Activation of Streptolysin S *in vitro* by Oligonucleotides

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When washed streptococci were incubated in a phosphate buffer containing $MgSO_4$ and maltose (Bernheimer's basal medium) and centrifuged, no hemolytic activity was detected in the supernatant. Although incubation of the spent medium with carrier oligonucleotide did not yield active hemolysin, the mixture turned to be significantly hemolytic, upon ethanol precipitation and dehydration. Oxygen stability, sensitivity to trypan blue, absence in the spent medium from strain C203U, as well as chromatographic properties demonstrated that the hemolytic activity was due to streptolysin S-oligonucleotide complex. The latent streptolysin S was detected in the streptococcal culture supernatant as well. These results indicate that, even in the absence of exogenous carrier, streptolysin S is produced extracellularly by hemolytic streptococci but suffers rapid denaturation, and that the carrier oligonucleotide serves as an effector for the toxin peptide to assume active conformation, through noncovalent interaction. Effects of several protein denaturants were investigated on the toxin activation *in vitro*.

Introduction

Production of the active form of streptolysin S (SLS), an oxygen-stable exotoxin of hemolytic streptococci, absolutely depends on the presence of specific carrier substance such as RNA or certain oligonucleotides [1, 2]. Extracellular form of the toxin is a complex composed of SLS polypeptide and the carrier [2], dissociation of which always results in complete inactivation of the toxin. Although the carrier substance has often been designated as inducer, the facts that streptococci cultured without supplementation of the carrier still retain intracellular and cell-bound SLS activities and are competent for the toxin production in resting state indicate dispensability of the carrier for synthesis of SLS messenger RNA [3]. The role of the carrier in translation or processing of SLS peptide, however remains to be elucidated. Above all, demonstration of free SLS peptide in the extracellular medium has been unsuccessful and whether SLS peptide is secreted by the cocci without the carrier or not is utterly unknown, owing to instability, minute amount and feeble antigenicity of the toxin.

In order to address these questions, a system has been devised to detect SLS in the carrier-free spent medium, by the oligonucleotide-dependent activation procedure. Taking advantage of the *in vitro* activation system, properties of the latent SLS and factors affecting renaturation of the toxin have been investigated.

Materials and Methods

Chemicals

RNase I core of yeast RNA was purchased from Sigma Chemical Co. 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]. Pronase was obtained from Kaken Co., Tokyo, Fraction V bovine serum albumin from Armour Pharmaceutical Corp., trypsin and α-chymotrypsin from Worthington Biochemical Corp., acetamide (zone purified) from Nakarai Chemicals Ltd., Kyoto, and antistreptolysin-O serum (ASLO) from Kitasato Institute Co., Tokyo. Other chemicals (reagent grade) were purchased from Wako Pure Chemical Ind. Ltd., Osaka.

Strains, media and culture technique

Strain Sa, an avirulent mutant of *Streptococcus pyogenes* was used in most experiments. In a certain case, Sv, Su, C203S, C203U and Blackmore were

Abbreviations: SLS, streptolysin S; BBM, Bernheimer's basal medium; HU, hemolytic unit.

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also used. The cocci were grown aerobically, but without shaking, in a peptone-meat infusion broth at 37 °C, for 15 h. The culture was centrifuged and, after removing the supernatant, the sedimented bacteria were washed three times with 0.15 M saline and suspended, at OD_{660} = 10, in Bernheimer's basal medium (BBM) [6] without the carrier. The resting cell suspension was incubated at 37 °C for 1 h, chilled and centrifuged. After an additional centrifugation, the supernatant (spent BBM) was used for the subsequent activation experiments or preserved at −20 °C. The culture supernatant was also centrifuged again at 0 °C and kept frozen at −20 °C.

Others

Measurement of bacterial density, titration of SLS and definition of hemolytic unit (HU) were as described previously [5, 6]. Gel permeation chromatography was performed on a Sephadex G-75 column (0.9 × 27 cm), using 0.1 M KCl as the eluent.

Results

Functional detection of inactive SLS secreted into the carrier-free medium

No hemolytic activity was demonstrable in the spent Bernheimer's basal medium (BBM) in which washed resting streptococci had been incubated in the absence of the carrier. Subsequent addition of the carrier oligonucleotide to the spent medium failed to yield any active hemolysin. The spent medium mixed with the oligonucleotide, however, contained hemolytic activity which was manifested after ethanol precipitation and dehydration. Thus, after mixing with oligonucleotide (1 mg/ml), pH of the spent BBM was adjusted to 8.0 and the mixture was allowed to stand for 15 min at 30 °C, and chilled at 0 °C. Then, pH of the mixture was adjusted to 4.6 and two volumes of chilled ethanol were added. The resultant precipitate was collected by centrifugation, washed sequentially with ethanol, ethanol-ether (1:1) mixture and ether, and dried in vacuo. Hemolytic activity of the dried sample, assayed after dissolving in the original volume of 0.15 M saline, was around 2×10^2 HU/ml (Table I), although somewhat fluctuated depending on each experiment. After ethanol precipitation and dehydration, the spent medium exhibited about 10 HU/ml of hemolytic activity, without mixing with oligonucleotide.

Table I. Oligonucleotide-dependent activation of the latent hemolysin in the spent medium. Washed streptococci suspended in BBM were incubated at 37 °C for 1 h and chilled at 0 °C. The bacteria were spun down and the supernatant was recentrifuged. The final supernatant (spent BBM) was divided into three 1 ml portions A, B and C, and 1 mg of oligonucleotide (RNase core) was added to B. After adjusting the pH to 8.0, samples A and B were incubated for 15 min at 30 °C, then chilled at 0 °C and pH was adjusted to 4.6 with acetic acid. Aliquot was removed for determination of the hemolytic activity (I), and then three volumes of chilled ethanol were added to the remaining portion. After standing for 30 min at 0 °C, the precipitate was collected by centrifugation, washed successively with ethanol, ethanol-ether (1:1) mixture and ether, and dried in vacuo. The dried sample C was dissolved in 0.1 ml of 10 mg/ml oligonucleotide solution and the volume was made to 1 ml with 0.1 M KCl. After adjusting pH to 8.0, the mixture was incubated for 15 min at 30 °C, chilled, acidified (to pH 4.6), precipitated with ethanol, and dehydrated as above. Each dried sample was dissolved in 0.15 M saline and their hemolytic activity was titrated (II).

Condition of activation	Hemolytic activity [HU/ml] I (before dehydration)	II (after dehydration)
A (without oligonucleotide)	< 0.5	10.2
B (oligonucleotide added to spent BBM)	0.8	224.3
C (oligonucleotide added to ethanol-precipitated fraction)	< 0.5	65.7

When the ethanol-precipitated dehydrated sample (prepared from the spent BBM without addition of oligonucleotide) was dissolved in oligonucleotide solution, its hemolytic activity was less than 0.5 HU/ml. Upon ethanol precipitation and subsequent dehydration, this inactive mixture turned to be moderately hemolytic (about 60 HU/ml). These results demonstrate that washed resting streptococci secrete into BBM a latent (or denatured) hemolysin which is activated in vitro by interaction with oligonucleotide. The latent hemolysin was inactivated by pronase or by α-chymotrypsin (data not shown).

The hemolysin activated *in vitro* was distinct from streptolysin O (SLO) in its stability to oxygen as well as in the profile of hemolysis. Its hemolytic activity was not significantly promoted by 10 mM cysteine. In order to characterize further the hemolysin, effects of several inhibitors were tested on its hemolytic activity. As presented in Table II, the hemolysin was sensitive to trypan blue, a specific inhibitor of SLS, but resistant to N-ethylmaleimide as well as to anti-

Table II. Effect of inhibitors on the hemolysin activated *in vitro*. Washed cells of strain Sa were incubated in BBM at 37 °C for 1 h and the spent medium prepared therefrom was mixed with 1 mg/ml oligonucleotide. The mixture was adjusted to pH 8.0, incubated for 15 min at 30 °C and chilled at 0 °C. Two volumes of chilled ethanol were added to the mixture acidified to pH 4.6 and, after 30 min-standing, the precipitate was collected, washed with ethanol, ethanol-ether mixture and ether and dried in vacuo. The hemolysin was dissolved in 50 mM Tris · HCl buffer (pH 7.6), incubated with the indicated inhibitor at 37 °C for 20 min and its hemolytic activity was determined after dilution.

Treatment with	Hemolytic activity [HU/ml]
Buffer only	288.5
Trypan blue (25 μg/ml)[a]	< 0.5
N-ethylmaleimide (10 mM)	295.1
ASLO (D = 10)	304.9
Pronase (100 μg/ml)	37.5
Trypsin (100 μg/ml)	282.2

[a] Hemolytic activity was determined in the presence of trypan blue, without preincubation.

serum against SLO. Like natural oligonucleotide-SLS complex, the hemolysin was inactivated by pronase but not by trypsin. In addition, its chromatographic patterns on a Sephadex G-75 column and a DEAE cellulose column were similar to that of oligonucleotide-SLS (data not shown).

Table III. Absence of the latent hemolysin in SLS-negative strain. Washed cells of each strain were incubated in BBM at 37 °C for 1 h and the spent medium was prepared from the suspension by repeated centrifugation. To the spent medium, oligonucleotide was added to 1 mg/ml, and pH of the mixture was adjusted to 8.0. After incubation for 15 min at 30 °C, each sample was chilled at 0 °C and divided into two portions. One portion was directly assayed for hemolytic activity (I), whereas the other portion was adjusted to pH 4.6 and mixed with two volumes of chilled ethanol. The resulting precipitate was collected, washed with ethanol, ethanol-ether mixture and ether. After drying in vacuo, each sample was dissolved in 0.15 M saline and the hemolytic activity was determined (II).

Strain	Hemolytic activity [HU/ml]	
	I (before dehydration)	II (after dehydration)
Sv	1.6	53.9
Su	0.9	309.2
C203S	0.9	81.9
C203U	2.1	0.1
Blackmore	1.2	57.1

Besides Sa, SLS-positive strains Sv, Su, C203S and Blackmore produced the latent hemolytic moiety into BBM (Table III). In the spent medium in which SLS-negative C203U cells had been incubated, however, no hemolytic activity was detectable upon addition of oligonucleotide and subsequent ethanol precipitation-dehydration. Taken together, it is evident that the latent or denatured hemolysin is nothing else than SLS.

Factors affecting oligonucleotide-dependent activation of SLS in vitro

Unlike *in vitro* transfer of native SLS peptide between different carriers [7, 8], oligonucleotide-dependent renaturation of the inactive SLS released into the carrier-free medium requires a dehydration process. In addition to hydration, it seems probable that intramolecular hydrogen- and/or hydrophobic-bonding prevents proper interaction of the SLS peptide with the carrier. In order to address this problem, effects of several protein denaturants were tested on the oligonucleotide-dependent activation of SLS peptide. As shown in Table IV, guanidine · HCl (6 M) facilitated construction of active SLS complex from the latent hemolysin fraction and carrier

Table IV. Effects of protein denaturants on SLS complex construction *in vitro*. Latent SLS collected from the carrier-free spent BBM by ethanol precipitation before (series I) or after (series II) mixing with oligonucleotide (1 mg/ml), was washed with ethanol, ethanol-ether mixture and ether and dried in vacuo. The hemolysin was dissolved in 0.1 ml of each denaturant and incubated for 15 min at 37 °C. To series I, 0.1 ml of 10 mg/ml oligonucleotide solution was added and incubated at 37 °C for 15 min. Volume of each mixture was made to 1.0 ml by gradual addition of 0.1 M KCl. After adjustment of pH to 8.0, the samples were incubated at 30 °C for 15 min and chilled at 0 °C. At this stage, hemolytic activity of the series I sample treated with guanidine · HCl was 57.2 HU/ml. From each mixture, SLS complex was collected by ethanol precipitation, washed with ethanol, ethanol-ether mixture and ether, dried, dissolved in 0.15 M saline and its hemolytic activity was titrated.

Treatment with	Hemolytic activity [HU/ml]	
	Series I	Series II
None	40.5	18.5
Acetamide (8 M)	11.8	115.2
Acetic acid (70%)	19.2	16.6
Formamide (100%)	20.9	20.7
Guanidine · HCl (6 M)	123.2	302.5
Sodium dodecyl sulfate (1%)	27.8	13.5
Urea (8 M)	56.2	11.3

oligonucleotide. In this denaturant, oligonucleotide-dependent activation of SLS took place without ethanol precipitation and dehydration. Guanidine chloride, at concentrations higher than 4 M, is known to break secondary structure of proteins, but not of nucleic acids. Acetamide (8 M) was somewhat effective, when added into dehydrated latent hemolysin fraction mixed with oligonucleotide. On the other hand, formamide (100%), urea (8 M), sodium dodecyl sulfate (1%) and acetic acid (70%) were ineffective or rather inhibitory for the renaturation. Aggregated carrier oligonucleotides are dissociated by urea or formamide but not by guanidine·HCl (unpublished results), and this disruption of micell structure may be responsible for ineffectiveness of the two denaturants. These results are consistent with the hypothesis that free SLS peptide suffers reversible denaturation through incorrect intramolecular bonding.

Activation of SLS secreted into culture supernatant

When streptococci were grown in peptone-meat infusion broth without supplementation of carrier substance, SH-independent extracellular hemolytic activity was negligibly low in the culture supernatant. Ethanol-precipitable fraction of the spent medium, however, turned to be significantly hemolytic after dehydration (Table V). This latent SLS activity was, like that in spent BBM, increased by interaction with oligonucleotide. Treatment with 6 M guanidine · HCl promoted the carrier-dependent renaturation of the apotoxin secreted into the culture supernatant. As the carrier, bovine serum albumin (Fraction V) was rather ineffective in this system.

Table V. Occurrence of the latent SLS in streptococcal culture supernatant. Streptococci, strain Sa, were grown in peptone-meat infusion broth at 37 °C for 15 h and the culture supernatant was obtained by repeated centrifugations. The latent hemolysin was collected by ethanol precipitation from the spent medium, before (series I) or after addition of 1 mg/ml of oligonucleotide or bovine serum albumin (series II). The samples, washed with ethanol and dried in vacuo, were incubated in 6 M guanidine · HCl at 37 °C for 15 min. Then, 1 mg/ml of oligonucleotide or serum albumin was added to series I, and each mixture was incubated further for 15 min at 37 °C. After adjusting pH to 8.0 and gradually diluting with 0.1 M KCl, the guanidine-treated samples were incubated for 15 min at 30 °C. From the mixture, SLS complex was precipitated with ethanol, dried in vacuo and hemolytic activity was titrated after dissolving in 0.15 M saline.

Carrier	Hemolytic activity [HU/ml]	
	Series I	Series II
None	11.6	20.4
Oligonucleotide	231.0	182.6
Serum albumin	25.6	43.2

Discussion

The data presented above clearly demonstrate that, even in the absence of the exogenous carrier, hemolytic streptococci excrete substantial amount of SLS into the medium. Unless coupled with specific effector (carrier), however, this apotoxin may undergo rapid inactivation possibly by incorrect folding. The fact that guanidine · HCl treatment of the denatured SLS promotes its oligonucleotide-dependent activation strongly suggests this notion. Involvement of intermolecular interaction in the denaturation effect is less likely, because inactivation of SLS is not prevented by reducing streptococcal density, hence concentration of the released apotoxin (unpublished observation). In addition to self-denaturation, there is another possibility that the latent SLS is by far susceptible to streptococcal protease(s). Taking this possibility into consideration, hemolytic activity of the SLS complex, constructed *in vitro* from completely inactive apotoxin and oligonucleotide, is significant, though yield of the active hemolysin is rather low.

Incubation of the secreted SLS with carrier oligonucleotide does not yield active hemolysin: hemolytic activity appears after ethanol-precipitable fraction of the inactive mixture was dried in vacuo. In this regard, it seems noteworthy that heat-inactivated RNA-SLS complex is partially reactivated by similar dehydration procedure [9]. When, however, the inactive SLS fraction (from the carrier-free spent medium) and oligonucleotide were incubated in 6 M guanidine · HCl, active SLS complex was formed without subsequent ethanol precipitation and dehydration. Interaction between the autodenatured apotoxin and oligonucleotide may yield an inactive complex similar to heat-denatured RNA- or oligonucleotide-SLS. Dehydration step or guanidine treatment is probably required to convert the inactive complex into active form. When streptococci are incubated in the medium containing sufficient amount of oligonucleotide, transfer of nascent SLS to the external carrier may be initiated before leaving

cell surface, without forming the abortive complex. The carrier functions neither as the inducer for SLS messenger nor as the simple transporter of SLS peptide, but as the effector which holds correct conformation of the apotoxin molecule.

Thus far, no assay system has been available for free SLS peptide. The experiments described here provide a clue for isolation and characterization of the apotoxin and enable further analysis of molecular interaction between SLS and the carrier.

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