resting cell system, however, the inhibitory effect of procaine was not so specific to SLS formation. When streptococci cultured in the presence of 25 mM procaine · HCl were washed and tested in the resting cell system, their capacity to produce SLS was 40 to 50% of that of the control cocci grown in the absence of the narcotic. Dibucaine, another tertiary amine local anesthetic, also decreased the SLS yield in streptococcal culture, without markedly affecting the cellular growth and the nuclease production (Fig. 1 D). The inhibitory effect of dibucaine was, as observed in various membrane systems [11], by far potent than procaine. As shown in Fig. 1 E, the selective inhibition of the SLS production was caused by

atropine as well, in streptococcal culture. Upon the addition of 15 mM atropine sulfate, the rate of the toxin production was promptly decreased, whereas the cellular growth continued rather normally. Significant difference was also observed between SLS formation and nuclease production, in sensitivity to chlorpromazine, an amphiphilic sedative (Fig. 1 F).

#### *Effect of aerobiosis on production of SLS and nuclease*

The amount of SLS produced into the culture medium was increased about 7.5-fold, when the streptococci were grown under aerobic condition (Table I). Although extent of the cellular growth per se was somewhat high in the aerobic culture, specific yield (amount of SLS/OD<sub>660</sub> of the culture) was five times the yield in the anaerobic culture. On the other hand, the degradation of RNA added as the carrier for SLS was hardly promoted by aerobiosis. In coincidence with the degree of acid-solubilization of the carrier RNA, the specific yield of the extracellular nuclease was not different between the two cultures (Table II). Additional data in Table III showed that the toxin production in the resting cell system was also promoted by aerobic incubation. (Streptococcal capacity for the produc-

Table I. Increased production of SLS in aerobic culture of streptococci. Hemolytic streptococci, strain Sa, were grown aerobically or anaerobically at 37 °C for 16 h, in peptone-meat infusion broth supplemented with 1% yeast RNA. After measuring the turbidity at 660 nm, the culture was centrifuged and amounts of SLS and the acid-soluble fraction in the supernatant were determined.

Culture condition	Extent of growth (OD <sub>660</sub> )	SLS formed		RNA acid-solubilized	
		HU <sup>a</sup> /ml	HU/OD <sub>660</sub>	OD <sub>260</sub> Units/ml	OD <sub>260</sub> Units/OD <sub>660</sub>
Aerobic	0.35	$1.49 \times 10^4$	$4.26 \times 10^4$	$2.79 \times 10^2$	$7.96 \times 10^2$
Anaerobic	0.23	$1.97 \times 10^3$	$8.58 \times 10^3$	$1.67 \times 10^2$	$7.28 \times 10^2$

<sup>a</sup> Hemolytic unit.

Table II. Production of the extracellular nuclease in aerobic and anaerobic cultures of hemolytic streptococci. Cells of *Streptococcus pyogenes* strain Sa were grown aerobically or anaerobically, in peptone-meat infusion broth, at 37 °C for 16 h. After measuring the turbidity at 660 nm, each culture was centrifuged and amount of the nuclease in the supernatant was determined, using calf thymus DNA and yeast RNA as the substrates [3].

Culture condition	Extent of growth (OD <sub>660</sub> )	DNase activity		RNase activity	
		Units/ml	Units/OD <sub>660</sub>	Units/ml	Units/OD <sub>660</sub>
Aerobic	0.36	89.8	249	3.94	10.9
Anaerobic	0.22	54.8	249	2.25	10.2

Table III. Effect of aeration on SLS production in resting streptococci. Washed streptococci were suspended in BBM supplemented with  $2 \text{ OD}_{260}$  units/ml of AF and incubated aerobically or anaerobically at  $37^\circ\text{C}$ , as specified in Materials and Methods. After 1 h, the mixture was centrifuged and SLS activity in the supernatant was titrated.

Condition of incubation	SLS formed		
	HU/ml	HU/ $\text{OD}_{660}$	
Aerobic	$7.82 \times 10^3$	$2.11 \times 10^3$	(100) <sup>a</sup>
Anaerobic	$4.26 \times 10^3$	$1.15 \times 10^3$	(54.5) <sup>a</sup>

<sup>a</sup> %.

tion of SLS in the resting cell system, however, suffered relatively rapid decay in the aerobic culture, as compared with that in the anaerobic culture.)

## Discussion

SLS and the extracellular nuclease are representative of streptococcal excretory proteins and, unlike streptolysin O, they are produced even by the resting cells suspended in salt solutions containing fermentable sugar. Apart from a requirement for the specific carrier, the mechanism of production is at least partly different between SLS and the nuclease. Thus, the production of SLS was relatively resistant to cerulenin, but considerably sensitive to procaine, dibucaine, atropine and chlorpromazine. In contrast, the nuclease production was inhibited by lower concentration of cerulenin, but slightly sensitive to procaine. Moreover, yield of the nuclease was reduced by dibucaine, procaine and chlorpromazine in parallel with growth inhibition, indicating that production of the extracellular nuclease was no more susceptible than synthesis of cellular bulk proteins, to these reagents.

The drugs used in the present study are known to affect the function of the biomembrane, through inhibition of the lipid synthesis (cerulenin) or inter-

action with lipid bilayer and/or membrane proteins (neuroactive amphiphiles). It seems therefore natural that higher concentrations of these drugs are inhibitory to streptococcal growth as well. As to transcription and translation, SLS polypeptide does not significantly differ from other cellular proteins [2, 12]. Therefore, the marked sensitivity of the extracellular SLS production to the membrane-active amphiphiles is suggestive of the involvement of distinct processing and/or transport mechanism for this toxin. In *E. coli*, neuroactive compounds having lipophilic moieties as well as ester or amide linkages inhibit proteolytic processing of precursors of certain proteins [9, 10]. Moreover, Gayda *et al.* [10] reported that several local anesthetics and atropine competitively inhibited amidase action of trypsin. Although the inhibitory effect of atropine on SLS production was not relieved by addition of  $\sim 2 \text{ mg/ml}$  trypsin, and the hydrolysis of benzoyl-arginine *p*-nitroanilide by trypsin was not inhibited by  $1 \text{ mM}$  dibucaine  $\cdot \text{HCl}$  (unpublished observation), it seems probable that the membrane-active amphiphilic compounds affect SLS production by inhibiting a putative processing protease indirectly, through modulation of membrane structure. Another possibility is the inhibition of porine-like membrane protein which might be required for SLS transport. For the excretion of the streptococcal nuclease, another processing protease or transport protein might be required, which is more sensitive to cerulenin-caused membrane alteration but less sensitive to the membrane-active amphiphilic compounds.

As demonstrated by the present investigation, the SLS yield was considerably increased by aerobiosis, whereas the production of the extracellular nuclease was not particularly promoted, as compared with cellular growth. The step of SLS production favored by aerobiosis remains to be elucidated, in relation to structure and function of streptococcal membrane.

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